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RESEARCH ARTICLE

Early life exposure to queen mandibular pheromone mediates persistent transcriptional changes in the brain of honey bee foragers

Tianfei Peng[1,2,*,¶], Anissa Kennedy[1,‡,¶,], Yongqiang Wu[1], Susanne Foitzik[1] and Christoph Grüter[1,§]**

ABSTRACT

Behavioural regulation in insect societies remains a fundamental question in sociobiology. In hymenopteran societies, the queen plays a crucial role in regulating group behaviour by affecting individual behaviour and physiology through modulation of worker gene expression. Honey bee (*Apis mellifera*) queens signal their presence via queen mandibular pheromone (QMP). While QMP has been shown to influence behaviour and gene expression of young workers, we know little about how these changes translate in older workers. The effects of the queen pheromone could have prolonged molecular impacts on workers that depend on an early sensitive period. We demonstrate that removal of QMP impacts long-term gene expression in the brain and antennae in foragers that were treated early in life (1 day post emergence), but not when treated later in life. Genes important for division of labour, learning, chemosensory perception and ageing were among those differentially expressed in the antennae and brain tissues, suggesting that QMP influences diverse physiological and behavioural processes in workers. Surprisingly, removal of QMP did not have an impact on foraging behaviour. Overall, our study suggests a sensitive period early in the life of workers, where the presence or absence of a queen has potentially life-long effects on transcriptional activity.

KEY WORDS: Sensitive period, Maturation, Division of labour, Signalling

INTRODUCTION

The behaviours of adult animals are often complex and determined by both genetics (i.e. gene expression differences and inheritance) and experience. Specifically, early life experiences contribute to the shaping and plasticity of behaviours in adulthood such as learning during critical periods of development or the persistence of behavioural habits (Beach and Jaynes, 1954). In humans and other social animals, the social environment during early development strongly affects social behaviours later in life (Laviola and Terranova, 1998; Niles et al., 2008). For example, differences in exposure to sensory cues in early life can elicit long-lasting responses in adult molecular physiology and behaviour (Kelley and Magurran, 2003; Lonnstedt et al., 2012; Patten, 1977).

Immediate transcriptomic responses to the social environment have been reported in many social insects (Huisken and Rehan, 2023; Manfredini et al., 2022; Rittschof and Robinson, 2013). However, how experiences during development or early adulthood influence molecular physiology later in life is largely unexplored. Indeed, in many species there are sensitive periods in life when animals are particularly sensitive to external influences or stimuli (English and Barreaux, 2020; Frankenhuis and Walasek, 2020; Knudsen, 2004). In humans, challenging conditions *in utero* and early childhood shape adult coping strategies, behaviour and health (Almas et al., 2020; Duchesne et al., 2017; Schulz, 2010).

Social insects, such as ants, social bees and wasps, provide excellent research models to address the question of whether early life experiences cause consistent and stable changes in adult behaviour and physiology. In many species, young females (workers) focus on tasks inside the colony, such as feeding the brood, whereas older workers perform tasks outside their nest, such as foraging (Grüter, 2020; Lindauer, 1952; Robinson et al., 1992; Seeley, 1982; Shorter and Tibbetts, 2009; Siegel et al., 2013). The behavioural maturation from nursing to foraging is affected by genetic factors such as parent-specific expression of genes from mothers (matrigenes) and fathers (patrigenes) (Bresnahan et al., 2023; Galbraith et al., 2016; Kocher et al., 2015). External parameters also affect this behavioural transition, such as sensory input from social and environmental stimuli (Ament et al., 2010; Grozinger et al., 2003; Leoncini et al., 2004).

One of the main signals from the social environment comes from the queen. The queen influences worker behaviour and colony dynamics through pheromones, which attract workers (Slessor et al., 1988), suppress reproduction in workers (Nunes et al., 2017; Van Oystaeyen et al., 2014) and other queens (Holman et al., 2013; Vargo, 1992), and alter the learning capacity of workers (Vergoz et al., 2007). In honey bee colonies, the queen signal is a five-compound pheromone mixture called queen mandibular pheromone (QMP), which acts as both releaser and primer pheromone. As a releaser, it elicits short-term effects that initiate immediate behavioural responses such as the promotion of retinue behaviour, swarm clustering, and drone attraction during mating (Grozinger et al., 2007), while suppressing aversive learning in young workers (Vergoz et al., 2007). As a primer, it elicits long-term effects that cause physiological changes that ultimately result in a behavioural response such as the inhibition of queen rearing, the inhibition of worker reproductive development (Hoover et al., 2003; Traynor et al., 2014), lowering of the sucrose response threshold (Pankiw and Page, 2003) and the modulation of worker activities such as the transition from nursing to foraging (Grozinger et al., 2007;

[1]Institute of Molecular and Organismic Evolution, Johannes Gutenberg University of Mainz, Biozentrum I, Hanns Dieter Hüsch Weg 15, 55128 Mainz, Germany. [2]College of Plant Science, Jilin University, Changchun 130062, PR China. *Present address: Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. ‡Present address: Department of Entomology, The Pennsylvania State University, University Park, PA 16801, USA. §Present address: School of Biological Sciences, University of Bristol, Bristol BS8 1TQ, UK.
¶These authors contributed equally to this work

**Author for correspondence (anissakennedy04@gmail.com)

A.K., 0000-0002-2585-4366

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Le Conte and Hefetz, 2008). Ample experimental evidence demonstrates behavioural changes induced by QMP exposure. However, there is limited evidence for upstream physiological changes that may modulate the subsequent behavioural changes induced by QMP.

In this experimental study, we used the honey bee as a model to investigate whether early life experience can alter gene expression and social behaviours in the longer term. We caged workers and created an environment simulating a lapse in QMP exposure to mimic the temporal queen absence that colonies experience during swarming or queen replacement. We expected long-term effects of the lapse in QMP exposure on the behaviour and gene expression of different age cohorts, possibly depending on an early sensitive period to QMP. Such responses are usually most pronounced during early life exposure and can carry over to influence phenotypes such as behaviour, physiology and morphology through adult life. Current models examine how mechanisms of behavioural plasticity allow animals to optimally respond to experience across the lifespan (English et al., 2016; Frankenhuis and Panchanathan, 2011; Panchanathan and Frankenhuis, 2016). Nevertheless, we still require a better understanding of the neurophysiological and transcriptional basis of these social effects to account for the complexities of individual behavioural phenotypes. Here, we tested whether the QMP signal affects gene expression profiles in different brain parts and whether the observed changes in gene expression were associated with subsequent foraging behaviour in different age cohorts experiencing a lapse in QMP signal.

The effects of QMP have been found to be most pronounced in young workers, which exhibit an increased sensitivity to QMP (Vergoz et al., 2009). However, the persistence of transcriptional changes in response to QMP exposure in young workers remains unknown. It is also unclear whether other adult life stages show a similar magnitude of change in gene expression patterns in response to QMP. We treated different age cohorts and tested whether our QMP treatment affected gene expression at forager age. We focused on foragers for two reasons. First, foragers play a fundamental role for the nutritional health of a colony, but it remains unknown whether and how QMP affects the gene expression of forager-aged workers. Second, queen replacement leads to a gap in brood production, and requires forager-aged workers to rear the brood of the new queen. Therefore, we expected workers that have experienced queenlessness at a young age to show strong changes in gene expression when reaching foraging age. We focused on gene expression in the mushroom bodies, antennal lobes and antennae to capture the entire pathway of odour perception, from pheromone binding in the antennae, to processing in the antennal lobes, to learning and memory and multimodal sensory integration in the mushroom bodies (Ganeshina and Menzel, 2001; Lin and Strausfeld, 2012; Menzel, 2012).

MATERIALS AND METHODS

Colony set-up

Three *Apis mellifera* Linnaeus 1758 observation colonies were established from three standard-sized colonies prior to the start of experiments from August to October 2019. Each observation colony contained approximately 2000–3000 workers of mixed ages from the Johannes Gutenberg University apiary in Mainz, Germany. Observation colonies were each headed by a naturally mated unrelated queen and each had three frames, brood, pollen and honey reserves.

Sample collection

Observation colonies were studied independently. We started experiments by first collecting brood frames and allowing workers to emerge overnight in an incubator at 34°C. Newly emerged bees were marked with enamel paint and numbered tags to indicate emergence day and source colony, respectively. This allowed us to track age-matched bees and account for variation in genetic composition among colonies throughout the experiment.

We staggered worker emergence to allow collection of forager-aged workers (at 19 days), nurse-aged workers (at 7 days) and newly emerged workers (at 1 day) at the same time (Fig. 1) (Seeley, 1982). We also introduced a subset of newly emerged workers directly into observation colonies for the duration of experiments. We used this cohort of workers as control foragers that never experienced QMP treatment in cages. Mixed cohorts of ca. 150 workers consisting of forager-aged workers (n=50), nurse-aged workers (n=50) and newly emerged workers (n=50) were evenly distributed and introduced into their designated cages (12 cm×12 cm) with or without synthetic QMP, equivalent to one queen (1/10 strip according to the manufacturer's instructions, Bee Equipment Ltd, Bridge, Kent, UK). Cohorts were kept in their cages for 2 days, with access to *ad libitum* queen candy (a putty-like mixture of powdered sugar and warm water). Caged bees were re-introduced into their respective observation colony after treatment and monitored for 3 days when bees reached 21 days (typical foraging age) (Fig. 1). After filming (see below), we collected bees from the different cohorts at 24 days old from observation colonies and immediately preserved them in liquid nitrogen. All samples were stored in −80°C freezer until further analysis.

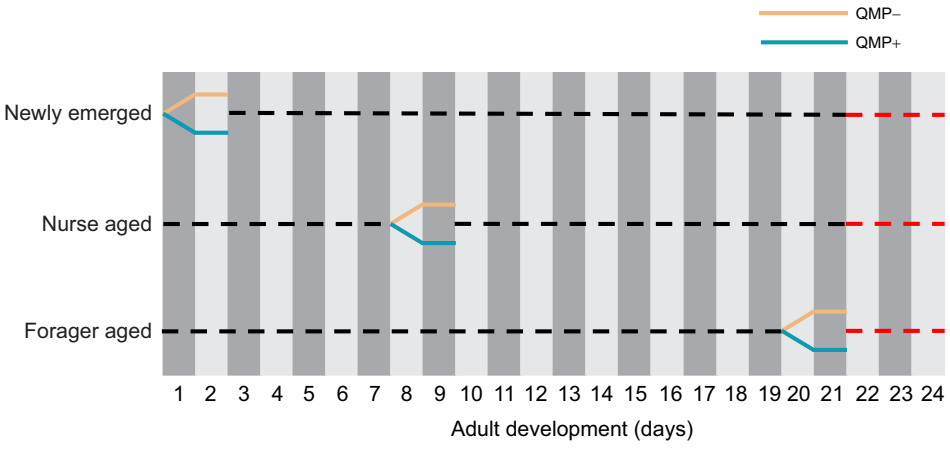

Fig. 1. Experimental design: sample collection and treatment overview. Honey bee workers were collected at 1 day (newly emerged), 7 days (nurse aged) and 19 days (forager aged). The dashed black line represents when workers were in the observation colony; the solid lines represent when workers were in the cage for treatment (exposed or not to queen mandibular pheromone, QMP+/−); the dashed red line represents when workers were filmed in the observation colony.

Video analysis

We filmed the entrance of the colonies for 8 h day^{-1}, from 09:00 h to 17:00 h, and noted the time of entry and departure of marked foragers. We logged behavioural observations using VLC Media Player and statistically analysed the differences between age and treatment groups using generalised linear models (GLM) with a negative binomial distribution. We performed pairwise comparisons between age groups using the general linear hypothesis test (GLHT) with Bonferroni correction for multiple testing.

Brain dissection and RNA extraction

The heads from n=42 workers were cut from the body and fixed on melted dental wax in a pre-chilled Petri dish over ice. The antennae were cut off and stored in 100 µl of Trizol™ (Invitrogen, Carlsbad, CA, USA). The mushroom bodies and antennal lobes were removed by making incisions through the antennal base, eyes, compound eye and ocellus (Kennedy et al., 2021a,b). The cuticles, glands, retina and tissue around the brain were removed, and the exposed tissues of the head were submerged in cooled bee saline (154 mmol l^{-1} NaCl, 2 mmol l^{-1} NaH_2PO_4, 5.5 mmol l^{-1} Na_2HPO_4, pH 7.2). Each dissection (one bee brain dissected into three tissues) was completed in less than 5 min to prevent degradation of RNA. Brain parts were stored in 100 µl of Trizol™ at −80°C for later RNA extraction using RNAeasy Mini Extraction Kit™ (Qiagen, Hamburg, Germany) according to the manufacturer's protocol. We extracted RNA from 36 caged bee samples, with two forager replicates per two QMP treatments (QMP+, QMP−), three ages (newly emerged, nurse aged, forager aged) and three colonies. We also collected two control (never caged) forager-aged bees per observation colony (three) for an additional six samples.

Transcriptome analysis

Aliquots of RNA from each sample were sent to Beijing Genomics Institute (BGI) for sequencing using BGISeq to get 100 base pair (bp) paired-end reads, obtaining ∼45 million clean paired reads per sample sequencing. Reverse transcription to cDNA was performed as part of the library preparation by BGI. Clean reads without adaptor sequences were provided by BGI, and quality checked using *FastQC* v.0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Clean reads were aligned using *HiSat2* v.2.1.0 (Kim et al., 2015) under default settings to the most recent honey bee reference genome assembly HvA3.1 (Howe et al., 2020), with a mapping ratio of more than 95% per sample. To quantify reading mapping to genes, we used *HtSeq* v.0.11.2 (Anders et al., 2015) to generate count tables under the default parameters. Count tables were generated separately for each sample and complied separately for each tissue (i.e. antennae, mushroom bodies and antennal lobes), treatment (i.e. QMP+/−) and life-stage when experiencing the treatment (i.e. newly emerged, nurse aged and forager aged).

Gene expression analysis

Gene expression differences were analysed between treatments (QMP+ versus QMP–) for each tissue (mushroom bodies, antennal lobes and antenna) and life stage (newly emerged, nurse aged and forager aged) using the R package *DESeq2* v.1.24.0 (Love et al., 2014). Before the analysis, an additional filtering step was added to ensure that only genes with counts of at least 9 reads in n−1 of the smallest sample size were used in the gene expression analysis. We analysed gene expression separately for each tissue and age. We tested the effect of treatment with QMP on gene expression by using the likelihood ratio test (LRT) approach whereby a full model with treatment (QMP+/−) and colony-ID as fixed factors is compared with a reduced model containing only colony-ID, taking into consideration colony effects. Genes were considered differentially expressed if the false discovery rate (FDR)-corrected P-value was <0.05. All analysis was completed in R v.4.1.0. We performed permutations to test whether the overlap in differentially expressed genes (DEGs) between tissues differed from what could be expected by chance. We generated a gene universe that had the same total number of genes (n=12, 320), which contained random gene IDs and zeros. We created a vector for the number of permutations we wanted to generate (n=1000). We performed automatic iterations with the number of DEGs in each gene list to get overlap values with our randomly generated list. We plotted histograms of the distribution generated by the permutations against the overlap to see whether what we found was within (expected by chance) or outside (not expected by chance) the normal distribution.

We performed both gene ontology (GO) and KEGG overrepresentation analysis using a background list of all detected genes in our dataset. We annotated their functions using the honey bee genome annotation that can be retrieved with the R package AnnotationHub (https://www.rdocumentation.org/packages/AnnotationHub/). We performed the enrichment analysis to contrast frequencies of functions among the DEGs compared with all expressed genes with the clusterProfiler package (Wu et al., 2021). To further investigate QMP treatment effects on specific genes, we focused on candidate genes involved in key individual and social behaviours and traits. We compiled lists of genes and molecular pathways associated with *foraging behaviour* and *division of labour* in honey bees (Amdam et al., 2012; Brito et al., 2021; Foret and Maleszka, 2006; Fussnecker and Grozinger, 2008; Ihle et al., 2010; Johnson and Jasper, 2016; Kennedy et al., 2021a,b; Linn et al., 2020; Nelson et al., 2007; Peng et al., 2021; Prato et al., 2021; Robinson, 1987; Schulz et al., 2003; Shpigler et al., 2010; Shpigler et al., 2021; Sullivan et al., 2000). Furthermore, we searched for genes that play roles in *ageing* (Corona et al., 2005; Haddad et al., 2007; Kennedy et al., 2021a,b; Kuszewska et al., 2017; Seehuus et al., 2006), *immunity* (Kennedy et al., 2021a,b; Richard et al., 2008) and *reproduction* (Hoover et al., 2003; Traynor et al., 2014), i.e. processes known to be affected by queen signals (see Table S1 for a complete list of the candidate genes). We cross-referenced our DEG lists, including the overlapped DEGs, to the candidate gene list to find genes of interest from previous studies.

RESULTS

Influence of QMP on gene expression

Workers treated with QMP as newly emerged bees showed the most pronounced differences in gene expression, despite the long time period between treatment and sampling: a total of 1398 genes were differentially expressed across all tissues (Fig. 2). In contrast, bees exposed to QMP treatment at nurse age or forager age only altered the expression of 80 and 67 genes across tissues, respectively (Fig. 2). Tissue-specific analyses revealed the strongest transcriptomic shifts in the mushroom bodies, with 760 DEGs (Fig. 2C). The vast majority of DEGs were significantly upregulated in bees that experienced a lack of QMP during the first days of their life (Fig. 2; 68% in mushroom bodies, 85.7% in antennal lobes, 91.7% in antennae). Interestingly, the opposite pattern was found in bees treated at nurse age (Fig. 2). Here, most DEGs were upregulated in QMP+ bees (Fig. 2; 94% in antennal lobes and 75.8% in antennae).

Control foragers that remained in observation colonies for the duration of experiments were used to test the effects of caging as well as the differences between natural and synthetic QMP exposure. We found that caging had strong short-term effects

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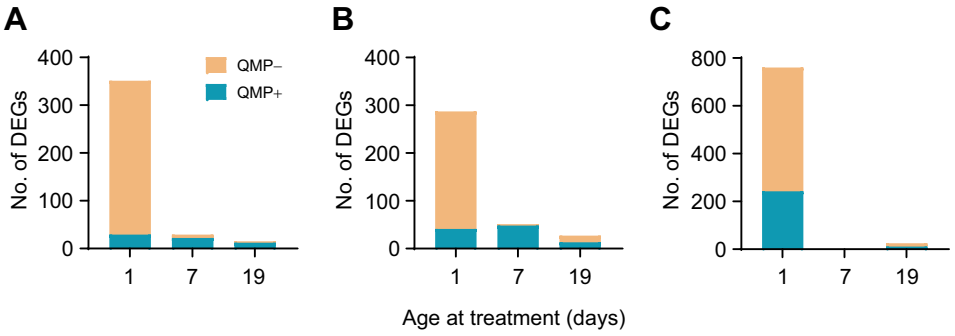

Fig. 2. The number of differentially expressed genes across the different tissues. (A) Antennae, (B) antennal lobes and (C) mushroom bodies. Bars indicate the total number of differentially expressed genes (DEGs) that were upregulated in each age group after 48 h QMP+/– treatment (1 day, newly emerged; 7 days, nurse aged; 19 days, forager aged). We analysed a total of 36 samples (12 each of newly emerged, nurses and foragers, *n*=6 QMP+, *n*=6 QMP–).

(observed in bees treated at nurse or forager age; 1526 and 2698 DEGs, respectively), but those effects disappeared over time (observed in bees treated after emergence; 67 DEGs; Table S3).

We found the most enriched GO terms of the DEGs in foragers treated with QMP as newly emerged bees. Many of these GO terms were associated with RNA processing and binding in the antennal lobes and in the mushroom bodies (Table S2). We also found a number of behaviourally relevant candidate genes when comparing our DEGs with lists of genes found previously in the honey bee to be linked to foraging, division of labour, ageing, reproduction and immunity genes (Table S1, Fig. S2).

DEGs across tissues and age

Some genes were differentially expressed in more than one tissue. For the DEGs of newly emerged bees, we found 15 genes overlapped between the antennae and antennal lobes, 67 genes overlapped between the antennal lobes and mushroom bodies, 115 genes overlapped between the antennae and mushroom bodies, and 102 genes overlapped between all tissues (Fig. 3). Permutations showed that this gene expression overlap between all tissues, between mushroom bodies and antennae, and between mushroom bodies and antennal lobes was more than what could be expected by chance (Fig. S1). We found five genes that overlapped between the antennae and antennal lobes for samples treated with QMP at nurse age. There were no DEGs in the mushroom bodies. The gene expression overlap for bees treated at nurse age was within the range of what could be expected by chance. We found no overlap of DEGs between any tissues for samples treated at forager age. All gene lists with a significant overlap were analysed for matches against the candidate gene list (see Table S1) as well as GO enrichment analysis. We did not find any GO enrichments or matches against the candidate gene list.

Only a few genes were differentially expressed across multiple age groups. In the antennal lobes, LOC107963983 (*pre-rRNA processing protein FTSJ3*) and LOC413582 (*allatostatin-A receptor*) overlapped for workers treated with QMP at newly emerged and forager stage. Meanwhile, in the mushroom bodies, LOC100576482 (*centromere protein J*), LOC552498 (*DNA repair protein complementing XP-C cells homolog*), LOC725689 (*golgin subfamily A member 4*), LOC102656444 (*protein dopey homolog PFC0245c-like*) and LOC100576346 (*myb-like protein D*) showed overlap for the same age groups.

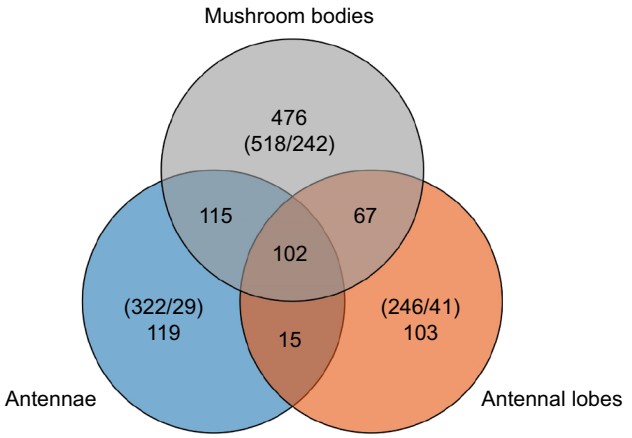

Fig. 3. Gene expression overlap for workers exposed to QMP treatment at 1 day post-emergence. Venn diagram representation of the overlap between DEGs of tissues (antennae, antennal lobes and mushroom bodies) for foragers treated with QMP at 1 day post-emergence. Numbers within each individual circle indicate the unique number of DEGs for that specific tissue, with numbers in parentheses corresponding to the number of upregulated genes in the treatment groups (QMP–/QMP+). Numbers in the overlap between circles indicate the number of shared DEGs between tissues.

Foraging behaviour

We found an overall effect of the age when foragers were treated with QMP on the number of foraging trips (GLM, negative binomial distribution, χ^2=17.91, P<0.001; Fig. 4A) and the average time spent foraging (LME, normal distribution, χ^2=12.25, P=0.002; Fig. 4B). However, we did not find an influence of QMP treatment on foraging behaviour (QMP+/–) (χ^2=0.259, P=0.6; χ^2=0.705, P=0.4, respectively) and there was no interaction between QMP treatment and age when treated (χ^2=2.603, P=0.272; χ^2=4.18, P=0.123, respectively; Fig. 4).

Pairwise comparisons showed that foragers treated with QMP at nurse stage made fewer foraging trips and spent on average less time foraging than those treated with QMP at forager stage (GLHT: z=−3.214, P<0.004 and z=−3.202, P=0.004, respectively; Fig. 4) and as newly emerged workers (GLHT: z=−2.813, P=0.013; Fig. 4), while there was no difference in total number of foraging trips between workers treated as newly emerged bees or nurses (GLHT: z=−2.120, P=0.068; Fig. 4A), irrespective of QMP treatment type (QMP+/–). Foragers treated with QMP as young foragers or newly emerged bees did not differ in total number of foraging trips (GLHT: z=−1.616, P=0.106; Fig. 4A) or average foraging duration (GLHT: z=−0.960, P=0.6; Fig. 4B).

DISCUSSION

Our results support the hypothesis that there is a period in the early adult life of a honey bee that is highly sensitive to the influence of the queen pheromone, which leads to long-term physiological changes. Transient deficiency of QMP had the greatest effects when workers were treated within days of emergence: it led to widespread transcriptional changes in the central and peripheral nervous systems. Moreover, caging of workers induced a transcriptional (potentially stress-related) response that reduced over time while QMP treatments

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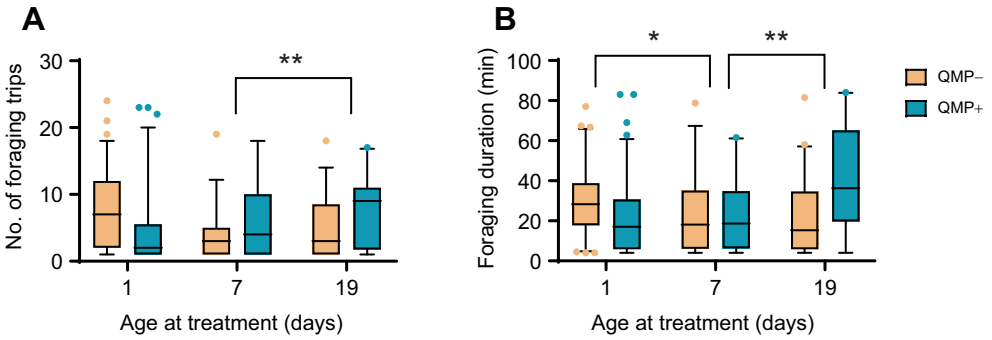

Fig. 4. Effect of QMP treatment on foraging activity. (A) Total number of foraging trips. (B) Average foraging duration. Box and whisker plots (median, upper and lower quartiles and 1.5× interquartile range) represent values for each age group (1 day, newly emerged; 7 days, nurse aged; 19 days, forager aged) after 48 h QMP+/– treatment. There was no difference in foraging activity between QMP treatment groups. Pairwise tests between age groups were performed using general linear hypothesis tests (GLHT) and differences were found in the total number of foraging trips (7 days versus 19 days GLHT: z=–3.214, **P=0.00393) and average foraging duration (1 day versus 7 days GLHT: z=–2.813, *P=0.01340; and 7 days versus 19 days GLHT: z=–3.202, **P=0.00385). We treated a total of 305 newly emerged workers (n=143 QMP+, n=162 QMP–), 129 nurses (n=69 QMP+, n=69 QMP–) and 88 foragers (n=30 QMP+, n=58 QMP–).

showed the opposite pattern, inducing transcriptional changes that were long lasting. This reveals that the molecular consequences of a lack of QMP exposure persist across all behavioural transitions until workers reach foraging age late in life.

QMP elicits complex behavioural and physiological responses in workers, as it acts both as a releaser and as a primer pheromone, and thus elicits responses on very different time scales (Grozinger et al., 2007; Slessor et al., 2005). The persistent effects of the lack of QMP exposure on gene expression profiles of workers treated directly after emergence (1398 genes in all tissues combined; Fig. 2) was most pronounced in the mushroom bodies (785 DEGs; Fig. 2). In contrast, there were only minor to moderate effects on transcription in bees treated as nurses or young foragers (80 genes and 67 genes, respectively; Fig. 2), suggesting that the time window for large-scale QMP effects closes very early in the life of a worker. Our findings align well with the observation that young workers are more attracted to QMP, which results in the retinue response (Rangel et al., 2016; Slessor et al., 1988, 2005), while older workers

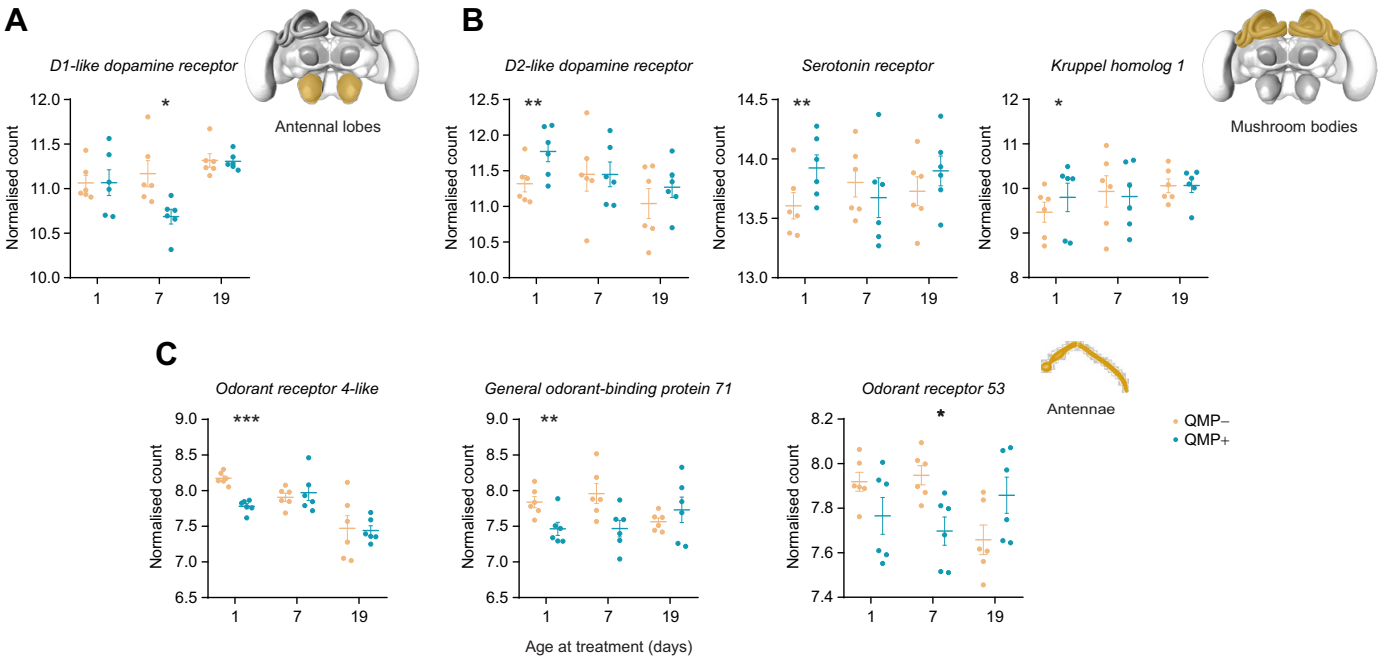

Fig. 5. Significant gene expression patterns for genes of interest. We searched the top DEGs of each gene list separately for genes associated with foraging and other behaviours (see Table S1). We show only the tissues where expression of the gene was significant, according to treatment group (QMP+/–). (A) In the antennal lobes, only *D1-like dopamine receptor* (*AmDop1*) expression was significant (P=0.044) in workers treated with QMP at 7 days (nurse aged). (B) In the mushroom bodies, *D2-like dopamine receptor* (*AmDop3*; P=0.004), *serotonin receptor* (*5-ht1*; P=0.003) and *Krüppel homolog 1* (*Kr-h1*; P=0.038) expression was significant in workers treated with QMP at 1 day post-emergence (newly emerged). (C) In the antennae, *odorant receptor 4-like* (LOC107966034; P<0.001) and *general odorant-binding protein 71* (LOC113218767; P=0.001) expression was significant in workers treated with QMP at 1 day post-emergence; *odorant receptor 53* (*Or53*; P=0.043) expression was significant in workers treated with QMP at 7 days. *P<0.05, **P<0.01, ***P<0.001 (after FDR correction). We used a total of 36 samples for gene expression analysis (12 each of newly emerged, nurse aged and forager aged, n=6 QMP+, n=6 QMP–).

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engaging in foraging are repelled by queen pheromones (Jarriault and Mercer, 2012; Vergoz et al., 2009). When the observed gene expression patterns were cross-referenced with similar QMP studies, we did not find a significant enrichment or over-representation of any particular gene class. However, we found genes associated with immunity, learning and regulation of social behaviours did overlap between studies (Fig. S2). This rather indicates a principal physiological response in central and peripheral nervous systems of worker bees to the presence/absence of the queen, involving many different molecular pathways.

In social insects, the social environment of the colony during development and throughout adult life shapes an individual's physiology and subsequent behaviour. In honey bees, the queen's pheromone is one of the most important social signals for the regulation of the intricate colony dynamics. Workers close to the queen in the colony would sense the most QMP, as they are in direct contact with the queen, while foragers are exposed less to QMP because they spend more time outside the colony or further away from the brood area. Therefore, different workers are exposed to variable amounts of QMP as they age and transition between tasks. This could partly explain why the molecular physiology of forager-aged workers is less influenced by QMP effects.

When we compared the gene expression profiles between different age groups, we found few gene expression overlaps between workers treated with QMP shortly after emergence and later in life. The limited gene expression overlap between QMP treatment during early versus late life stages suggests that the effect of QMP on gene expression is time sensitive and predominantly affects newly emerged bees. Temporary periods of queenlessness are natural situations in the colony life cycle. During swarming, the old queen leaves with the swarm before the new queen emerges. Queen replacement can also occur outside the swarm season when there is queen loss. Workers that do not experience QMP during certain developmental stages can develop 'selfish' traits. These so-called 'rebel workers' develop their ovaries, frequently migrate to foreign colonies and generate a notably higher number of male offspring in colonies with a queen (Kuszewska et al., 2018; Kuszewska and Woyciechowski, 2015). Future studies could explore the link between natural queenlessness and transcription during early life, larval and pupal stages and investigate whether these putative effects are also demonstrated throughout adult behaviours.

QMP in high doses is reported to repel workers (Moritz et al., 2002, 2001) and can make workers more aggressive (Vaitkevièienë and Budrienë, 1999). However, QMP exposure during early adult life has been implicated in suppressing aversive learning in insects by regulating the expression of D1-like dopamine receptor, D2-like dopamine receptor and octopamine receptor genes (Beggs et al., 2007; Vergoz et al., 2009). We found that QMP absence during early life led to changes in the expression of D2-like dopamine receptor (*AmDop3*) and D1-like dopamine receptor (*AmDop1*) in the mushroom bodies and antennal lobes, respectively (Fig. 5; Table S1). The effects of QMP on aversive learning may serve to prevent young workers that attend the queen from forming an association between the queen and any unpleasant effects of her pheromone (Vergoz et al., 2007). The prediction that QMP acts on dopamine receptors to prevent the formation of aversive olfactory memories is consistent with the observation that young workers are more attracted to QMP (Vergoz et al., 2009, 2007).

Dopamine receptors have a crucial role in a broad range of behaviours, such as motor function, sensory processing, arousal and reward signalling (Elsik et al., 2014; McQuillan et al., 2012; Mishra et al., 2018). *Amdop3* is widely expressed in the brain in both adults and pupae, with a unique pattern of expression compared with the other subtypes, *Amdop1* and *Amdop2* (Suenami et al., 2016). Homovanillyl alcohol (HVA), a major component of QMP, has been shown to reduce the concentration of brain dopamine levels in the centres associated with learning and memory (i.e. mushroom bodies). HVA selectively activates *Amdop3*, which blocks aversive learning in workers (Beggs and Mercer, 2009), possibly promoting the retinue response as seen in the upregulation of *Amdop3* in the mushroom bodies of workers treated with QMP 1 day after emergence. As we also find an upregulation of *Amdop1* in the antennal lobes of bees treated at nurse age, this could suggest an early trigger of non-nursing behaviours. We also found a serotonin receptor (*5-ht1*) to be upregulated in the mushroom bodies of foragers treated with QMP 1 day after emergence.

We found that QMP presence induced an upregulation of the transcription factor *Krüppel homolog 1* (*kr-h1*) in the mushroom bodies of bees treated shortly after emergence. This transcription factor has been linked to hormone-mediated social organisation in honey bees, bumble bees and ants (Gospocic et al., 2021; Grozinger and Robinson, 2007; Shpigler et al., 2010). In young workers (<1 week), QMP exposure activates nursing genes and represses

Table 1. Effects of QMP on functional categories of differentially expressed genes

Tissues	Treatment age (post-emergence)	Function	Gene
Mushroom body	1 day	Ageing	*DNA repair proteins*
			telomere associated proteins
		Division of labour	*krüppel homolog 1*
		Memory	*metabotropic glutamate receptor*
		Foraging	*serotonin receptor*
			D2-like dopamine receptor
Antennal lobes	1 day	Ageing	*telomere associated proteins*
			DNA repair proteins
		Learning	*Allatostatin A receptor*
	7 days	Foraging	*dopamine receptor, D1*
	19 days	Learning	*Allatostatin A receptor*
Antennae	1 day	Odour binding	*odorant receptor 4-like*
			general odorant-binding protein 71
		Ageing	*DNA repair proteins*
	7 days	Odour binding	*odorant receptor 53*

Workers were collected at 1 day (newly emerged), 7 days (nurse aged) and 19 days (forager aged) post-emergence and treated with queen mandibular pheromone (QMP) for 48 h. For tissue location, see Fig. 4.

foraging genes, suggesting that QMP delays the transition from nursing to foraging by acting on *kr-h1* in the mushroom bodies (Grozinger et al., 2003). Although we did not find behavioural evidence to suggest that foraging activity was suppressed, future studies could further investigate nursing behaviours and age of foraging onset after exposure to queenlessness.

QMP has varied effects on brain transcriptional activity depending on the behavioural state of a worker (Grozinger et al., 2003; Kocher et al., 2010), and our study shows that QMP information is processed differently at different adult stages. Accordingly, we found that a lapse in QMP exposure caused a down-regulation in the expression of odour binding proteins (*odorant receptor 4-like* and *general odorant-binding protein 71*) and receptors (*odorant receptor 53*) in the antennae of foragers treated at 1 day post-emergence and nurse age (Fig. 5, Table 1). A variety of odorant binding proteins (OBPs, ca. 21) and odorant receptors (ORs, ca. 180) have been characterised in the honey bee (Foret and Maleszka, 2006; Zhang et al., 2016), but their roles in odour discrimination are still poorly understood. While we present further evidence for the role of some OBPs and ORs in the context of QMP signal detection, more research is needed to understand their functioning in the context of odour differentiation and subsequent behavioural regulation. In summary, we demonstrate a highly sensitive period for molecular physiology in the early adult life of honey bee workers to the chemical signal of the queen. Moreover, these transcriptomic responses in the brain and antennae to the queen signal are long-lasting and can still be detected later in life. We build on current knowledge of the effects of social and maternal factors by presenting data showing not only that QMP attracts young workers and affects gene expression but also that these gene expression changes can persist into late foraging age. We show that QMP has the largest effect on the mushroom bodies, suggesting that there could be an impact on learning and memory. Future studies are needed to explore the subsequent behavioural changes such as age of foraging onset, foraging preferences for nectar or pollen, and nursing behaviours.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.P., A.K., C.G.; Methodology: T.P., A.K., C.G.; Validation: T.P., A.K., C.G.; Formal analysis: T.P., A.K., C.G.; Investigation: T.P., S.F., C.G.; Resources: T.P., S.F., C.G.; Data curation: T.P., A.K., Y.W., C.G.; Writing - original draft: A.K.; Writing - review & editing: T.P., A.K., S.F., C.G.; Visualization: T.P., A.K., Y.W., C.G.; Supervision: S.F., C.G.; Project administration: S.F., C.G.; Funding acquisition: T.P., S.F., C.G.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request. All sequencing data are deposited in the Sequence Read Archive (SRA) database under the BioProject ID PRJNA792844. All R scripts used in the analysis are available from GitHub: https://github.com/Hansptf/Early-Life-Exposure-to-Queen-Mandibular-Pheromone.git

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