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Gene expression differences in relation to age and social environment in queen and worker bumble bees

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ABSTRACT

Eusocial insects provide special insights into the genetic pathways influencing aging because of their long-lived queens and flexible aging schedules. Using qRT-PCR in the primitively eusocial bumble bee *Bombus terrestris* (Linnaeus), we investigated expression levels of four candidate genes associated with taxonomically widespread age-related pathways (*coenzyme Q biosynthesis protein 7*, *COQ7*; *DNA methyltransferase 3*, *Dnmt3*; *foraging*, *for*; and *vitellogenin*, *vg*). In Experiment 1, we tested how expression changes with queen relative age and productivity. We found a significant age-related increase in *COQ7* expression in queen ovary. In brain, all four genes showed higher expression with increasing female (queen plus worker) production, with this relationship strengthening as queen age increased, suggesting a link with the positive association of fecundity and longevity found in eusocial insect queens. In Experiment 2, we tested effects of relative age and social environment (worker removal) in foundress queens and effects of age and reproductive status in workers. In this experiment, workerless queens showed significantly higher *for* expression in brain, as predicted if downregulation of *for* is associated with the cessation of foraging by foundress queens following worker emergence. Workers showed a significant age-related increase in *Dnmt3* expression in fat body, suggesting a novel association between aging and methylation in *B. terrestris*. Ovary activation was associated with significantly higher *vg* expression in fat body and, in younger workers, in brain, consistent with vitellogenin's ancestral role in regulating egg production. Overall, our findings reveal a mixture of novel and conserved features in age-related genetic pathways under primitive eusociality.

Keywords: Aging, DNA methylation, Epigenetics, Gene expression, Social environment, Social insect

1. Introduction

The occurrence of aging in organisms raises important questions at both evolutionary and mechanistic levels (Hughes and Reynolds, 2005; Parker, 2010; Flatt et al., 2013; Gems and Partridge, 2013). Aging is defined as the deterioration in organismal survivorship, fecundity and performance with age. At the mechanistic (proximate) level, much progress have been made in recent years in characterizing the genetic pathways that underpin aging, including those involved in nutrient sensing, energy metabolism, stress and growth (Kenyon, 2010; Gems and Partridge, 2013). The eusocial insects (those with a worker caste), comprising principally the eusocial Hymenoptera and termites, provide a particularly informative case in terms of understanding the genetic pathways and mechanisms that influence aging. First, eusociality is associated with phenotypically flexible aging and longevity. Specifically, the queen and worker castes, which arise from the same genome, exhibit widely differing schedules of aging and longevity, with queens typically far outliving workers (Keller and Genoud, 1997; Keller and Jemielity, 2006; Bourke, 2007; Parker, 2010). Second, eusociality in insects is associated with reversals in conventional life history patterns, as exemplified by positive associations between longevity and lifetime reproductive success observed in queens of eusocial insects (Lopez-Vaamonde et al., 2009; Heinze et al., 2013). In queens of the ant *Cardiocondyla obscurior*, aging-related gene expression changes have been found to occur in a direction opposite to that found in Drosophila, consistent with a reversed (positive) association of longevity and fecundity under eusociality (Von Wyschetzki et al., 2015). Third, in eusocial insects, aging can be regulated by the social environment and even reversed within the lifespan of individuals (Huang and Robinson, 1996; Amdam et al., 2005; Smedal et al., 2009; Amdam, 2011; Woodard et al., 2013). All these traits point, in eusocial insects, to a large degree of flexibility and responsiveness in the genetic pathways that influence aging.

Several major, well-characterized genetic pathways associated with aging in other organisms have been shown to be linked to aging in eusocial Hymenoptera (Parker et al., 2004; Corona et al., 2005, 2007; Amdam, 2011; Von Wyschetzki et al., 2015). For example, in workers of the honey bee (*Apis mellifera*), a pathway involving juvenile hormone and vitellogenin has been shown to affect aging and the temporal division of labor (changes in task with time) in workers (Amdam et al., 2004; Nelson et al., 2007; Münch and Amdam, 2010; Bloch and Grozinger, 2011; Page et al., 2012). Since vitellogenin is ancestrally a yolk protein, this suggests that its original role in the regulation of reproduction has been co-opted to social ends during the course of social evolution (Amdam et al., 2004; Remolina and Hughes, 2008; Flatt et al., 2013), but whether a similar process of co-option has occurred in other eusocial Hymenoptera remains unclear (Bloch and Grozinger, 2011; Amsalem et al., 2014; Von Wyschetzki et al., 2015). In *A. mellifera*, DNA methylation has been found to covary with task and age in workers (Herb et al., 2012; Lockett et al., 2012) and with age and caste in larvae (Foret et al., 2012; Shi et al., 2013). Evidence from other eusocial Hymenoptera and termites also suggests a role for DNA methylation in the regulation of caste-specific longevity (Yan et al., 2015). To test whether or not these processes and phenomena are general requires additional studies of genetic mechanisms of aging, including epigenetic effects of the social environment and effects associated with reproduction, in eusocial insects.

We investigated gene expression as a function of age, social environment and reproductive status for a set of candidate genes associated with taxonomically widespread age-related genetic pathways in queens and workers of the bumble bee *Bombus terrestris* (Linnaeus). We followed a tissue-specific approach, quantifying gene expression in brain, fat body or ovary, because previous studies suggest that relevant pathways are localized within these tissues

(Grozinger et al., 2007; Thompson et al., 2008; Foret et al., 2009; Page et al., 2012). *B. terrestris* has a more primitive form of eusociality (characterized by lower queen-worker dimorphism in the reproductive system) than the advanced eusocial *A. mellifera* and shares with it a common primitively eusocial ancestor (Cardinal and Danforth, 2011). The potential contrast with *A. mellifera* renders the genetic pathways underpinning aging in *B. terrestris* of particular interest. *B. terrestris* forms annual colonies of a single queen and 100-200 worker daughters. Following eclosion (emergence from the pupa) in the previous year and overwintering diapause, *B. terrestris* queens typically live about 6 months (Goulson, 2010), while workers live 1-2 months as adults in laboratory colonies (Holland and Bourke, 2015). Colonies produce first workers and then (in the reproductive phase) males and new queens. During this second part of the colony cycle, some workers activate their ovaries to become reproductive, egg-laying workers (Duchateau and Velthuis, 1988; Bloch, 1999; Zanette et al., 2012).

We selected four candidate genes, *coenzyme Q biosynthesis protein 7* (*COQ7*), *DNA methyltransferase 3* (*Dnmt3*), *foraging* (*for*) and *vitellogenin* (*vg*), as they combined coverage of a range of putative functions associated with aging with a gene structure suitable for the design of gene expression assays. *COQ7*, also known as *clk-1*, encodes a biosynthesis protein involved in electron transport in the mitochondrial respiration pathway. Mutants for this gene exhibit increased longevity in *Caenorhaebits elegans* (Felkai et al., 1999) and mice (Liu et al., 2005). In *A. mellifera*, *clk-1* expression decreases with age in queens but not workers (Table 1). Mitochondrial respiration is thought to contribute to aging via production of reactive oxygen species (Larsen and Clarke, 2002). However, because there is also evidence against a direct role for oxidative damage in aging (Van Remmen et al., 2003; Parker et al.,

2004), we sought to test whether the expression of a gene in the mitochondrial respiration pathway is associated with age in *B. terrestris*.

Dnmt3 encodes the DNA methyltransferase enzyme essential for creating *de novo* DNA methylation marks on the genome. DNA methylation is known to change with age in mammals (Wilson et al., 1987; Issa, 2003) including humans (Horvath, 2013). In *A. mellifera*, associations of DNA methylation patterns with age in workers (Lockett et al., 2012) show that a link between DNA methylation and aging also occurs in eusocial Hymenoptera. In *B. terrestris*, recent evidence points to an association between DNA methylation and worker reproduction (Amarasinghe et al., 2014). In *A. cerana*, *Dnmt3* expression changes with age (in workers) and caste (Table 1) and, in *A. mellifera*, there is experimental evidence for its role in downregulating queen development (Kucharski et al., 2008). However, *Dnmt3* expression has not previously been investigated in *B. terrestris*.

In Drosophila, the *for* gene encodes a cGMP-dependent protein kinase and underpins a polymorphism in foraging behavior (Osborne et al., 1997). Foraging kinase also influences whether energy is stored as lipids or carbohydrates and interacts with the insulin pathway (Kent et al., 2009). Consistent with its association with foraging behavior in Drosophila, *for* has been found to be overexpressed in foraging workers compared to nurse workers in several species of eusocial Hymenoptera, including *A. mellifera* and *B. terrestris*, although the pattern is not universal (Table 1). In addition, *for* expression has been found to decrease with age in *B. terrestris* queens and workers (Table 1). In queens, this occurred only in individuals from which workers were removed, implying the presence of an interaction between age and social environment (Woodard et al., 2013). Since foundress *B. terrestris* queens forage externally only up to the time when their first workers eclose (Goulson, 2010),

we predicted that *for* expression would be higher in foundress queens experimentally deprived of workers.

The *vg* gene encodes an insect version of a yolk protein. In *C. elegans*, *vg* expression provides a potential example of hyperfunction (Blagosklonny, 2012), whereby *vg* is not downregulated after reproduction as expected, but maintains relatively high expression levels into later life, resulting in detrimental effects (DePina et al., 2011; Gems and Partridge, 2013). In eusocial Hymenoptera, *vg* is known to have developed novel functions, particularly with respect to regulation of temporal division of labor in workers, but when in social evolution such functions arose is unclear (Amdam et al., 2004; Corona et al., 2007; Nelson et al., 2007; Münch and Amdam, 2010; Bloch and Grozinger, 2011; Wurm et al., 2011; Page et al., 2012). *Vg* may act by regulating microRNAs (Nunes et al., 2013). *Vg* expression is also associated with age and reproductive status in eusocial Hymenoptera (Table 1). In *B. terrestris*, *vg* was recently found to be associated with worker aggression independently of worker ovarian activation (Amsalem et al., 2014), but relationships with queen and worker age remain unclear.

We performed two experiments. In Experiment 1, to test effects of queen age, queens of different relative ages were removed sequentially from colonies and gene expression in brain and ovary was assayed using quantitative real-time PCR (qRT-PCR). Demographic data were also collected from these colonies to investigate associations of gene expression with queen productivity. In Experiment 2, to test effects of queen age, worker age, worker reproductive status and the social environment, queens were reared in either a 'social' treatment (allowed to head colonies) or an 'asocial' treatment (deprived of workers). Gene expression in brain was assayed for queens removed at different relative ages using qRT-PCR. In the social treatment

colonies in Experiment 2, marked workers were also sampled at different ages and gene expression in brain and fat body was assayed using qRT-PCR.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Queen gene expression as a function of relative age

Queen gene expression as a function of relative age was investigated in both Experiments 1 and 2. In Experiment 1, queens were sampled only over the reproductive phase of the colony cycle and in Experiment 2 they were sampled over the colony cycle as a whole.

For Experiment 1, we obtained 58 colonies (each containing a single queen with workers and brood) of *Bombus terrestris terrestris* from a commercial supplier (Syngenta Bioline Bees B.V., Weert, The Netherlands) in two cohorts (Cohort 1: 48 colonies obtained on 22 January 2010, mean ± s.d. number of workers = 24 ± 5; Cohort 2: 10 colonies obtained on 11 March 2010, mean ± s.d. number of workers = 46 ± 13). Colonies were transferred to wooden nest-boxes, fed *ad libitum* with pollen and artificial nectar and kept in standard conditions (28°C, 60% relative humidity, constant darkness) until the experiment was terminated at the end of the colony cycle (defined as occurring when there were no mature larvae or pupae and fewer than ten adult workers present). In daily monitoring, all newly-eclosing individuals were counted and sexual offspring (males and gynes or young queens) were removed.

Colonies in Cohort 1 were randomly assigned to one of three treatment groups (Groups 1-3). Where necessary, sample sizes were subsequently maintained by adding colonies from Cohort 2 to the groups. The treatment involved the removal of the colony queen from colonies at increasing intervals (approximately 20, 35 and 50 days in Groups 1, 2 and 3,

respectively) following the estimated date of the switch point, i.e. the date when the colony queen switches from laying diploid to laying haploid (male) eggs. (Hence the switch point marks the start of the reproductive phase of the colony cycle (Duchateau and Velthuis, 1988; Holland et al., 2013).) Sample sizes (both cohorts pooled) for treatment groups were 17, 8 and 9 colonies in Group 1, Group 2 and Group 3, respectively. However, because some queen tissues did not yield sufficient RNA for genetic analysis, final sample sizes for tissues were lower than the final number of queens obtained (brains: N = 16, 8 and 8 samples in Groups 1, 2 and 3, respectively; ovaries: N = 10, 7, 3 samples in Groups 1, 2 and 3, respectively). The actual date of the switch point for each colony was back-calculated at the end of the experiment as the date of first male eclosion minus males' egg-to-adult developmental time of 26 days (Lopez-Vaamonde et al., 2003). The mean (± s.d.) actual numbers of days after the switch point on which queens in the treatment groups were removed were 20 ± 6 days, 33 ± 3 days and 54 ± 6 days for Groups 1, 2 and 3, respectively. In sum, therefore, the treatment yielded a sample of colony queens of differing ages relative to their switch points.

For Experiment 2, we obtained 150 mated and hibernated foundress *B. terrestris terrestris* queens, with brood but no workers, from the same commercial supplier on 18 November 2010. Queens were initially housed in plastic boxes lined with paper, fed *ad libitum* with pollen and artificial nectar, and kept in standard conditions. On arrival, queens were randomly assigned to either a 'social' or 'asocial' treatment (see 2.1.2). In both treatments, when the first worker eclosed, the colony was transferred into a larger wooden box identical to those used in Experiment 1. On transfer, each queen was randomly assigned to one of four removal treatments; these consisted of queen removal from the colony 20, 40, 60 or 80 days after the eclosion of the first worker, respectively. The numbers of queens that survived to successfully rear their first worker and were therefore assigned to the treatment groups were,

for social queens, 5, 5, 5 and 12 queens in the 20, 40, 60 and 80 day removal groups, respectively, and, for asocial queens, 5, 5, 6 and 11 queens in the 20, 40, 60 and 80 day removal groups, respectively. However, because several queens died before their assigned collection day, final queen sample sizes were as in Table S1A. For the same reason, the single queen surviving at 70 days in each treatment was sampled at 70 days instead of 80 days, to ensure against the queen dying before the planned sampling date (Table S1A).

Demographic data were collected from each colony in Experiment 1. Within colonies, queens were assumed to have produced all males eclosing up to 26 days following the queen's removal. This was justified because workers produce only a small percentage (c. 2%) of males in queenright colonies, i.e. colonies with the queen present (Lopez-Vaamonde et al., 2004; Zanette et al., 2012). However, because queen removal might have truncated total male production by queens, for the present study male production by queens was quantified as the daily rate of eclosion of adult males in the 7 days preceding queen removal. 'Female production' was defined as the number of workers plus the number of gynes eclosing between the assignment of colonies to treatments and the termination of the experiment. Because all queens were removed following their switch points, queen removal would not have truncated queens' female production.

2.1.2. Queen gene expression as a function of social environment

In Experiment 2, to test the effects of the social environment on the expression of aging-related genes in queens, queens were assigned to social or asocial treatments before assignment to removal groups. In the social treatment (control), workers were removed from the colony within 24 h of eclosion and then immediately returned. Hence, in the social treatment, workers were retained within colonies and colonies were allowed to develop in the

normal way. In the asocial treatment, all workers were removed within 24 h of eclosion and not returned. Hence, in the asocial treatment, queens were not allowed to head normally developing colonies but remained in a permanently solitary state (Holland et al., 2013). In both treatments, queens were then removed at sequential intervals (Table S1A) as described above (see 2.1.1).

2.1.3. Worker gene expression as a function of age

In the social treatment in Experiment 2, the newly-eclosed workers that were removed from the colonies were marked using individually numbered plastic discs before return. These known-age, marked workers were then sampled from their colonies at sequential intervals, namely at 10, 20, 30 and 45 days after their individual dates of eclosion (mean ± s.d. = 2.8 ± 1.9 workers sampled per colony from 10 colonies). Workers were sampled only from colonies that were queenright and had >5 workers present. Sampled workers were predominantly those eclosing early in the colony cycle (median [range] of sampled workers = 7th [1st – 27th] to eclose). This procedure therefore yielded samples of workers of known absolute adult age. Sample sizes of removed workers were 11, 10, 7 and 1 workers in the 10, 20, 30 or 45 day removal groups, respectively, but, because some samples did not yield sufficient RNA, final worker sample sizes were as given in Table S1B.

2.1.4. Worker gene expression as a function of reproductive status

To allow us to investigate how workers' reproductive status affected the expression of aging-related genes, the ovarian activity of workers removed from the colonies in the social treatment in Experiment 2 (see 2.1.3) was assessed during ovarian dissections (see 2.2.1). Following the criteria of Duchateau and Velthuis (1989), workers with an ovariole in which either the oocyte was clearly larger than the trophocyte follicle or a developed egg was

present were categorized as having active ovaries; otherwise workers were classified as having inactive ovaries.

2.2. Molecular methods

2.2.1. Tissue dissection and sample preparation

In both Experiments 1 and 2, following removal, all queens and workers were chilled at -20°C for 5 min and then dissected on ice. In Experiment 1, queens' heads and abdomens were removed and in Experiment 2, queens' heads and workers' heads and abdomens were removed. Tissue samples were preserved in RNAlater or AllProtect reagents (Qiagen Ltd, Manchester, UK). Each sample was kept at 4°C for 24 h to allow the protectant to permeate, then stored at -80°C. Prior to RNA extraction, samples were thawed and removed from protectant solution, then brains, ovaries or fat bodies were dissected from samples under a dissection microscope, for the removal of non-target tissue. In dissections of brains, care was taken to dissect out brain tissue alone, as previous studies have suggested that *vg* may be expressed not only in brain but also in the adjacent hypopharyngeal glands (Corona et al., 2007; Toth et al., 2007, 2010). Hence it is unlikely that contamination by surrounding tissue affected our measurement of *vg* gene expression in brain. Dissected tissues were stored at -80°C until RNA extraction.

2.2.2. RNA extraction and cDNA synthesis

RNA was extracted from each tissue sample individually by grinding with TRI reagent (Applied Biosystems, Paisley, UK) and ceramic beads, followed by column-based purification (RNeasy kit, Qiagen) as described by Lockett et al. (2010). The RNA yield for each sample was determined by spectrophotometry (NanoDrop). The quality of each RNA extract was confirmed using agarose gel electrophoresis, as other methods are ineffective in

insect RNA (Greenberg, 1969). Tissues from which RNA was extracted were, in Experiment 1, queen brain and queen ovary, and, in Experiment 2, queen brain, worker brain and worker fat body. After quality checks, cDNA was synthesized from each sample individually, using 500 or 1000 ng RNA per synthesis and a poly(T) primer as described by Lockett et al. (2010)

2.2.3. Gene selection and qRT-PCR assay design

Candidate genes were selected based on a putative function associated with aging (see Introduction) and suitable genetic structure (at least one intron) for the design of TaqMan assays (Applied Biosystems). *Arginine Kinase* (*ArgK*) was selected as the reference gene as its expression is known to be stable with age in *B. terrestris* (Hornáková et al., 2010).

B. terrestris gene sequences were assembled and structures were determined based on sequences in *A. mellifera* (Honeybee Genome Sequencing Consortium, 2006) and *B. terrestris* (Munoz-Torres et al., 2011; Sadd et al., 2015). TaqMan assays were designed manually according to the manufacturer's recommendations and to span exon boundaries (Table S2). TaqMan assays were validated experimentally to confirm that they amplified cDNA and not genomic DNA.

2.2.4. Quantitative real-time PCR (qRT-PCR)

Each sample was analyzed individually. TaqMan qRT-PCRs were performed in duplicate (technical replication) in 10 µL volumes with Brilliant III Ultra-Fast qRT-PCR master mix (Agilent, Stockport, UK) on a RotorGene cycler (Qiagen). qRT-PCR cycling comprised 3 min at 95°C, then 40 cycles of 5 s at 95°C and 15 s at 60°C. Cycling was performed blindly with respect to treatment group and tissue by qStandard (University College London, UK).

A calibrator cDNA sample was constructed to allow inter-plate comparisons and act as a reference sample from which gene expression differences could be calculated. The brains of 10 additional *B. terrestris* queens were dissected and their RNA was extracted and used to synthesize cDNA following the methods described above. The calibrator sample was used to construct standard curves to calculate reaction efficiency (Pfaffl, 2001) for each gene in each qRT-PCR run. Gene expression was quantified as an expression ratio, i.e. expression of the target gene relative to expression of the reference gene (*ArgK*), both expression levels being relative to the calibrator sample, using equation 1 in Pfaffl (2001). Data files for the qRT-PCR data are available on figshare (doi:10.6084/m9.figshare.1594826).

2.3. Statistical methods

All gene expression data were analyzed using R v. 2.14.1 (R Development Core Team, 2011), with generalized linear mixed models (GLMMs) using package lme4 (function 'lmer', (Bates et al., 2012)). GLMMs were fitted with Gaussian errors and identity link function. For all models, we initially fitted a model that included all specified fixed terms and their interactions, and then sequentially removed non-significant terms (assessed using likelihood ratio tests, $\alpha = 0.05$) to generate a final model containing only significant terms (Crawley, 2005). Although queen and worker removals took place at discrete time steps in both experiments, both queen and worker age were treated as continuous variables in all analyses.

For Experiment 1 (queen data from brain and ovary), $\log_2$ expression ratio was fitted as the dependent variable, and queen relative age (days between switch point and queen removal), female production (total number of queen-produced female offspring, i.e. workers and gynes) and male production (queen's mean daily rate of production of male offspring in the 7 days preceding queen removal) were fitted as fixed effects. Two-way interactions between queen

age and each measure of queen offspring production were also fitted as fixed effects. Sample identity (to control for technical replication of expression ratios) nested within cohort was fitted as a random effect.

For Experiment 2 (queen data from brain), $\log_2$ expression ratio was fitted as the dependent variable, with queen relative age (days between first worker eclosion and queen removal), social environment (social or asocial) and their interaction fitted as fixed effects. Sample identity was fitted as a random effect.

Finally, for Experiment 2 (worker data from brain and fat body), $\log_2$ expression ratio was fitted as the dependent variable, with worker age (days between worker's eclosion and removal), reproductive status and their interaction fitted as fixed effects. Sample identity was fitted as a random effect.

3. Results

3.1. Queen gene expression as a function of relative age

There was a significant age-related change in gene expression in *COQ7* in ovary of queens, with *COQ7* expression increasing significantly with queens' relative age in Experiment 1 (LR = 5.28, d.f. = 1, $P = 0.022$; Fig. 1; Table S3), but not in brain in either Experiment 1 or 2 (Tables S3, S4). For *Dnmt3*, *for* and *vg*, there were no significant age-related changes in gene expression in queens in either brain or ovary in Experiment 1 or brain in Experiment 2 (Figs S1, S2; Tables S3, S4).

In Experiment 1, for *COQ7* in brain, queens showed significantly higher expression with decreasing male production (LR = 4.40, d.f. = 1, $P = 0.036$; Table S3). This association was

absent for *COQ7* expression in ovary and there was no significant relationship between gene expression and male production for *Dnmt3* and *for* in either brain or ovary or for *vg* in ovary (Table S3).

In Experiment 1, there was a significant interaction between queen age and female production for all four genes in brain (LR = 4.37–6.40, d.f. = 1, P = 0.011–0.036; Table S3). Queens showed a relationship between expression levels of all four genes and female production, with this relationship becoming increasingly positive as queen age increased (Fig. 2). This interaction was not present in ovary (Table S3). There were no significant interactions between queen age and male production in either brain or ovary, except for *vg* in brain, in which queens showed an interaction between queen age and male production similar to that between queen age and female production (LR = 12.6, d.f. = 1, $P < 0.001$; Table S3).

3.2. Queen gene expression as a function of social environment

There was a significant effect of the social environment on expression of *for* in brain in Experiment 2, with asocial queens showing significantly higher *for* expression than social queens (LR = 8.44, d.f. = 1, P = 0.004; Fig. 3; Table S4). Social environment had no significant effect on gene expression in brain in any of the other genes, and there was no significant interaction between queen age and social environment in *for* or in any of the other genes examined (Fig. S2; Table S4), although there was a general pattern of a decline in *for* expression with age in asocial but not social queens (Fig. S2). In addition, *vg* expression was elevated and highly variable in 20-day queens in the asocial treatment (Fig. S2), the high variation perhaps contributing to a lack of a statistically significant difference in *vg* levels between social and asocial queens.

3.3. Worker gene expression as a function of age

There was a significant age-related change in expression of *Dnmt3* in fat body of workers, with *Dnmt3* expression increasing significantly with workers' age (LR = 9.41, d.f. = 1, P = 0.002; Fig. 4; Table S5). This change was absent in brain (Fig. S3; Table S5). There were no significant age-related changes in gene expression for workers in any other gene in either brain or fat body (Figs S3, S4; Table S5). Although in these cases (excepting *vg* in brain), gene expression tended to be lowest in workers aged 20 days (Figs S3, S4), post-hoc pairwise Tukey contrasts (with worker age treated as a factor) also found that differences between worker age classes were not significant (e.g. brain; all $P > 0.3$).

3.4. Worker gene expression as a function of reproductive status

There was a significant effect of workers' reproductive status on gene expression for *vg* in fat body, with ovary-active workers showing significantly higher *vg* expression than ovary-inactive workers (LR = 4.24, d.f. = 1, P = 0.039; Fig. 5A; Table S5). For *vg* in brain, there was a significant interaction between worker age and reproductive status (Table S5), with ovary-active workers showing high expression initially and decreasing expression with age, relative to ovary-inactive workers that showed low expression initially and stable or only slightly increasing expression with age (LR = 5.81, d.f. = 1, P = 0.016; Fig. 5B).

There were no significant effects of workers' reproductive status on gene expression, or significant interactions between worker age and reproductive status, in any other gene in either brain or fat body (Figs S3, S4; Table S5).

4. Discussion

We investigated expression changes as a function of age, social environment and reproductive status in four candidate genes (*COQ7*, *Dnmt3*, *for* and *vg*), each associated with taxonomically widespread age-related pathways, in queens and workers of the bumble bee *Bombus terrestris*. We found expression patterns both confirming and challenging those previously described for these genes in eusocial Hymenoptera, as well as some completely novel expression patterns, as we now discuss.

4.1. Queen gene expression as a function of relative age

We found that *COQ7* expression increased significantly with relative age in queen ovary (Experiment 1), but not in brain in either Experiment 1 or 2. The result for *COQ7* contrasts with patterns in *A. mellifera* queens, in which *COQ7* expression decreased with age in brain, thorax and abdomen (Table 1). Although *A. mellifera* was studied over a different timeframe (Corona et al., 2005), this suggests that *COQ7* does not vary with aging in the same way across the two species. *B. terrestris* differs from *A. mellifera* in many aspects of its social biology, but particularly in having an annual colony cycle (Goulson, 2010), in which, unlike the case in the perennial *A. mellifera*, the end of queen life is essentially predetermined and hence predictable. This difference conceivably accounts for the contrasting findings with respect to changes in *COQ7* expression levels, and perhaps points to increases in metabolic rate and/or stress in the reproductive system of older *B. terrestris* queens as they get closer to their predetermined end of life. The difference could also be explained if the downregulation of *COQ7*, with subsequent reduction in reactive oxygen production, is an adaptation for the greater queen lifespan of *A. mellifera* compared to *B. terrestris.*

Expression did not change with relative age of queens in *Dnmt3*, *for* or *vg* in either brain or ovary. For *Dnmt3*, changes with age in queens have not previously been investigated in

eusocial Hymenoptera (Table 1). Our results suggest that, although the regulation of DNA methylation by *Dnmt3* is associated with female development as a queen in *A. mellifera* (Kucharski et al., 2008), this does not necessarily imply changes with age in the adult *B. terrestris* queen. Expression of *for* has previously been found to decrease with age in brain of *B. terrestris* queens, at least when deprived of workers (Woodard et al., 2013). We found no corresponding age-related decrease in *for* expression in Experiments 1 and 2 and no interaction of queen age and social environment affecting *for* expression in Experiment 2. In the study of Woodard et al. (2013), the comparison made was between queens before and 2 days after the eclosion of their first workers ('early-' and 'late-stage' queens, respectively), whereas the youngest queens in our experiment were at least 20 days post-first worker eclosion (see Materials and methods). This difference in the relative age of queens conceivably accounts for the different results of our study and that of Woodard et al. (2013). Expression of *vg* has previously been found to increase with age in the head of queens in *A. mellifera* (Corona et al., 2007) and *B. terrestris* (Amsalem et al., 2014). As regards *A. mellifera*, it needs noting that our comparisons concerned relative age of queens, with age being measured relative to events in the colony cycle (as *B. terrestris* queens in our study had an unknown period in diapause and as foundresses). Studies of *A. mellifera* have involved chronological age of queens, given *A. mellifera* queens lack diapause and a solitary foundress stage. This factor may account for some of the differences observed between *A. mellifera* and *B. terrestris* queens. As regards the study of *B. terrestris* by Amsalem et al. (2014), the contrast might again stem from our having investigated differences between queens of older relative age, since Amsalem et al. (2014) compared colony queens with much younger virgin queens.

A novel set of findings concerned the association of gene expression in brain of queens with the production of adult offspring (male or female). This was manifested as a negative association of *COQ7* expression with male production and, most strikingly, with a consistent pattern whereby *COQ7*, *Dnmt3*, *for* and *vg* all showed a positive association of expression with female production (for *vg*, also with male production) at greater queen ages. It is unclear why these relationships should exist, and particularly the increasingly positive relationship of gene expression and female production with age. Previous studies have not reported similar associations, since most did not investigate productivity correlates of gene expression. The generality of the effect suggests that queens may vary in overall quality and that this affects both (a) their expression levels of genes within genetic pathways related to aging and reproduction and (b) their productivity. Such a phenomenon may be linked with the reversed (positive) association of fecundity and longevity found in eusocial insect queens (Lopez-Vaamonde et al., 2009; Heinze et al., 2013; Von Wyschetzki et al., 2015), as it suggests that greater longevity of more productive (and hence fecund) queens is linked to greater expression levels of the candidate genes. For *Dnmt3*, the results suggest that DNA methylation is increasingly altered in the brain of queens as the production of new queens proceeds (since colonies produce first workers then new queens). This suggests a link with pheromonal changes that are believed to occur in *B. terrestris* queens at the onset of new queen production (Cnaani et al., 2000; Alaux et al., 2006; Lopez-Vaamonde et al., 2007).

4.2. Queen gene expression as a function of social environment

As predicted, queens reared in an asocial environment (lacking any contact with adult workers) exhibited higher expression of *for* in brain than control (social) queens allowed to head colonies of workers in the usual way. This suggests that the behavioral change from external foraging to non-foraging shown by foundress *B. terrestris* queens in nature when

their first worker eclose (Goulson, 2010) is associated with downregulation of the *for* gene. As queens in the present experiment were not able to leave the nest-boxes, our results further suggest that it is the presence of adult workers that provides queens with the cue to cease foraging. The large variation in *vg* expression of 20-day old asocial queens also suggests that some queens may have responded to the absence of workers by simultaneously increasing *vg* expression and their egg-laying rate, although this possibility remains to be tested. Woodard et al. (2013) compared *B. terrestris* early- and late-stage queens with and without workers and found no effect of worker presence or absence on *for* expression. A possible reason for this difference with our study is that, in the study of Woodard et al. (2013), the early-stage queens were all assayed for gene expression after 7-12 days of treatment (by adding or not adding workers), whereas queens in our experiment were assayed after 20-70 days (see Materials and Methods), perhaps leading to greater expression differences. Since colony foundation in *B. terrestris* can take many weeks (AFGB, personal observations), a long timeframe for the present experiment appears reasonable. Moreover, the late-stage queens in the study of Woodard et al. (2013) had all already experienced first worker eclosion for 2 days before worker removal, which may have led to the lack of observed difference in *for* expression in these queens. Overall, our results suggest that, in eusocial Hymenoptera, the role of *for* in regulating foraging in workers may have been based on a pre-existing role of *for* in regulating foraging in foundress queens.

Our finding that there were no expression changes in *COQ7*, *Dnmt3* and *vg* with social environment in brain of *B. terrestris* queens is consistent with a previous result showing that *B. terrestris* queens do not alter the timing of their switch point (laying of first male egg) as a function of the social environment (Holland et al., 2013), which suggests that at least some events in the colony cycle are endogenously timed in queens and not cued on changes in the

social environment. The lack of a significant association between *Dnmt3* expression and social environment does not necessarily conflict with the known correlation between specific DNA methylation marks and social environment in *A. mellifera* workers (Lockett et al., 2012), since localized methylation changes in the genome may occur without large-scale changes in *Dnmt3* expression and levels of DNA methyltransferase.

4.3. Worker gene expression as a function of age

We found that *Dnmt3* expression increased with age in fat body of workers, but not in brain. As *Dnmt3* expression has not previously been investigated in *B. terrestris*, this is a novel finding. Amarasinghe et al. (2014) found methylation differences in heads of ovary-active and ovary-inactive *B. terrestris* workers. Vitellogenin is synthesized in fat body (Bloch and Grozinger, 2011) and plays a role in regulating reproductive status in *B. terrestris* workers (see below). Combining these findings suggests a possible link between age, methylation and reproductive status in *B. terrestris* workers, especially as in unmanipulated colonies worker ovary activation tends to correlate positively with worker age (Duchateau and Velthuis, 1989).

In contrast to *Dnmt3* expression, *COQ7*, *for* and *vg* expression did not change with age in either brain or fat body of workers. For *COQ7*, this finding is consistent with lack of change in expression of this gene with age in *A. mellifera* workers (Corona et al., 2005). Our finding in *for* contrasts with that of Tobback et al. (2011), who found a decrease in expression with age in heads of *B. terrestris* workers. For *vg*, although age did not have a main effect on expression, there was a significant interaction of age and reproductive status, which is discussed below.

The results also show that queens and workers did not overlap in genes showing expression changes with age or relative age, as only *COQ7* increased expression with relative age in queens (in ovary but not brain; Fig. 1), whereas only *Dnmt3* increased expression with age in workers (in fat body but not brain; Fig. 4).

4.4. Worker gene expression as a function of reproductive status

Of the four genes investigated, only *vg* showed expression differences correlated with reproductive status in workers, being overexpressed in fat body of ovary-active compared with ovary-inactive workers (independently of age). This is consistent with findings of Amsalem et al. (2014) for *vg* expression in heads of *B. terrestris* workers. More broadly, it is consistent with the ancestral role of vitellogenin in regulating egg production in insects and with the fat body being the site of vitellogenin synthesis (Bloch and Grozinger, 2011). This result may therefore reflect the more primitive eusociality of *B. terrestris*, from which one would not necessarily expect *B. terrestris* to exhibit the co-option of the vitellogenin pathway to non-reproductive uses found in *A. mellifera* workers (Amdam et al., 2004; Nelson et al., 2007), or not as completely (Amsalem et al., 2014).

For *vg*, we also found an interaction in brain of workers between age and reproductive status, such that ovary-active workers showed higher expression of *vg* than ovary-inactive workers when workers were young, with *vg* expression then decreasing with age in ovary-active workers. Amsalem et al. (2014) also investigated *vg* expression in *B. terrestris* workers and found that social interactions (aggression) predicted *vg* level more strongly than reproductive status. Our results complement those of these authors, since, although we did not measure worker aggression, we isolated the effects of age and reproductive status (Fig. 5B). The observed decline in *vg* expression with age in ovary-active *B. terrestris* workers might seem

surprising given an association of age and ovarian activation in unmanipulated workers (Duchateau and Velthuis, 1989). This suggests that vitellogenin could be involved more in the establishment of ovary-active status in workers than in its maintenance. This age-related decline also has possible relevance to the hyperfunction theory of aging (Blagosklonny, 2012). In *C. elegans*, this theory has been used to explain aging in older individuals as a maladaptive overshooting of *vg* expression (DePina et al., 2011; Gems and Partridge, 2013). In eusocial Hymenoptera, in cases in which *vg* downregulation mediates the transition in workers from nurses to foragers as they grow older, an overshoot in downregulation might occur; analogously with the *C. elegans* case, this might lead to aging, the difference being that this would stem from excessive downregulation (not upregulation) of *vg*.

As neither *COQ7* nor *for* expression are known to be correlated with workers' reproductive status in eusocial Hymenoptera (Table 1), the absence of such a correlation in our data matches previous understanding. The lack of correlation of *Dnmt3* with workers' reproductive status is perhaps surprising, given the discovery of methylation differences between ovary-active and ovary-inactive workers in *B. terrestris* (Amarasinghe et al., 2014). However, as discussed in the preceding section and earlier in this section, the *vg* pathway, methylation, age and reproductive status might be linked in *B. terrestris* workers in complex ways that remain to be completely unpicked.

4.5. Conclusions

Our findings reveal that, within age-related genetic pathways, *B. terrestris* queens and workers exhibit both novel features and features that are conserved with respect to those of either non-social insects or the advanced eusocial honey bee. Novel features included an increasingly positive association of *COQ7*, *Dnmt3*, *for* and *vg* expression with female

production in brain of queens as queen age rose (Fig. 2), possibly linked to the positive association of fecundity and longevity found in eusocial insect queens. In addition, queens experimentally deprived of workers showed higher *for* expression (Fig. 3), as predicted if *for* is downregulated when foundress queens cease foraging on first worker eclosion. In workers, a novel feature was the increase in *Dnmt3* expression with age in fat body (Fig. 4), suggesting new links of methylation with aging. Conserved features included a lack of age-related expression change in *COQ7* in brain and fat body of workers, matching results from *A. mellifera*. Ovary-active *B. terrestris* workers exhibited higher *vg* expression than ovary-inactive ones in fat body (Fig. 5A), and in brain when younger (Fig. 5B), suggesting that vitellogenin's ancestral role of regulating egg production in insects is conserved in *B. terrestris*. This finding in a primitively eusocial bee is as expected if the co-option of the *vg* pathway in regulating the temporal division of labor among workers in *A. mellifera* is a derived feature of advanced eusociality. Overall, our findings demonstrate that *B. terrestris* provides a highly informative model for the elucidation of how taxonomically widespread age-related genetic pathways interact with aging, social environment and reproduction during eusocial evolution.

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Figure legends

Fig. 1. Effect of queen relative age on *COQ7* gene expression in ovary of *Bombus terrestris* queens. Queen relative age is days between switch point and queen removal. Data are from Experiment 1 (GLMM, LR = 5.28, d.f. = 1, $P = 0.022$; Table S3). $N = 20$ queens. Line is trend line of relationship predicted from the model (Table S3).

Fig. 2. Effect of interaction between queen relative age and female production on *COQ7* gene expression in brain of *Bombus terrestris* queens. Queen relative age (values in upper row) is days between switch point and queen removal. Female production is sum of workers and gynes or new queens produced. Data are from Experiment 1 (GLMM, LR = 6.40, d.f. = 1, $P = 0.011$; Table S3). $N = 16$, 8 and 8 queens in the three age-classes, respectively. Lines are trend lines of relationships predicted from the model (Table S3). The figure is illustrative, as there were similar significant interactions for *Dnmt3*, *for* and *vg* (Table S3).

Fig. 3. Effect of social environment (social v. asocial treatment) on *for* gene expression in brain of *Bombus terrestris* queens. Data are from Experiment 2 (GLMM, LR = 8.44, d.f. = 1, $P = 0.004$; Table S4). $N = 9$ queens (social treatment) and 10 queens (asocial treatment). Horizontal bar, median; box, quartiles; whiskers, range.

Fig. 4. Effect of worker age on *Dnmt3* gene expression in fat body of *Bombus terrestris* workers. Worker age is days between worker's eclosion and removal. Data are from Experiment 2 (GLMM, LR = 9.41, d.f. = 1, $P = 0.002$; Table S5). $N = 21$ workers. Line is trend line of relationship predicted from the model (Table S5).

Fig. 5. Relationship of worker's reproductive status and *vg* gene expression in *Bombus terrestris* workers. (A) Effect of worker's reproductive status (ovary active v. ovary inactive) on *vg* gene expression in fat body of *B. terrestris* workers (Experiment 2, GLMM, LR = 4.24, d.f. = 1, $P = 0.039$; Table S5). $N = 8$ workers (ovary-active) and 13 workers (ovary-inactive). Horizontal bar, median; box, quartiles; whiskers, range. (B) Effect of interaction between worker age (days between worker's eclosion and removal) and reproductive status (ovary active v. ovary inactive) on *vg* gene expression in brain of *B. terrestris* workers (Experiment 2, GLMM, LR = 5.81, d.f. = 1, $P = 0.016$; Table S5). $N = 2$, 4 and 3 ovary-active workers and 8, 5 and 5 ovary-inactive workers in the three age-classes, respectively (with the 30-d ovary-inactive sample including one 45-d old worker; Table S1B).

Table 1. Expression changes in the four candidate genes as a function of age, caste or reproductive status in previous studies of eusocial Hymenoptera

Species, phenotype	Tissue	Correlate	Relevant gene/protein expression change
COQ7 (*Coenzyme Q biosynthesis protein 7*)			
Apis mellifera, queen	Brain, thorax, abdomen	Adult age	Gene expression level in brain, thorax and abdomen decreases with age (Corona et al., 2005).
A. mellifera, worker	Brain, thorax, abdomen	Adult age	Gene expression level does not change with age in brain, thorax or abdomen (Corona et al., 2005).
A. mellifera, queen v. worker	Brain	Adult caste	At old age, gene expression level in brain is lower in queens than in workers (Corona et al., 2005).
Dnmt3 (*DNA methlytransferase 3*)			
A. cerana cerana worker	not specified	Adult age	Gene expression level is higher in 30-day-old workers than in 1- and 7-day-old workers (Liu et al., 2012).
A. cerana cerana, queen v. worker	not specified	Adult caste	Gene expression level is higher in 1-day-old queens than in 1-day-old workers (Liu et al., 2012).
A. cerana cerana, queen v. worker	not specified	Adult caste	Gene expression level does not differ between laying queens and laying workers (Liu et al., 2012).
A. cerana cerana, queen v. worker pupa	not specified	Pupal caste	Gene expression level is higher in queen pupae than in worker pupae (Liu et al., 2012); contrasts with findings of Kucharski et al. (2008).
A. mellifera, worker	Whole body	Reproductive status	Gene expression is higher in ovary-active workers than in ovary-inactive workers (Cardoen et al., 2011).
A. mellifera, female larva	Larval stage injection	n/a	RNAi knockdown of *Dnmt3* increases chance of queen development (Kucharski et al., 2008). Suggests there would be lower *Dnmt3* gene expression level in queen-

			destined compared to worker-destined larvae.
A. mellifera, female larva	Whole larva	n/a	With increasing duration of royal jelly feeding to female larvae, *Dnmt3* gene expression and DNMT3 activity decrease and chance of queen development increases (Shi et al., 2011). Again suggests there would be lower *Dnmt3* gene expression level in queen-destined compared to worker-destined larvae.
for (*foraging*)			
A. mellifera worker	Head	Foraging behavior	Gene expression is higher in forager workers than in nurse workers (Ben-Shahar et al., 2002).
A. mellifera worker	Head	Worker task	Gene expression is higher in the heads of undertaker worker than in workers performing other in-hive roles (Ben-Shahar et al., 2003).
Bombus terrestris, queen	Brain	Adult age	Gene expression is lower in late-stage queens without workers than in to early-stage queens without workers (Woodard et al., 2013).
B. terrestris, worker	Head	Foraging behavior	Gene expression is higher in forager workers than in nurse workers (Tobback et al., 2011).
B. terrestris, worker	Head	Adult age	Gene expression level decreases with age (Tobback et al., 2011).
B. terrestris, female	Brain	Adult caste and reproductive status	Gene expression level does not differ between queens, gynes, foundresses and workers (Woodard et al., 2014).
Pheidole pallidula, worker	Brain	Worker caste	Major workers have higher activity of PKG product of *for* than minor workers (Lucas and Sokolowski, 2009).
Pogonomyrmex barbatus, worker	Brain	Age and foraging behavior	Gene expression level is higher in callows (young adults) than in foragers (Ingram et al., 2005).
Polistes metricus, female	Brain	Adult caste, stage and foraging behavior	Gene expression level is higher in foundresses and workers (compared to queens and gynes), these being the actively foraging and provisioning females (Toth et al., 2007, 2010).
Vespula vulgaris,	Brain	Foraging behavior	Gene expression level is higher in nurse workers than in foraging workers (Tobback

worker			et al., 2008).
vg (*vitellogenin*)			
A. mellifera, queen	Head, thorax	Adult age	Gene expression level increases with age in head and thorax (Corona et al., 2007).
A. mellifera, queen	Abdomen	Adult age	*Vg* transcription drops at age 1 week then stays steady up to at least age 1 year (Corona et al., 2007).
A. mellifera, worker	Haemolymph, abdomen	Adult age, strain	Vitellogenin titre and *vg* expression are both higher in high-pollen hoarding strain than in low-pollen hoarding strain workers and both increase with worker age (Amdam et al., 2004).
A. mellifera, worker	Thorax, abdomen	Adult age	*Vg* transcription is low at ages 1 day and 1 month, and peaked at age 1 week, in thorax and abdomen (Corona et al., 2007).
A. mellifera, worker	Abdominal injection	Foraging behavior and longevity	RNAi knockdown of *vg* results in earlier foraging onset and shortened longevity (Nelson et al., 2007).
A. mellifera, worker	Head	Reproductive status	Gene expression level is the same or lower in ovary-active compared to ovary-inactive workers, depending on choice of reference gene (Grozinger et al., 2007).
A. mellifera, worker	Whole body	Reproductive status	Gene expression is higher in ovary-active workers than in ovary-inactive workers in one of two colonies (Cardoen et al., 2011).
B. terrestris, queen	Head	Adult stage	Gene expression level is higher in colony queens than in virgin queens (Amsalem et al., 2014).
B. terrestris, worker	Head	Adult age and reproductive status	Gene expression level is higher in ovary-active workers (10-day-old, queenless) than in ovary-inactive workers (4-day-old, queenright) (Amsalem et al., 2014).
B. terrestris, worker	Head	Foraging behavior	Gene expression level does not differ between nurse and forager workers (Amsalem et al., 2014).
B. terrestris, worker	Head, fat body	Aggressive behavior	In groups of queenless workers, gene expression level is higher in more aggressive workers in head and fat body (Amsalem et al., 2014).

B. terrestris, queen v. worker	Head	Adult caste	Gene expression level is higher in colony queens than in ovary-active workers (10-day-old, queenless)(Amsalem et al., 2014).
B. terrestris, female	Brain	Adult caste and reproductive status	In a comparison of queens, gynes, foundresses and workers, gene expression level is associated with reproduction but not provisioning (Woodard et al., 2014).
Cardiocondyla obscurior, queen	Whole body	Adult age	Gene expression level is higher in 4-week-old queens v. 18-week-old queens (Von Wyschetzki et al., 2015)
Lasius niger, queen v. worker	Whole body	Adult caste	Gene expression level is higher in queens than in workers (Gräff et al., 2007).
Polistes canadensis, queen v. worker	Whole body	Adult caste	Gene expression level is higher in queens compared to workers (Sumner et al., 2006).
P. metricus, female	Brain/head	Adult caste and reproductive status	Gene expression level is highest in queens, intermediate in gynes and lowest in foundresses and workers (Toth et al., 2007, 2010).
Solenopsis invicta, queen	Whole body	Reproductive status	Gene expression level is higher in dealate queens than alate queens (Tian et al., 2004).

Fig. 1

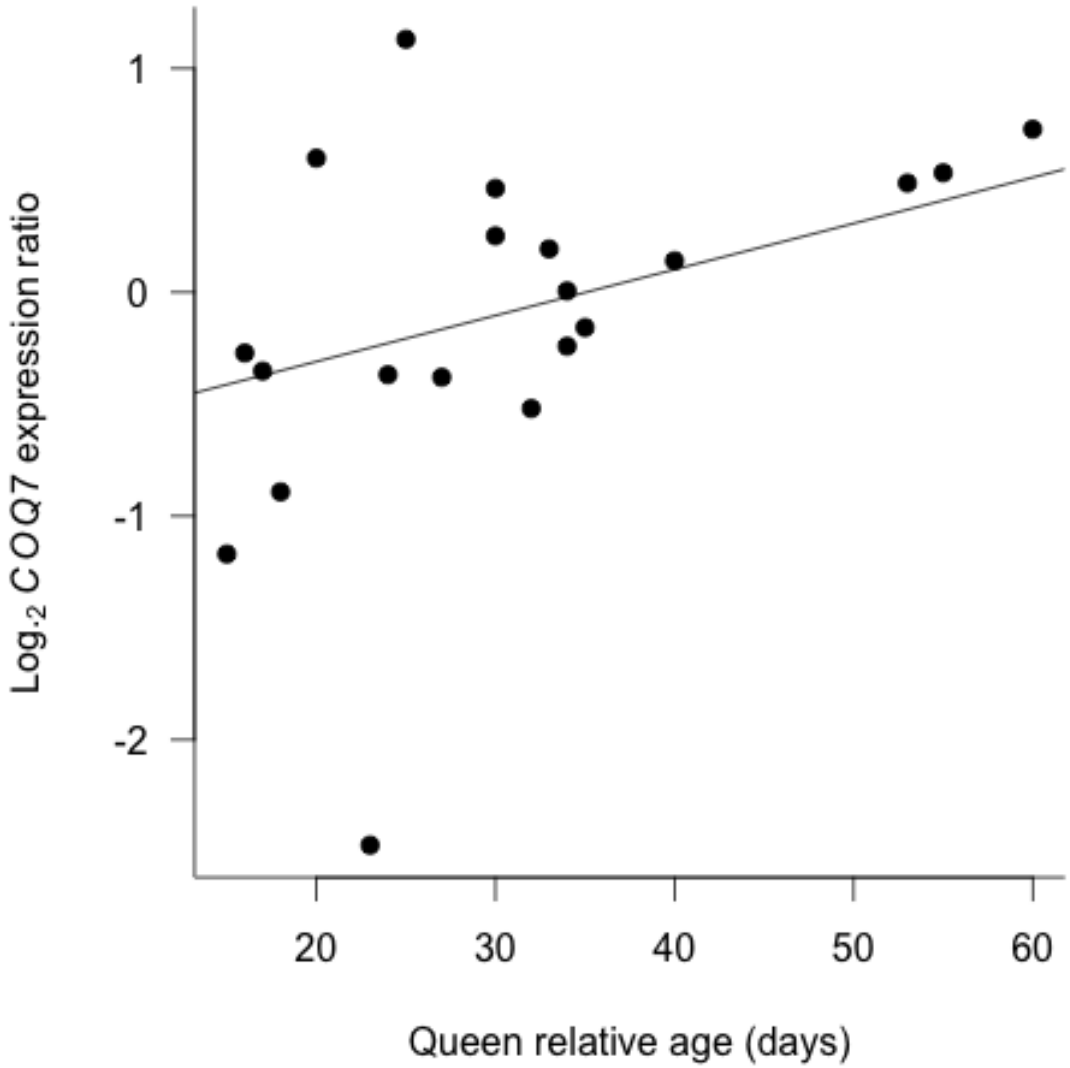


Fig. 2

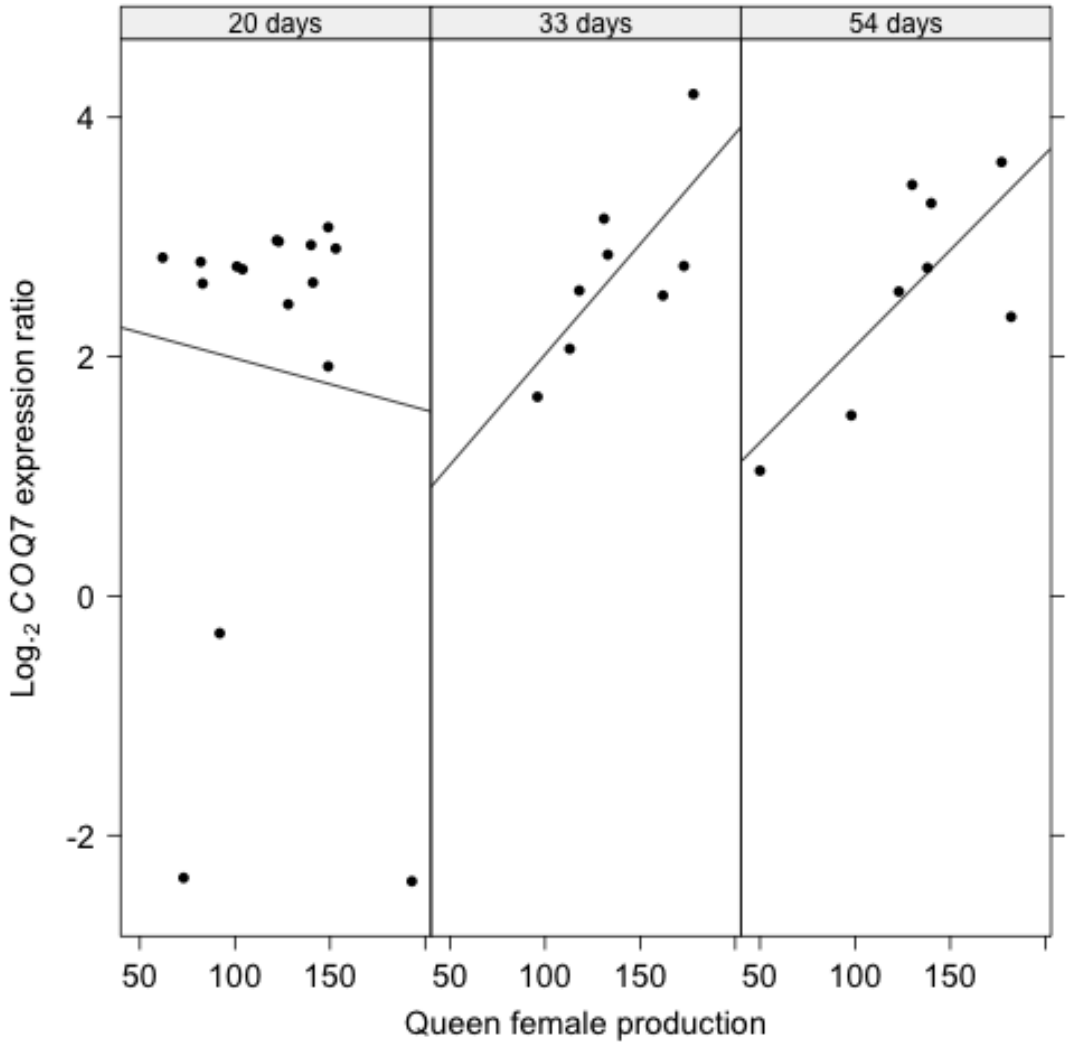


Fig. 3

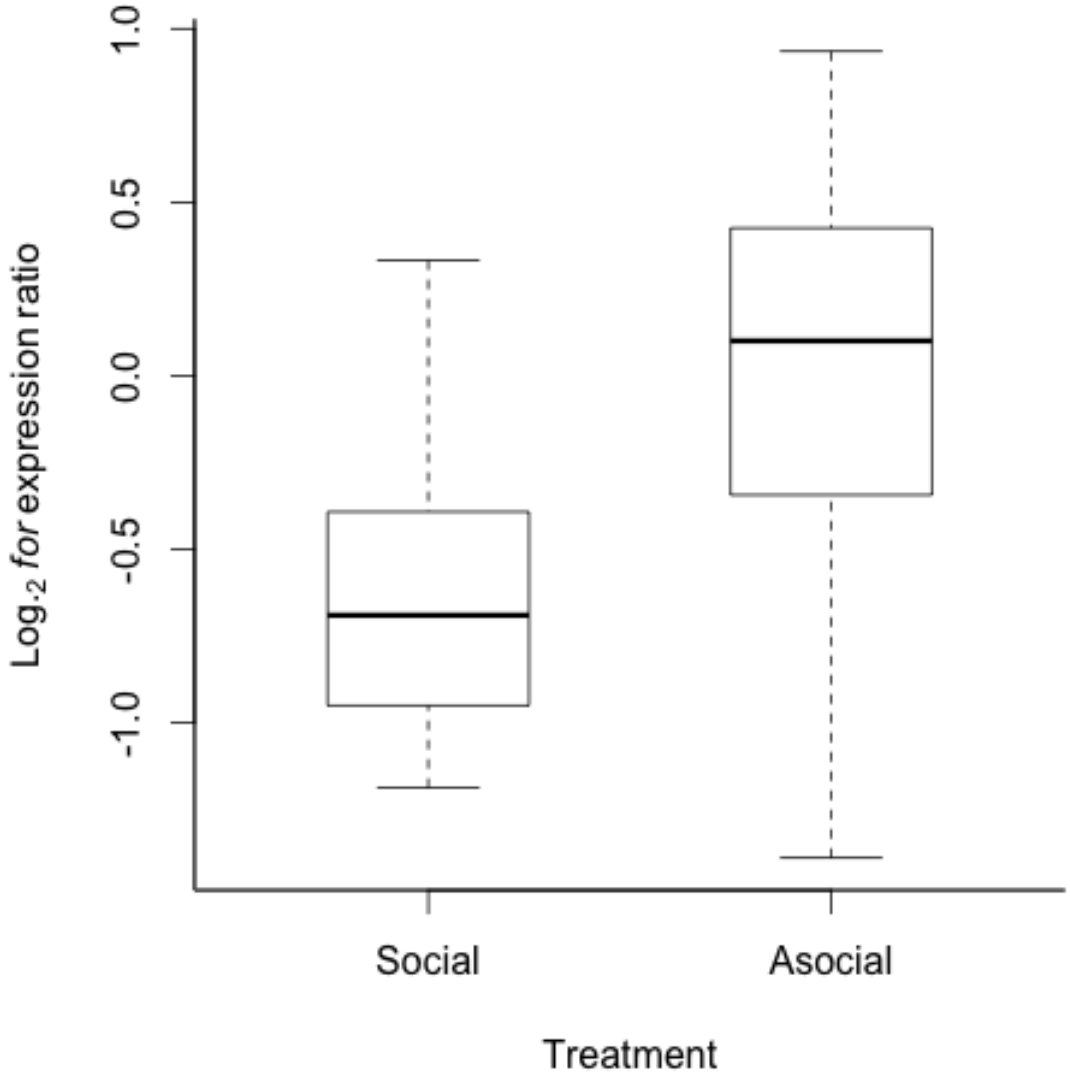


Fig. 4

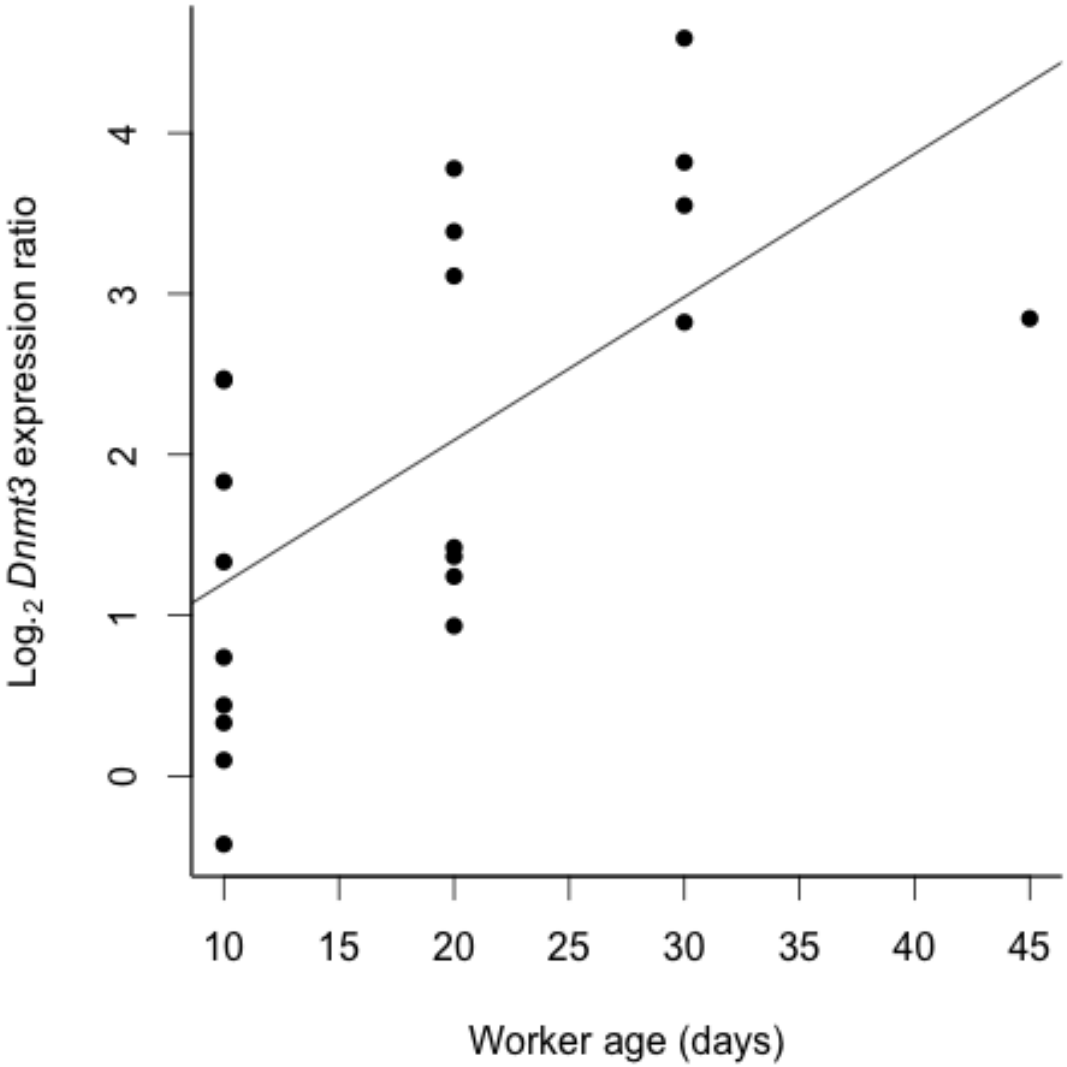
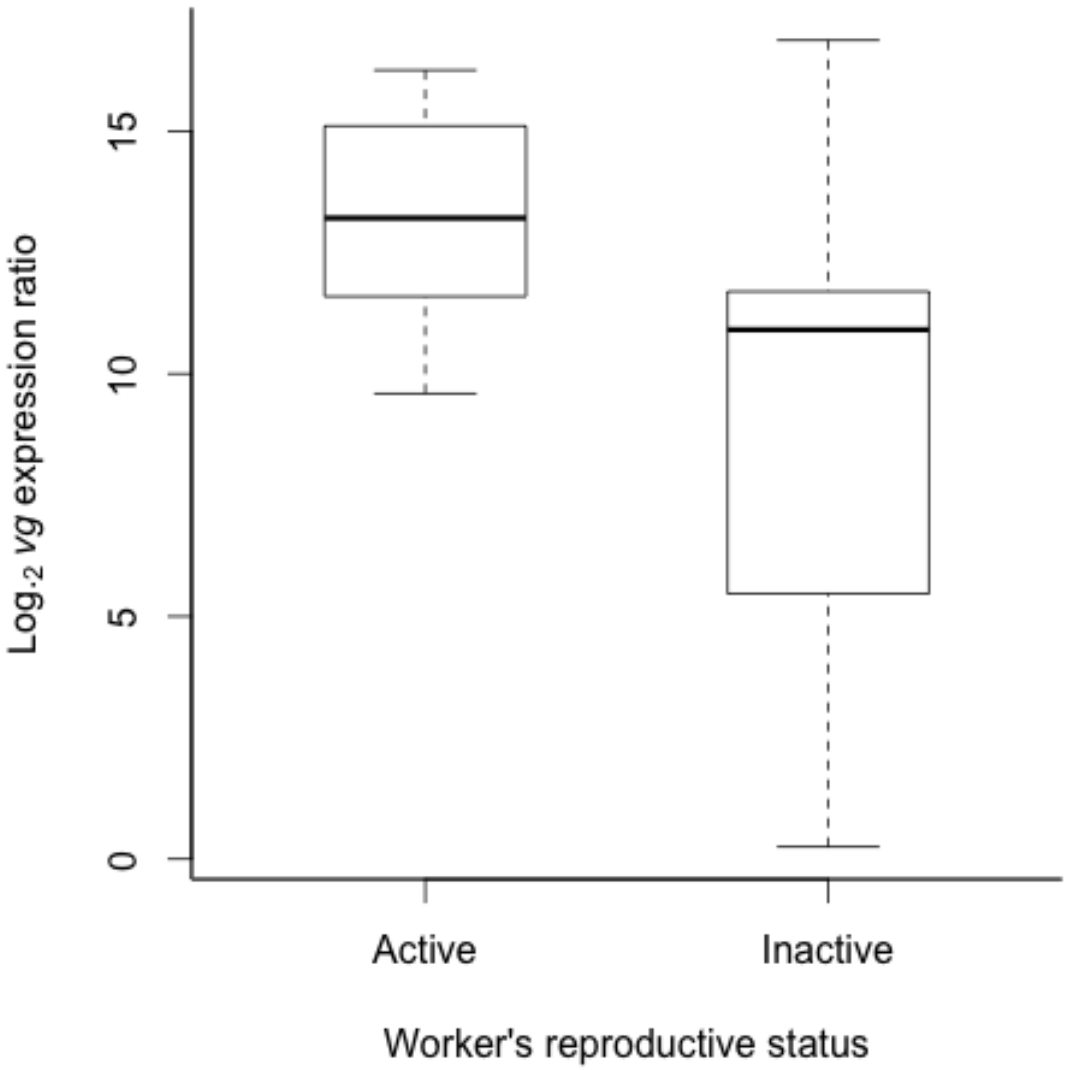


Fig. 5

A



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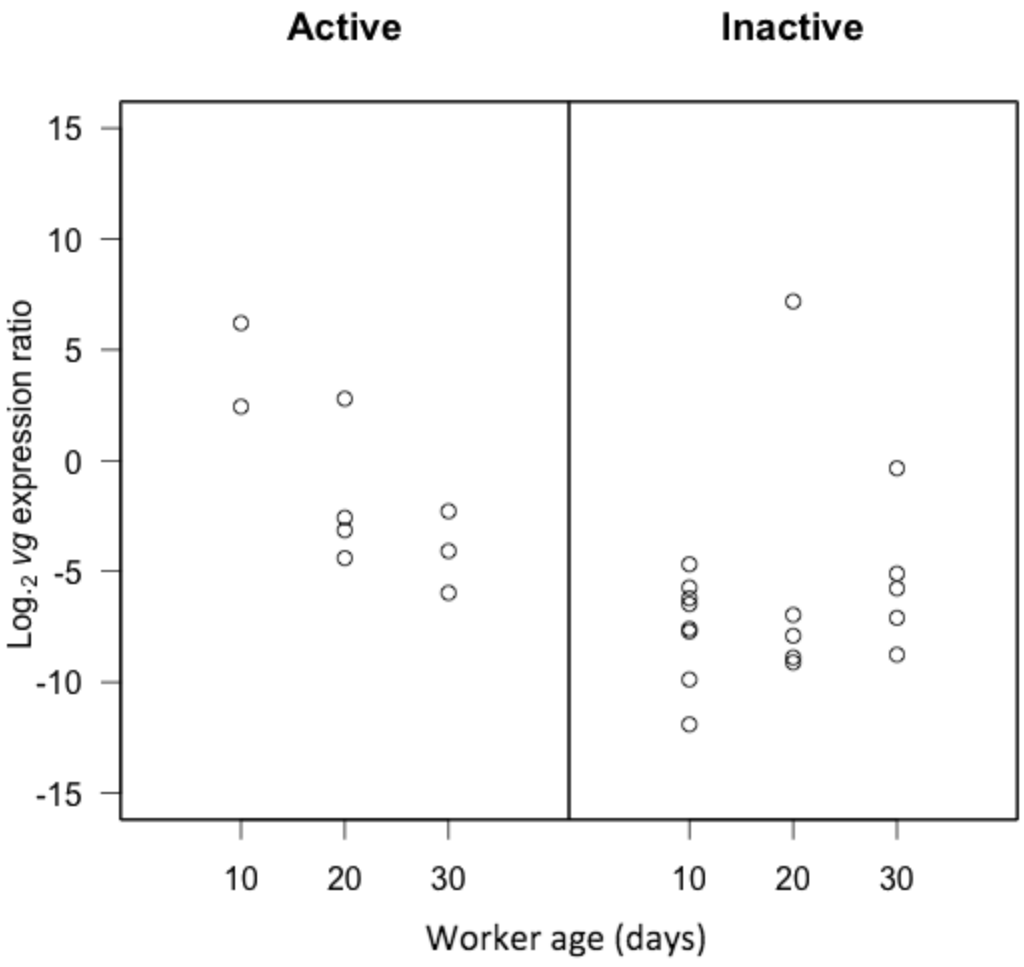

EXG-15-395: 'Gene expression differences in relation to age and social environment in queen and worker bumble bees', Lockett GA, Almond EJ, Huggins TJ, Parker JD, Bourke AFG

Highlights:

- We investigated expression levels of aging-related genes in a bumble bee.
- In queens, expression levels increased with female productivity.
- Queens without workers showed higher expression of the gene, *foraging*.
- In workers, *vitellogenin* expression covaried with age and reproductive status.
- Overall, we found novel and conserved features in age-related genetic pathways.