Queen pheromone modulates the expression of epigenetic modifier genes in the brain of honey bee workers

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Abstract

Pheromones are used by many insects to mediate social interactions. In the highly eusocial honey bee (*Apis mellifera*) queen mandibular pheromone is involved in the regulation of reproduction and the rate of ageing of workers. The molecular mechanisms by which queen mandibular pheromone acts remain largely unknown. Here we investigate how genes responsible for epigenetic modifications to DNA, RNA and histones expressed in the brain of honey bee workers respond to the presence of queen mandibular pheromone. Several of these genes are upregulated in workers exposed to queen mandibular pheromone. This suggests that epigenetic mechanisms are mediated by pheromonal communication between queens and workers, and that the interaction of pheromone signalling and epigenetics may mediate some of the neural plasticity that underlies ageing-associated traits in social insects. We suggest that pheromonal communication systems, such as those used by social insects, evolved to respond to environmental clues by making use of epigenomic machineries.

Keywords: DNMTs, HATs, HDACs, SIRT, honeybees, aging

Introduction

The transition to social living in the eusocial insects required that the reproductive interests of individual workers be subsumed by the collective interests of the colony (Ratnieks 1988; Maynard-Smith & Szathmáry 1995; Ratnieks *et al.* 2006). In particular, workers are normally sterile, though they do switch to laying eggs when their queen dies (Ronai *et al.* 2016c). Furthermore, because insect societies are self-organized, it was necessary for systems to evolve so that tasks are distributed among workers in ways that enhance colony-level productivity (Gordon 1996; Bonabeau *et al.* 1997; Page 1997; Visscher 2003). Both the regulation of worker fertility and the efficient allocation of tasks among workers required the evolution of effective inter- and intra-caste communication systems that can respond rapidly to changes in colony needs. Communication between nestmates mainly occurs via pheromones (Free 1987; Meer & Preston 2008), chemical signals that are produced by one individual, and cause changes in the behaviour or physiology of another (Billen & Morgan 1998; Holman 2010).

In the western honey bee (*Apis mellifera*) a key pheromone is queen mandibular pheromone (QMP). This pheromone is a blend of fatty acids secreted by the mouthparts of the queen that regulates several important traits of workers, including their reproduction (Ronai *et al.* 2016b), foraging behaviour (Pankiw *et al.* 1998), learning capacity (Vergoz *et al.* 2007), nestmate recognition (Fan *et al.* 2010) and age at onset of foraging (Pankiw *et al.* 1998). Furthermore, phenotypic variation in these traits is associated with differential gene expression in the brains of workers (Grozinger *et al.* 2003; Fussnecker *et al.* 2011). Nonetheless, little is known about the intermediate steps between QMP production by the queen, the regulation of gene expression in workers, and changes in their behaviour (Brockmann *et al.* 1998; Huang *et al.* 1998; Ronai *et al.* 2015, 2016a).

Epigenetic mechanisms are a likely mediator between the social environment and global gene expression responses (Cardoso-Junior *et al.* 2018). Several classes of epigenetic mechanism have been described in honey bees that are potentially associated with environmental cues (Wang *et al.* 2006; Dickman *et al.* 2013; Yan *et al.* 2014, 2015). For example, DNA methylation, a reversible chemical modification on carbon 5 of cytosines in a CpG context, is associated with behavioural maturation in the brains of honey bee nurses and foragers (Herb *et al.* 2012). DNA methylation is catalysed and maintained by the DNA methyltransferase (DNMT) family of enzymes (Bestor 2000; Allis & Jenuwein 2016). Interestingly, the expression of genes associated with the maintenance of DNA methylation levels after DNA replication (*Dnmt1a* and *Dnmt1b*), as well *de novo* DNA methylation patterns (*Dnmt3*), are modulated by different social stimuli in honey bees (Kucharski *et al.* 2008; Holman *et al.* 2016; Cardoso-Junior *et al.* 2018; Cardoso-Júnior *et al.* 2018). In addition, the expression of *Dnmt2* (also called *Trdmt1*), a gene whose enzyme product methylates RNA substrates (Jeltsch *et al.* 2016), is affected by different social contexts in honey bees (Herb *et al.* 2012; Cardoso-Junior *et al.* 2018), suggesting that epigenetic alterations of DNA and RNA nucleotides are affected by environmental stimuli.

Another epigenetic mechanism, histone post-translational modifications (HPTMs) change chromatin structure by altering the physiochemical affinity between DNA and histones. HPTMs are associated with honey bee queen-worker differentiation (Wojciechowski *et al.* 2018) and are likely triggered by their different larval diets (Spannhoff *et al.* 2011). This demonstrates that, like DNA methylation, differential amounts of HPTMs are sensitive to environmental signals (e.g. food, pheromones) in honey bees and other social insects (Simola *et al.* 2013, 2016; Cardoso-Júnior *et al.* 2017).

HPTMs are produced by histone modifier proteins (Allis & Jenuwein 2016), which can be divided into three functional classes. “Writer” enzymes, such as lysine

acetyltransferases (KAT), promote acetylation of lysine residues (Allis & Jenuwein 2016). Acetylation reduces the affinity between DNA and nucleosomes, which in turn induces chromatin relaxation and is often associated with increased gene expression (Albaugh *et al.* 2011). "Eraser" enzymes, such as histone deacetylases (HDACs), remove acetyl groups from lysine residues, resulting in chromatin compaction (heterochromatin) and, consequently, inhibition of gene expression (Allis & Jenuwein 2016; Pasyukova & Vaiserman 2017). Notably, there is a histone deacetylase protein class that comprises NAD^+-dependent enzymes, the Sirtuins (SIRT). SIRT enzymes are key regulators of ageing in several species (Satoh *et al.* 2017). Thus, it is possible that SIRT genes may play a role in QMP modulation of ageing for honey bee worker. Finally, "reader" enzymes recognise epigenetic modifications and induce chromatin remodelling through the recruitment of protein complexes (Allis & Jenuwein 2016). A proteome study (Dickman *et al.* 2013) has shown that the histone tails of honey bees are extensively modified by epigenetic marks, indicating that writer, eraser and reader enzymes are all coded by the honey bee genome.

As QMP promotes behavioural maturation in honey bee workers (Pankiw *et al.* 1998) we hypothesised that the proteins associated with DNA methylation, RNA methylation and HPTM, particularly acetylation/deacetylation processes, respond to QMP exposure. These epigenetic mechanisms can serve as a proxy to understand the regulation of global changes in gene expression in a complex social environment. Specifically, we investigated the expression of epigenetics genes in the brains of honey bee workers exposed or not exposed to QMP.

Material and Methods

Biological material

To obtain age-matched adult honey bee workers we collected brood frames from four queenright *A. m. ligustica* source colonies and kept them in an incubator overnight at 34.5 °C. From each source colony, newly-emerged workers were randomly allocated to two cages (n = 150 bees per cage, eight cages in total). Four cages (QMP^+) contained a 0.5 queen equivalent per day QMP strip (Phero Tech Inc. Canada), which is an effective queen mimic in cage experiments with young workers (Ronai *et al.* 2015; Cardoso-Júnior *et al.* 2019). The other four cages (QMP^-) contained no QMP strip. Pollen, honey and water were provided *ad libitum*. Food was replenished when necessary, and the number of dead workers was recorded each day, which was nearly the same in both control and QMP-treated cages (data not shown). Cages were kept in an incubator at 34.5 °C for four days. Workers were collected on dry ice at Day 0 (directly from the brood comb), Day 1 and Day 4. Day 1 was chosen to investigate genes with a quick response to the QMP treatment, and Day 4 was chosen to identify the genes that are still influenced by the QMP exposure after the majority of QMP-sensitive genes had returned to their basal expression line (Grozinger *et al.* 2003). We then dissected the brains of the workers on dry ice (Vergoz *et al.* 2012).

Identification of the honey bee DNA methyltransferases and histone modifiers

We identified the nucleotide and histone modifiers in the honey bee genome (Amel_4.5) (Elsik *et al.* 2014) searching manually for the names of each epigenetic gene in GenBank (NCBI Resources Coordinators 2018) (Table S1) based on a large list of histone-modifier genes present in eukaryotes (Yun *et al.* 2011; Jęśko *et al.* 2017). We filtered this list by selecting those associated with acetylation and deacetylation processes. From this specific list we identified the proteins that are predicted to reside in the nucleus, checking their amino acid sequence using Protcomp software version 9.0 (Softberry). The genes were characterised following a previously described workflow (see (Ronai *et al.* 2016b)), briefly: gene

information was obtained from GenBank; protein information from UniProt (UniProt Consortium 2015); insect orthologues (genomes of 116 species) from OrthoDB hierarchical catalogue v9.1 (Zdobnov *et al.* 2017); and TBLASTN to the *Drosophila melanogaster* genome deposited in GenBank.

Gene expression quantification and bioinformatics analysis

Each sample consisted of a single brain. We extracted total RNA from the brains through maceration in TRIzol (Invitrogen) and a Direct-zol™ RNA Miniprep kit (Zymo Research). The RNA was treated with Turbo DNase Kit (Thermo Fisher Scientific) and quantified with a Qubit 2.0 Fluorometer (Invitrogen). The cDNA was synthesized from 600 ng of RNA using a SuperScript™ III Reverse Transcriptase Kit (Invitrogen) with oligo(dT) primer. Then, the cDNA was diluted to 5 ng/µL in ultrapure water.

The expression of the four nucleotide modifiers genes (*Dnmt1a*, *Dnmt1b*, *Dnmt2* and *Dnmt3*) and eleven histone modifiers genes (*Kat2a*, *Kat3b*, *Kat6b*, *Kat7*, *Kat8*, *Hdac1*, *Hdac3*, *Sirt1*, *Sirt6*, *Sirt7* and *Rcs1*) was quantified by reverse transcription quantitative real-time PCR (RT-qPCR) according to (Brito *et al.* 2010; Cardoso-Júnior *et al.* 2019). RT-qPCR assays were set up with 2.5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad), 1.25 pmol of each primer, 1 µL diluted cDNA (5 ng) in a total volume of 5 µL using a CFX384 Real-Time System (Bio-Rad). For each biological replicate (n = 8 per source colony and treatment) three technical replicates were conducted. RT-qPCR cycle conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. At the end of the RT-qPCR protocol a melting curve analysis was run to confirm a single amplification peak. Primer efficiencies (Table S2) were calculated based on an amplification curve of 10 points obtained through serial dilution of mixed cDNA samples. The expression of the genes of interest was normalised against the expression of two reference genes (*Rpl32* and *Ef1α*), whose expression was found stable according to *BestKeeper* software (Pfaffl *et al.* 2004). Relative expression levels were calculated as in Brito *et al.* (2010), using a relative gene expression formula that takes into account the efficiency of each primer. The genes, primer sequences and efficiencies are listed in Table S2.

Statistical analysis

To compare the expression of the QMP^+ and QMP^- treatments at Day 1 and Day 4 we used a generalised linear mixed model (GLMM) with 'source colony' as random effect and treatment and age as fixed effects. To model the gene expression data, we used link = identity, family = Gaussian. Where necessary, a transformation $\log^{10}$ function was applied (see Table S3 for details). We used Day 0 data as a baseline of gene expression data. GLMM analyses were performed in R (R Core Team 2018) loading the packages lme4, car and lsmeans. An adjusted *p*-value (Tukey correction for each gene) lower than 0.05 was considered significant for all statistical tests.

Results

Using the protein sequences of the 15 genes studied here, we first acquired *in-silico* evidence that each gene was an authentic epigenetic modifier of DNA, RNA or histone tails (Table S1). Next, RT-qPCR assays show that in 1-day-old honey bee workers the expression of eight genes associated with epigenetic processes (*Dnmt3*, *Kat2a*, *Kat6b*, *Kat8*, *Hdac1*, *Hdac3*, *Sirt 1* and *Rcs1*) was affected by exposure to QMP (GLMM, $p < 0.05$, Figure 1 and Table S3). However, only four genes (*Dnmt1b*, *Dnmt2*, *Kat3b* and *Sirt7*) continued to be differentially expressed at the age of four days (GLMM, $p < 0.05$, Figure 1 and Table S3). Age was statistically significant for 14 of the 15 epigenetics genes (GLMM, $p < 0.05$, Figure

1 and Table S3), the only exception being *Kat 7*. A significant interaction between treatment and age was found for *Hdac1*, *Sirt1* and *Kat6b* ($p < 0.05$, Table S3).

Discussion

Our study shows that QMP affects the expression of 12 genes that are associated with epigenetic processes in the brain of honey bee workers across a short time scale. Rapid activation of these genes within 24 hours of exposure to QMP suggests that epigenetic mechanisms are a likely mediator between queen pheromone signalling and the regulation of gene expression in honey bee workers. In particular, we show that genes associated with epigenetic mechanisms and involved in ageing are strong candidates for mediating gene expression changes in response to QMP. QMP alters foraging onset in honey bee workers, a key marker of behavioural maturation in this species (Robinson 1992; Pankiw *et al.* 1998; Amdam & Omholt 2002). Thus, our results suggest that genes associated with epigenetic processes underlie the effects of QMP on behavioural maturation in honey bees.

We detected differential expression of three *DNMTs*. The expression of *DNMT*s changes with chronological age and is sensitive to diverse social stimuli that affect ageing in the honey bee (Holman *et al.* 2016; Cardoso-Junior *et al.* 2018) and ants (Holman *et al.* 2016, 2019; Kay *et al.* 2016) workers. Interestingly, the expression of the RNA methyltransferase *Dnmt2* is upregulated at both day 1 and day 4 of QMP treatment. While the role of DNMT2 is currently unknown in the honey bee, it is the evolutionarily most conserved and supposedly most ancient of the DNMT enzymes (Jeltsch *et al.* 2016). DNMT2 is present in insects that have lost several or all of the other DNMTs, as is the case in drosophilids (Vieira *et al.* 2018). Hence, our results now suggest a potential role of *Dnmt2* in mediating pheromonal communication in a eusocial insect. In the fruit fly *D. melanogaster*, DNMT2 plays a critical role regulating the biogenesis of microRNAs and tRNA stability upon environmental stress (Wang *et al.* 2006; Schaefer *et al.* 2010; Durdevic *et al.* 2013). Moreover, DNMT2 is associated with ageing in *Drosophila* (Lin *et al.* 2005), suggesting that QMP may drive alterations in the non-coding RNA pathways in honey bee workers.

Another two ageing-associated genes (Lee *et al.* 2014; Satoh *et al.* 2017) influenced by QMP exposure were *Sirt1* and *Sirt7*. SIRT1 activity is known to be modulated by resveratrol, a plant polyphenol that mimics caloric restriction effects (Feige *et al.* 2008; Smith *et al.* 2009) and alters lifespan in honey bees (Rascon *et al.* 2012) and other species (Howitz *et al.* 2003; Wood *et al.* 2004; Valenzano *et al.* 2006). Therefore, these results show that via their pheromone queens modulate the expression of ageing-associated genes of honey bee workers, a process that possibly facilitates the behavioural maturation changes associated with QMP exposure (Pankiw *et al.* 1998). The precise epigenetic mechanisms by which each of these genes act in reprograming neural networks involved in modulating ageing process upon QMP signalling remains to be elucidated.

We have determined that two HDAC-encoding genes (*Hdac1* and *Hdac3*) are differently expressed in response to QMP treatment. However, unlike *Sirt1* and *Sirt7*, *Hdac1* and *Hdac3* are only indirectly related to ageing in honey bees. The two genes were first reported (Wang *et al.* 2014) to be differentially expressed in honey bee workers treated with a diet supplemented with 10-hydroxy-2-decenoic acid (10-HDA), an abundant component of royal jelly that inhibits HDAC activity in mammalian cells and is thought to play a role in honey bee caste determination (Spannhoff *et al.* 2011). While the effects of 10-HDA on honey bee ageing have been not yet investigated, 10-HDA has been shown to affect lifespan in other organisms, including *D. melanogaster* and the nematode *Caenorhabditis elegans* (Honda *et al.* 2011, 2015), suggesting that QMP makes use of environment-sensitive pathways, such as histone (de)acetylation, to promote altered behavioural maturation changes in the brain of honey bee workers.

Another gene promoting HPTM, *Kat8*, was also found differentially expressed in honey bees exposed to QMP versus those not exposed. *Kat8* was previously identified as being differentially spliced in the transcriptome of *Lasius flavus* ants treated with pheromones of their own queen (Holman *et al.* 2019), suggesting that (de)acetylation processes may be regulated by queen pheromones not only in the honey bee but also in other eusocial hymenopterans. In line with this hypothesis, acetylation was reported as an important regulator of foraging activities in the ant *Camponotus floridanus* (Simola *et al.* 2016). Together, these results highlight the role of epigenetic mechanisms in chromatin reprogramming upon exposure to environmental cues.

Our identification of new QMP-responsive genes (Grozinger *et al.* 2003; Fussnecker *et al.* 2011; Holman *et al.* 2019) can be attributed to the differences in methodological strategies adopted in these studies, including QMP dosage, age of workers at the time of sampling and the tissues analysed. For example, our data suggests that *Dnmt1a* expression is not affected by QMP exposure, contrasting with data from a previous study (Holman *et al.* 2016). While we analysed the expression of *Dnmt1a* in brains of workers exposed to QMP for one day, the previous study focused on *Dnmt1a* expression after 3 days of QMP treatment in whole-body RNA preparations. Such contrasting results indicate a tissue and temporal specificity in the transcriptional response to QMP, highlighting the dynamics and complexity of social interaction between queens and workers.

Pheromonal modulation of gene expression in honey bee workers changes over time (Grozinger *et al.* 2003; Fussnecker *et al.* 2011). Gene expression in QMP^- workers was relatively stable from Day 0 to Day 1 when compared to QMP^+, suggesting QMP *actively* promotes gene expression within 24 hours. Also, we found that only some epigenetic modifier genes (4 out of 15) are persistently regulated by QMP-treatment over different time-points, indicating that their expression is dynamically switched on and off (Grozinger *et al.* 2003).

In conclusion, our study provides evidence that genes associated with epigenetic mechanisms are differentially expressed in the brain of honey bee workers in response to queen pheromone. Hence, we propose that epigenetic mechanisms are key responders to the honey bee social environment. These critical environment-sensitive mediators are likely candidates to drive transcriptomic changes triggered by queen pheromones, both as a short-term response indicating queen presence (a “releaser” pheromone attribute of QMP) and a long-term response related to behavioural maturation and ageing (a “primer pheromone characteristic of QMP). In general terms, this suggests that efficient epigenomic machineries that are capable of responding quickly as well as persistently to social cues, are an inherent component of the complex communication systems of social insects and substantially contributed to their ecological and evolutionary success.

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Figure 1. Heat maps representing the relative expression of epigenetic mechanisms-associated genes in the brains of 0-, 1- and 4-days old honey bee workers, exposed or not to QMP (QMP^+/QMP^-). a) Transcript levels of DNA methyltransferases. b) Expression of histone modifiers genes. The colour grading for each box represents the mean gene expression level of the biological replicates (N = 32; N=8 from each of the four colonies) for each of the three time points. Different letters represent statistical significance after Tukey correction for each time point, 1- or 4-days after QMP exposure (GLMM test, $p < 0.05$). Day 0 was used as baseline for gene expression.

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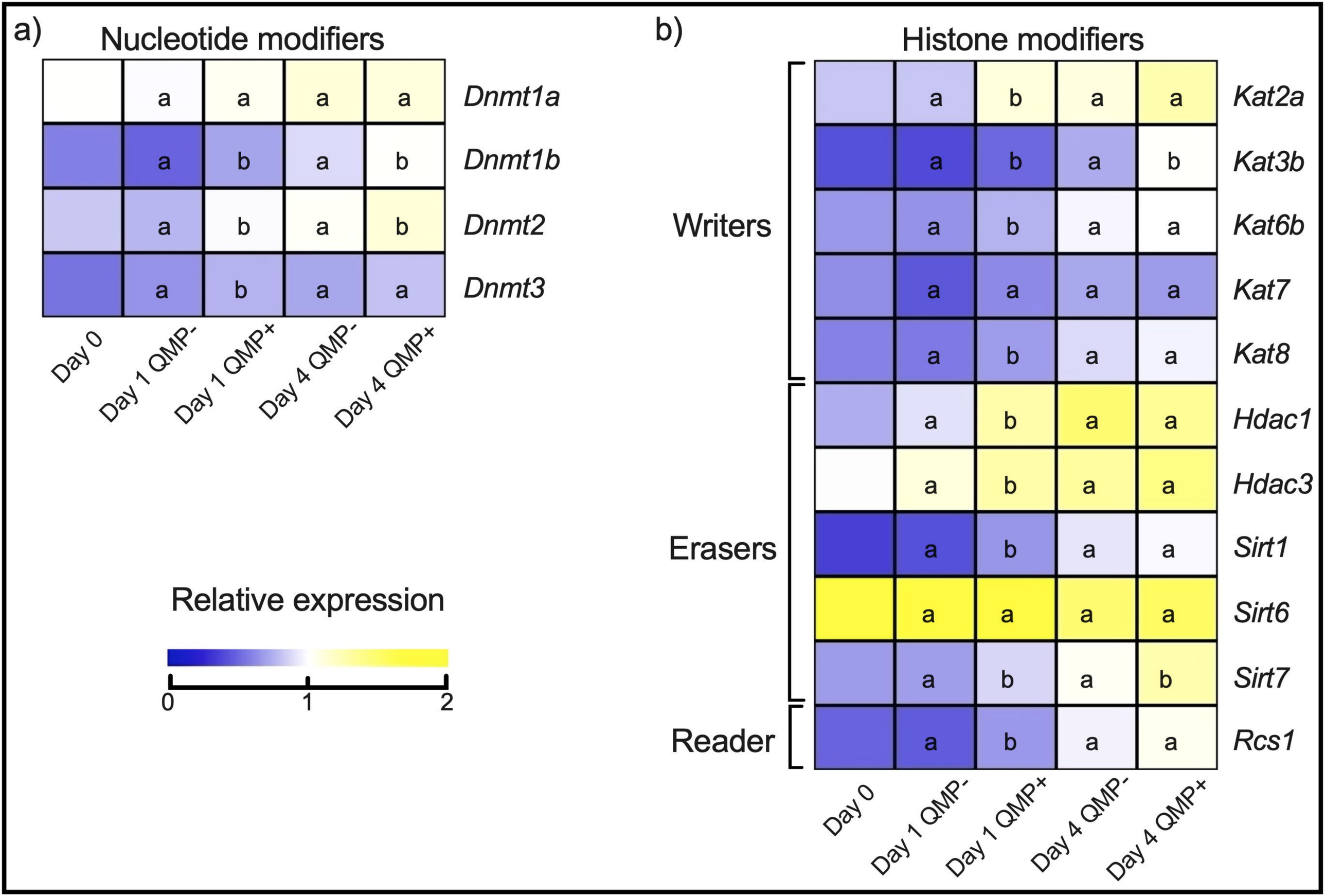
a)
Nucleotide modifiers
a a a a Dnmt1a
a b a b Dnmt1b
a b a b Dnmt2
a b a a Dnmt3
Day 0
Day 1 QMP-
Day 1 QMP+
Day 4 QMP-
Day 4 QMP+
Relative expression
0
1
2
b)
Histone modifiers
Writers
Erasers
Reader
a b a a Kat2a
a b a b Kat3b
a b a a Kat6b
a a a a Kat7
a b a a Kat8
a b a a Hdac1
a b a a Hdac3
a b a a Sirt1
a a a a Sirt6
a b a b Sirt7
a b a a Rcs1
Day 0
Day 1 QMP-
Day 1 QMP+
Day 4 QMP-
Day 4 QMP+