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DNA methylation affects the lifespan of honey bee (*Apis mellifera* L.) workers – Evidence for a regulatory module that involves vitellogenin expression but is independent of juvenile hormone function

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ABSTRACT

The canonic regulatory module for lifespan of honey bee (*Apis mellifera*) workers involves a mutual repressor relationship between juvenile hormone (JH) and vitellogenin (Vg). Compared to vertebrates, however, little is known about a possible role of epigenetic factors. The full genomic repertoire of DNA methyltransferases (DNMTs) makes the honey bee an attractive emergent model for studying the role of epigenetics in the aging process of invertebrates, and especially so in social insects. We first quantified the transcript levels of the four DNMTs encoding genes in the head thorax and abdomens of workers of different age, showing that *dnmt1a* and *dnmt3* expression is up-regulated in abdomens of old workers, whereas *dnmt1b* and *dnmt2* are down-regulated in heads of old workers. Pharmacological genome demethylation by RG108 treatment caused an increase in worker lifespan. Next, we showed that the genomic DNA methylation status indirectly affects vitellogenin gene expression both *in vitro* and *in vivo* in young workers, and that this occurs independent of caloric restriction or JH levels, suggesting that a non-canonical circuitry may be acting in parallel with the JH/Vg module to regulate the adult life cycle of honey bee workers. Our data provide evidence that epigenetic factors play a role in regulatory networks associated with complex life history traits of a social insect.

1. Introduction

DNA methylation by chemical modification of cytosines on CpG dinucleotide sites has been suggested as an important factor in gene regulatory networks involved in aging, with strong evidence coming from methylome analyses that contrasted human newborns and centenarians (Heyn et al., 2012). Nonetheless, the full understanding on how DNA methylation impacts on aging remains far from conclusive, especially since the best studied animal models for aging, *Caenorhabditis elegans* and *Drosophila melanogaster*, lack important components of the DNA methylation machinery. In the absence of DNA methyltransferase (DNMT) enzymes, *C. elegans* completely lacks CpG methylation (Gabor Miklos and Maleszka, 2011), and in *D. melanogaster*, which has only a *dnmt2* ortholog, but no homologs of the *dnmt1* and *dnmt3* genes in its genome, DNA methylation is restricted to only few CA- and CT-rich loci in certain developmental contexts (Kunert et al., 2003; Takayama et al., 2014).

Having a complete repertoire of DNMTs encoded in its genome (Wang et al., 2006) the honey bee, *Apis mellifera*, has emerged as an interesting model for investigating the role of DNA methylation in invertebrates. Since CpG methylation is largely restricted to gene bodies, and is not widespread over promoter regions, identifying and connecting DNA methylation with specific gene functions, such as alternative splicing, could thus be a simpler task than is the case with mammals (Lyko et al., 2010; Wang et al., 2006). Furthermore, the phenotypic plasticity exhibited in the adult females, which includes drastic morphological and functional caste differences associated with reproduction and longevity, also shows great potential for studies on aging and senescence (Cash et al., 2005; Withers et al., 1993).

Adult honey bee workers typically last only 30–45 days, but can live for up to six month as winter (*diutinus*) bees in temperate climates, allowing colonies to survive through a long winter season (Page and Peng, 2001; Winston, 1987). This variability in worker lifespan has been shown to be associated with their flight activity,

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essentially with the transition from intranidal tasks, especially brood care, to the more risky tasks of foraging. This major transition in the life cycle of an adult worker involves both dietary and physiological changes, including the switch from a carbohydrate/protein to a pure carbohydrate diet, associated with a drop in hemolymph vitellogenin (Vg) levels and an increase in the hemolymph juvenile hormone (JH) titer (Hartfelder and Engels, 1998). This physiological transition, which promotes foraging behavior, is conceptually formulated as a dual repressor model (Amdam et al., 2005; Amdam and Omholt, 2002), wherein high Vg levels in young workers suppress JH synthesis, and consequently maintain a basal JH titer (Guidugli et al., 2005). With the transition to foraging, the JH levels increase and suppress *vg* gene expression (Elekonich et al., 2001). This mutually repressive Vg/JH association in honey bees, which is a novelty in insects, has conceptionally been associated with the apparent disruption of the reproduction/lifespan trade-off, a paradigm that permeates life history theory of animals (Flatt et al., 2013; Rodrigues and Flatt, 2016).

More recently, another important life history link has been evidenced, a link between JH and DNA methylation that affects honey bee caste development during the larval stages. (Foret et al., 2012; Kucharski et al., 2008) have shown by RNA interference that the knockdown of *dnmt3* gene function affected the size of the *corpora allata*, the glands that produce JH, and induced a queen-like phenotype, especially so in the ovary. Subsequently, Foret et al. (2012) demonstrated that genes of the JH signaling pathway were differentially methylated in queen and worker larvae. Hence, a question that emerged was whether DNA methylation might also play a role in the already well-established Vg/JH regulatory circuitry that underlies the behavioral transition in adult honey bee workers.

We investigated this by monitoring the expression of the DNMT genes in different age classes of honey bee workers, and we could show that *dnmt3* transcript levels in the abdomen are positively associated with age. Next, by treating adult workers with RG108 we pharmacologically inhibited DNMT activity. Such a reduction in global DNA methylation resulted in an increase in worker longevity. This lifespan increase was also found associated with an increase in *vg* expression, but without affecting JH levels in hemolymph. Based on these data we propose a non-canonical circuitry involving DNA methylation and Vg, as well as other not yet characterized components. We consider that this circuitry acts in parallel with the already well-established Vg/JH module that regulates life history plasticity in honey bees.

2. Material and methods

2.1. Bees

Workers of Africanized *A. mellifera* hybrids were obtained from the Experimental Apiary of the University of São Paulo at Ribeirão Preto, Brazil. Brood frames containing ready-to-emerge worker brood were kept in an incubator (34 °C) and checked daily for bees that had emerged within a 1–20 h interval. These bees, which were considered 0-day-old, were paint-marked and re-introduced into the colony of origin, and were sampled once they had reached the desired age (1 day, 3 days, 1 week, or 1 month).

2.2. RNA extraction and quantitative analysis of transcript levels

Upon sampling, the bees were immediately anesthetized on ice and the head, thoracic and abdominal body segments were separated. These were then directly transferred into TRIzol reagent (Invitrogen) and snap frozen for storage at −80 °C. In all experiments, the stinger and gut were removed from the abdomens. For each age class, five individual replicates were prepared. RNA extraction was done using a TRIzol (Invitrogen, Carlsbad, CA) protocol following the manufacturer's instructions, and 8 μL of each RNA sample was treated with RNase-free DNase (Invitrogen). RNA concentrations and quality were measured spectrophotometrically (NanoView Plus; GE Healthcare Life Sciences, Pittsburgh, PA). First-strand cDNA synthesis was done using 2 μg aliquots of DNase-treated RNA, Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen). The obtained cDNA was then 10x diluted in deionized (Direct-Q, Millipore) water.

Quantitative PCR (RT-qPCR) assays were carried out with 1 μL of diluted cDNA, 5 μL of SYBR Green Power Master Mix (Invitrogen), 1.25 pmol of each specific primer (Table S1 – Supplementary Material) and water to complete the 10 μL final volume. The assays were run in a StepOnePlus system (Applied Biosystems, Foster City, CA) with three technical replicates per biological replicate under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After each assay, a melting curve analysis was run to monitor product specificity. The detection threshold was adjusted manually for each primer set. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression of the following genes was assessed: *dnmt1a*, *dnmt1b*, *dnmt2*, *dnmt3*, *vitellogenin*, *foxo*, *krüppel-homolog 1* and *tor*. The ribosomal protein encoding gene *rp49* (also known as *rpl32*) was used in all RT-qPCR reactions as endogenous control for normalization; the gene has previously been validated for use in honey bee RT-qPCR assays (Lourenço et al., 2008). Primer efficiency was calculated as E = 10[−1/slope], based on a 1 : 10 dilution series of a cDNA sample. An internal sample of each primer was used for calibration of the entire group. For further information, see Table S2 (Supplementary material).

2.3. RG108-treatment of bees: survival and food intake

In a pilot experiment, 1 μL of a 0.2 mM solution of the DNA methylation-inhibiting reagent RG108 (Cayman Chemicals, Ann Arbor MI) diluted in DMSO (Merck, Darmstadt, Germany) was topically applied on the thorax of 50 newly emerged workers. Fifty control bees were treated with DMSO only. RG108 was chosen for this experiment due to its slow decay rate and proven effectiveness with honey bees (Biergans et al., 2015; Brueckner et al., 2005), including topical treatment experiments on the thorax (Lockett et al., 2010). Following treatment, the bees were kept in 8 × 11 × 13 cm cages in queenless condition in an incubator (34 °C, 70% RH). They received sucrose (50%) and water *ad libitum*. Subsequently, the experiment was repeated four times with an increased number of bees per cage (n = 80). In these experiments, we also quantified food intake by weighing the sucrose solution remaining in the feeding tube after each 24 h interval and dividing total consumption by the number of alive bees.

2.4. In vitro and in vivo effects of RG108 on aging-related gene expression, vitellogenin and JH levels

To test whether DNA methylation affects the expression of aging-related candidate genes, we quantified the expression of vitellogenin (*vg*), forkhead box O (*foxo*) and target-of-rapamycin (*tor*), as well as the JH-response gene krüppel-homolog 1 (*kr-h1*). For the *in vitro* assays, five abdomens from 3-day-old workers were each cut in half and incubated for 6 h in Grace's insect medium (Sigma-Aldrich, St. Louis, MS) supplemented with 0.2 mM of RG108 in DMSO (experimental group) or DMSO only (control group), in a pairwise test design. RNA extraction, cDNA synthesis and RT-qPCR assays were done as described above.

Since *vg* expression was significantly affected by RCG108