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Faculty of Chemistry, Biotechnology and Food Science

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Oslo, May 2023

Erik M. K. Rasmussen

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Abbreviations

5caC - 5-carboxylcytosine

5fC- 5-formylcytosine

5hmC - 5-Hydroxymethylcytosine

5mC - 5-Methylcytosine

BER - Base excision repair

CpG - 5'—Cytosine—phosphate—Guanine—3'

DCC - Double Cohort Colony

DHF - Dihydrofolate

DNMT - DNA Methyl Transferase

TET – Ten – eleven translocation methylcytosine dioxygenase

ELISA - Enzyme-linked Immunosorbent Assay

ESI – Electrospray ionization

FDR - False Discovery Rate

GC - Guanine Cytosine

HAT - Histone Acetyltransferase

HDAC - Histone Deacetylase

HPLC - High Performance Liquid Chromatography

HRP - Horse Radish Peroxidase

LC - Liquid Chromatography

LC-MS/MS - Liquid Chromatography tandem Mass Spectrometry

m/z – Mass-to-charge ratio

MeDIP-seq - Methylated DNA Immunoprecipitation sequencing

MS – Methionine synthase

PCR - Polymerase Chain Reaction

qPCR - quantitative PCR

SAM - *S*-Adenosyl methionine

SCC - Single Cohort Colony

SCFA – Short-chain fatty acid

SNP - Single Nucleotide Polymorphism

WGBS - Whole Genome Bisulfite Sequencing

List of papers

I. Münch, D., Baker, N., Rasmussen, E. M. K., Shah, A. K., Kreibich, C. D., Heidem, L. E. & Amdam, G. V. (2013). Obtaining Specimens with Slowed, Accelerated and Reversed Aging in the Honey Bee Model. *J Vis Exp* (78): e50550. doi: doi:10.3791/50550.

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IV. Rasmussen, E. M. K., Seier, K. L., Pedersen, I. K., Kreibich, C., Amdam, G. V., Münch, D. & Dahl, J. A. (2021). Screening bioactive food compounds in honey bees suggests curcumin blocks alcohol-induced damage to longevity and DNA methylation. *Scientific Reports*, 11 (1): 19156. doi: 10.1038/s41598-021-98614-4.

Abstract

Intergenic DNA methylation is widespread in both the animal and plant kingdoms. Its presence in exons is believed to be dynamic and suggests its involvement in the regulation of alternative splice variants. In addition, the relatively recently discovered enzymatically oxidized forms of 5mC, such as 5fC, 5caC, and 5hmC, seem to be involved in active demethylation. However, their role is poorly understood.

Gene body methylation could be influenced by bioactive food compounds, a class of non-macromolecular compounds that are mainly plant-derived. Ethanol, a versatile solvent of many bioactive food compounds, is one of the most detrimental substances abused in Europe and a leading cause of death in the US. Nonetheless, the effects of chronic ethanol consumption on gene body methylation remain largely unstudied.

Using the honey bee as a model organism, this thesis seeks to investigate the effects of bioactive food components and ethanol on survival and intragenic DNA methylation. Additionally, by including enzymatically oxidative 5mC derivatives, this work seeks to extend the understanding of invertebrate DNA methylation in a general and social context.

Honey bees are a good model for studying intragenic methylation, as DNA methylation is mostly confined to gene bodies, as opposed to mammals, where the bulk of DNA methylation is located at promoters and repeat elements.

My findings indicate that gene body methylation can be influenced externally by bioactive food compounds. Specifically, ethanol induces damage to lifespan and DNA methylation, but these changes are blocked by curcumin, the main compound isolated from the rhizome of the turmeric plant. These DNA methylation changes were present in genes involved in fertility, temperature regulation, and tubulin transport. Additionally, my data indicate that isovaleric acid, valproic acid, cyanocobalamin, and folic acid can also modulate the honey bee's lifespan. Furthermore, by employing a diversity of honey bee workers of similar chronological age, we quantified levels of 5mC, 5hmC, 5fC, and 5caC in brain and abdominal tissue, identifying higher levels of 5hmC in the fat body than in the brain. However, 5fC and 5caC were not detected, meaning that these modifications, if at all present, are below the detection limits of our assay.

This thesis adds to the growing understanding of DNA methylation, possible external influencers, and its oxidative derivatives in honey bee workers.

Abstrakt

Intragen DNA-metylering er utbredt i både dyre- og planteriket. Dets tilstedeværelse i eksoner antas å være dynamisk og å regulere uttrykk av alternative spleisede genvarianter. I tillegg går man ut ifra at de relativ nylig oppdagede enzymatisk oksiderte formene av 5mC, som 5fC, 5caC og 5hmC deltar i aktiv demetylering. Men deres rolle i denne prosessen er uavklart.

Genkroppmetylering kan bli påvirket av bioaktive matforbindelser, en klasse ikke-makromolekylære stoffer som hovedsakelig er plantederiverte. Etanol, et allsidig løsemiddel for mange bioaktive matforbindelser, er et av de mest skadelige stoff som blir misbrukt i Europa og er en ledende dødsårsak i USA. Effekten av kronisk etanolinntak på genkroppmnetylerig har stort sett ikke blitt studert.

Ved å bruke honningbien som modellorganisme vil denne avhandlingen undersøke effektene av bioaktive matforbindelser og etanol på overlevelse og intragen DNA-metylering. I tillegg, ved å inkludere de enzymatisk oksiderte 5mC derivatene søker denne avhandlingen å utvide forståelsen av invertebrat DNA-metylering i en generell og sosial kontekst.

Honningbier er en god modell for å studere intragen metylering da DNA-metylering er stort sett begrenset til genkropper, i motsetning til pattedyr hvor hoveddelen av DNA-metylering er lokalisert til promotere og repeterte elementer.

Mine funn indikerer at genkroppmetylering kan bli påvirket eksternt av bioaktive matforbindelser. Mer spesifikt kan etanol indusere skade til overlevelse og DNA-metylering, men disse forandringene blir blokkert av kurkumin, hovedkomponenten isolert fra rhizomet av gurkemeie. Disse DNA-metyleringsforandringene var til stede i gener involvert i fertilitet, temperaturregulering og tubulintransport. Videre indikerer mine data at isovalerinsyre, cyanokobalamin og folsyre også kan endre honningbiens levelengde. Videre, ved å benytte en mengde forskjellige honningbiearbeidere med samme kronologiske alder kvantifiserte vi nivåer av 5mC, 5hmC, 5fC og 5caC i hjerne- og abdominalvev, og identifiserte høyere nivåer av 5hmC i abdominalvev enn i hjerne. 5fC og 5caC ble ikke oppdaget, noe som indikerer at disse modifikasjonene, hvis til stede overhodet, er under deteksjonsnivået på vårt assay.

Hovedmålet med denne avhandlingen er å gi enn bredere forståelse av DNA-metylering, mulige eksterne påvirkere, og det oksidative derivater i honningbie-arbeidere.

1.1 Introduction

1.2 Honey bee biology

The well-ordered society of the European honey bees (*Apis mellifera* Linnaeus) has fascinated humankind since ancient history. The ancient Greek philosopher and scientist Aristotle included the thoughts and hypotheses of the inner workings of the honey bee colony in his works (Aristotle, 1862). The honey bee has been used as a model organism to advance our understanding of science in numerous fields, including ethology, neurobiology, sociogenomics, and aging. The Nobel Laureate Karl von Frisch stated, "The bee's life is like a magic well: the more you draw from it, the more it fills with water." Research by von Frisch and others over the last two centuries has greatly contributed to the field of ethology by increasing our understanding of how the social structures of the honey bee colony are regulated and maintained (Frisch, 1923; Gruter & Farina, 2009).

In neurobiology, research by Randolph Menzel and colleagues has led to a more unified understanding of how and when bees interpret color (Menzel & Backhaus, 1989). Gene Robinson coined the term "sociogenomics," which is a relatively new research field particularly concerned with how the environment influences behavioral developmental plasticity (Robinson, 1998; Shpigler et al., 2017; Suryanarayanan, 2019). Research by Gro Amdam and colleagues has considerably increased our knowledge of aging and how honey bees regulate it through hormonal and social control (Amdam et al., 2004; Amdam, Gro V. et al., 2005).

Honey bee colonies are complex social structures with around 30,000 workers in a colony, and they require precise regulation on many different levels (Seeley, 1995). These levels include the colony level, behavioral regulation at the individual level, and nutritional homeostasis at the cellular level. The multiple levels of regulation are linked to eusociality, another hallmark feature of honey bee societies. Eusociality is the phenomenon where multiple generations of adult individuals are present in a hive, cooperate in caring for larvae, and are divided into reproductive (queen) and non-reproductive (worker) castes (Wilson & Hölldobler, 2005). In honey bees, closely related half-sisters (e.g., 0.75–0.25 by haplo-diploid reproduction) make up the majority of individuals within a colony (Seeley, 1995). This consequently leads to thousands of individuals supporting just one reproducing queen. These siblings also have very different phenotypes, causing very different life histories. From early

on in the research history, it was clear that these phenotypic differences were caused by mechanisms other than common allelic heterogeneity.

One mechanism that might partially explain some of the observed differences and that has gained popularity in recent years is epigenetics. Epigenetics is "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Adrian Bird 2007). Major players in epigenetics are post-translationally modified histone tails and cytosine modifications in DNA. These adaptations and modifications can be hereditary, adding another layer of complexity to classical DNA inheritance.

One type of regulation at the colony level is the regulation of reproduction. Reproduction is assigned to a single female (the queen), while worker tasks such as brood rearing, nectar foraging, comb construction, and hive defense are performed by essentially sterile female sister workers (Winston, 1991). All larvae are fed a highly nutritious food blend called royal jelly. However, larvae destined to become workers are fed less nutritious floral pollen after the third instar, while larvae destined to become queens are continued to be fed royal jelly until pupation. Royal jelly contains fatty acids that can influence the epigenome by altering specific histone modifications (Spannhoff et al., 2011). The male honey bees' (drones) purpose is solely reproduction.

Another layer of regulation is the regulation of worker tasks. A honey bee colony needs to have a balance of bees performing different worker tasks to avoid wasting resources. Adult worker honey bees usually start by caring for the brood (nursing) and progress to foraging (food collection of nectar, pollen, or water) weeks later (Winston, 1991). This progression is dynamic according to the needs and conditions of the hive, so older foragers can revert to nursing tasks if conditions within the hive favor it. Foraging is a demanding task for honey bee workers compared to hive bees, who do not have to spend energy on flying, navigation, and predator evasion, among others. This leads to more rapid senescence in foraging bees compared to hive bees even when their chronological age is the same (Tolfsen et al., 2011). Advanced senescence manifests itself as a functional decline in cognitive abilities, including learning and memory, increased physical damage (e.g., wing wear and hair reduction on the thorax), increased mortality risk, and increased cellular senescence (Münch et al., 2013). Foragers can sense the time it takes for them to unload their foraging cargo and use it as a measure of the relative number of nurse bees in the hive (Seeley, 1995). Some foragers will revert to nursing tasks if the waiting time to unload their cargo is too long.

By exploiting the mechanism behind natural reversion, researchers can disentangle aging from social tasks, making it possible to obtain age-matched individuals with different social tasks (Huang & Robinson, 1996; Robinson et al., 1992). In practice, this is done by removing nurse bees from combs with brood, forcing some of the foragers returning from forage trips to take care of the brood. By using elaborate marking schemes, researchers can keep control of which worker bees that do not deviate from their natural task progression and which foragers have reverted to nursing tasks. Amazingly, reverted nurses also display cognitive improvement compared to their age-matched foragers, where the yolk precursor protein Vitellogenin is a key modulator in the regulation of aging (Amdam et al., 2004; Baker et al., 2012). Thus, reverted nurses will have senesced less than continued foragers, although physical damage persists.

Although the generation of reverted nurses is very similar, there are distinct methodological differences between these previously mentioned publications (Baker et al., 2012; Huang & Robinson, 1996; Robinson et al., 1992). Robinson and colleagues (1992) used an observation hive, which greatly facilitates observations of nursing behavior, but may not be accessible to every prospective research groups. Huang and Robinson (1996) divided the generation of bees with different aging characteristics into separate experiments, greatly increasing the capacity for the generation of focal bees, while simultaneously making some inferences harder due to the nature of the different experimental conditions. Specifically, for the generation of reverted nurses, the authors established a colony comprised solely of foragers from a typical colony, thus generating numerous reverted nurses, but without precise control of chronological age which might limit the conclusions that can be drawn. Baker and colleagues (2012) used colonies solely consisting of close to minimal amounts of focal nurse bees (Seeley, 1995). This unnaturally accelerates forager transition and additionally makes the hive susceptible to robbing, if performed later in the season when nectar sources are scarce. Collectively, we reasoned, the scientific community could benefit from a more uniform, reproducible approach, which is based on a detailed reversion protocol with a reliable indicator of senescence, as chronological aging can be a confounding factor.

To assess cellular senescence, the biomarker lipofuscin is often used. Lipofuscin is often considered a hallmark of aging (Di Guardo, 2015; Terman & Brunk, 2004). Although mainly consisting of proteins and lipids, it is believed that Schiff bases and other unknown compounds give lipofuscin its auto-fluorescing properties, making it easy to visualize using microscopy methods. The origins of lipofuscin are not clear but are believed to come from

autophagocytosed cellular material that cannot be exocytosed or degraded and thus accumulate over time. In honey bees, lipofuscin can be found in several cell types, such as trophocytes in the fat body and neurons in the brain (Gray & Woulfe, 2005; Hsieh & Hsu, 2011a). This makes lipofuscin an easily exploitable biomarker of cellular senescence in honey bees.

Central to the regulation of individual behavior is the brain. The honey bee brain is relatively small, with about 960,000 neurons, but it is still capable of orchestrating complex behavior (Menzel & Giurfa, 2001; Witthöft, 1967). In addition, the honey bee brain can be electrophysiologically manipulated relatively easily, while the bee is engaged in learning and responding (Homberg, 1984; Menzel & Müller, 1996). These features, along with moderate body size, have made the honey bee brain a popular object of study among biologists and neurobiologists. The honey bee brain has been extensively mapped with a comprehensive three-dimensional atlas available for researchers (Brandt et al., 2005; Rybak et al., 2010).

A tissue also important for maintaining social behavior roles is the fat body. The fat body acts as nutrient storage and is the site of production for many vital hormones, including juvenile hormone, vitellogenin, and insulin-like peptides (Amdam et al., 2004; Ament et al., 2008; Corona et al., 2007). This makes the fat body a valuable driver in the regulation of temporal castes when workers progress from nursing to foraging (Amdam et al., 2004). In addition, the fat body itself has been shown to undergo large changes when workers progress from nursing to foraging (Chan et al., 2011). In adults, the fat body is situated as a thin cell layer just underneath the abdominal cuticle (Snodgrass, 1910). It consists mainly of two cell types: trophocytes and oenocytes (Dean & Locke, 1985). The trophocytes are fat cells with numerous vacuoles and globules of fatty acids (Snodgrass, 1910). They are functionally homologous to white adipose tissue in mammals. Trophocytes are mesodermal in origin (Wheeler, 1892). They arise from the embryonic cell layer, which is between the cell layer forming the outer body wall and the cell layer forming the embryonic alimentary canal. Oenocytes are generally larger than trophocytes (Koschevnikov, 1900). They are functionally homologous to mammalian liver cells (Boehm, 1964), and they play a role in wax secretion and lipid synthesis (Makki et al., 2014; Rösch, 1930). Unlike trophocytes, oenocytes are derived from the internal proliferation of ectodermal cells (Wheeler, 1892).

1.3 Honey bee genome sequencing

Having a map of the genome at a single-base resolution is highly valuable for model animals in molecular biology. The first draft sequence of the honey bee genome was published in 2006 (The Honeybee Genome Sequencing Consortium), with an improved draft published in 2014 (Elsik et al., 2014), and in 2019, by the use of a hybrid sequencing approach, a further improved draft was published with much-enhanced sequencing statistics (Wallberg et al., 2019). Sequencing revealed some characteristic points of the honey bee genome:

- Higher adenine + thymine content than comparable insects
- Fewer genes for innate immunity than the fruit fly (*Drosophila melanogaster*)
- More resemblance to vertebrate genomes concerning genes regarding RNA interference and DNA methylation

The last point regarding DNA methylation was a revelation for the research community. Because *Drosophila melanogaster* lacks the key enzymes necessary for DNA methylation, it was assumed that all insects had lost this feature. However, part of the epigenetic machinery governing DNA methylation soon proved to be functional (Wang et al., 2006).

1.4 Honey bee epigenetics

As mentioned before, epigenetics includes the posttranslational modification of core histones and enzymatic nucleotide modifications in DNA. Histone tails can be modified in different ways. For example, an addition of a methyl group to the 9th lysine of histone 3 (H3kme3) leads to chromatin compaction, and transcriptional repression of underlying genes. On the other hand, acetylation of the same lysine on the same histone (H3K9ac), leads to opening of the chromatin and transcriptional activation of underlying genes. Thus, histone acetylation marks tend to surround promoters and gene bodies of actively transcribed genes. Acetylation marks are added enzymatically by histone acetyl transferases (HATS) and removed actively by histone deacetylases (HDAC). Small organic compounds that inhibit HDAC activity are termed HDAC inhibitors (HDACi). Several studies have demonstrated crosstalk between DNA modifications and histone modifications, indicating that these layers of regulation are interconnected (Dhayalan et al., 2010; Du et al., 2015; Meissner et al., 2008; Mikkelsen et al., 2007; Nan et al., 1998; Ooi et al., 2007). For instance, DNA methylation can recruit HDACs (Tan & Nakielny, 2006) Although not part of Adrian Birds' definition of epigenetics (see Section 1.2, above), many researchers have also included RNA modifications in their definition of epigenetics.

One type of nucleotide modification in DNA is the addition of a methyl group to the 5' carbon of cytosine, which is catalyzed by a group/family of enzymes called DNA methyltransferases (DNMTs). DNMT1 is the enzyme that methylates newly synthetized DNA during replication, while DNMT3 is responsible for methylating loci *de novo*, with some aid from DNMT1 (Fatemi et al., 2002). DNMT2, on the other hand, is primarily a t-RNA methylase (Goll et al., 2006). Multiple studies of 5-methylcytosine and DNMT3 in honey bees have indicated a role in social behavior (Herb et al., 2012; Kucharski et al., 2008; Lyko et al., 2010). Deep sequencing studies have revealed that these changes in social behavior might be linked to the expression of different splice variants facilitated by the methylation of intron–exon borders (Herb et al., 2012). In mammals and honey bees, most methylated cytosines are found preceding a guanine, more commonly denoted as a CpG dinucleotide (Law & Jacobsen, 2010; Lyko et al., 2010). Since DNA is double-stranded, this means that a CpG site can be methylated on both strands, or hemi-methylated, meaning it is methylated on only one strand. The honey bee genome is sparsely methylated (approximately 0.5% of CpGs) compared to mammalian genomes (from 25–80% of CpGs, depending on the tissue and developmental stage) (Heyn et al., 2012; Lyko et al., 2010; Okae et al., 2014). This scarce methylation pattern has the advantage of more easily discriminating between subtle differences in methylation intensities in honey bees, which is often the case with intragenic methylation.

In mammals, the removal of 5mC is thought to happen mainly via two pathways (Pastor et al., 2013): either by failing to maintain methylation during DNA replication or by active removal with the help of the Ten -eleven translocation methylcytosine dioxygenase (TET) and the base excision repair (BER) pathway. In mammals, the TET family of dioxygenases is responsible for oxidizing 5mC to 5-hydroxymethylcytosine (5hmC), which can be further oxidized to 5-formylcytosine (5fC) and eventually to 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009) (Figure 1). 5caC, and to some extent 5fC, is recognized by the thymine DNA glycosylase (TDG) and can potentially be removed by this enzyme, leading to a restoration of unmodified cytosine (Maiti & Drohat, 2011). The honey bee genome encompasses a single TET homologue (mammals have three, although not all, are active in all stages of development) and a TDG homologue (Pastor et al., 2013; Wallberg et al., 2019; Wojciechowski et al., 2014). Although 5hmC is an intermediate in active demethylation, several studies in mammals have uncovered 5hmC-specific binding proteins, suggesting that 5hmC has a specific function in addition to being implicated in active demethylation (Frauer et al., 2011; Méllen et al., 2012; Spruijt et al., 2013; Zhou et al., 2014).

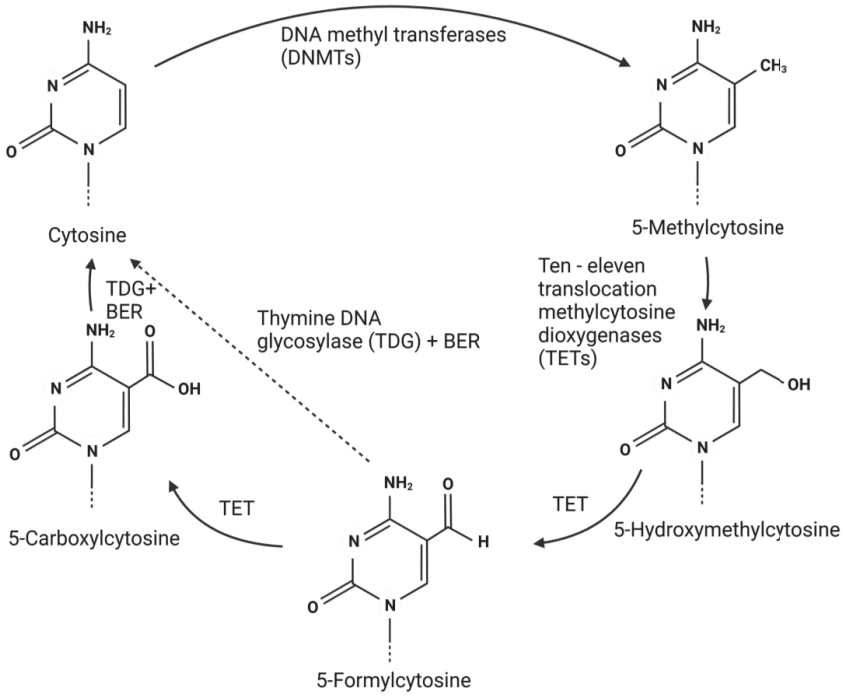

Figure 1: Active demethylation of 5mC, as depicted in mammals. BER- Base excision repair pathway . Created with www.biorender.com

In honey bees, the TET homologue has been found to be catalytically active, and two studies have confirmed the existence of 5hmC (Cingolani et al., 2013; Wojciechowski et al., 2014). As with 5mC, 5hmC seems to be present at a much lower concentration (~ 100–1000 fold) compared to mammals (Wojciechowski et al., 2014). However, the estimation of the global 5hmC content varied greatly between the reported studies.

1.5 Bioactive food compounds and possible effects on health and the epigenome

The lifespan of an animal can be affected by external factors like social interaction and diet, which, apart from macronutrients, include micronutrients that can be vital to health. These factors can also influence the epigenome. In honey bees, dietary differences can severely impact the lifespan of the bee.

As mentioned previously (Section 1.2), the differential feeding of workers and queens results in individuals with relatively extreme differences in expected lifespan (weeks versus years). These phenotypic differences were originally believed to be heavily influenced by differences

in DNA methylation, however, later findings have questioned the contribution of DNA methylation in maintaining caste differences (Herb et al., 2012; Kucharski et al., 2008; Lyko et al., 2010). As for royal jelly, attempts to identify a single compound responsible for the induction of queens have been made, nevertheless, none have been universally accepted by the scientific community (Buttstedt et al., 2016; Kamakura, 2011; Kamakura, 2016; Kucharski et al., 2015; Spannhoff et al., 2011).

Bioactive food compounds are usually smaller molecules, or sometimes present in an inactive form in larger complexes and needs to be metabolized, either by the host itself or by the gut microbiota. An example of compounds that also span both definitions are B-vitamins. Although many animals can synthesize most B-vitamins themselves, the direct dietary contribution and the indirect contribution from gut microbes producing B-vitamins are often non-negligible. Many B-vitamins are important players in one-carbon metabolism, acting as either co-enzymes or as methyl donor precursors (Anderson et al., 2012). For example, folic acid is a precursor that feeds early into the pathway by first being converted to dihydrofolate (DHF). DHF is then enzymatically converted several times to 5-methyl-tetrahydrofolate, which acts as a methyl donor for methionine synthase (MS) which generates methionine, which can be further processed into S-Adenosyl methionine (SAM), the direct methyl donor for DNA methylation (Banerjee & Matthews, 1990). Cobalamin acts as a co-enzyme for MS and is thus crucial for maintaining the integrity of the DNA methylome. Deficiencies in cobalamin have been linked to aberration of DNA methylation in adult rats and in their offspring, likely increasing the risk of attaining cancer and metabolic diseases (Tanwar et al., 2020). Cobalamin is usually not supplemented directly, but in the form of cyanocobalamin. Cyanocobalamin is readily converted to the active form of B12 is most animals (Hannibal et al., 2009; Watanabe & Nakano, 1997). Supplementation of cyanocobalamin has been shown to reduce atrophy in humans with mild cognitive impairment (Smith et al., 2010). Deficiencies in folate can lead to neural tube defects in the human fetus. Thus, women who are trying to conceive are advised to take folate supplements up until the first trimester (MRC Vitamin Study Research Group 1, 1991). Moderate folate deficiency has also been associated with changes in DNA methylation patterns in zebrafish, and these patterns are inherited to the next generation (Skjærven et al., 2016; Skjærven et al., 2018). This suggests that folate deficiency can leave a robust mark on the methylome.

Examples of bioactive food compounds that do not need prior metabolization to exert effects include resveratrol. Resveratrol is a polyphenol found in grapes, plums, apples, blueberries,

and peanuts (Koushki et al., 2018). Potential health benefits of resveratrol have been extensively studied in humans (Singh et al., 2019). Nevertheless, clear effects have been difficult to establish (Sahebkar et al., 2015; Vang et al., 2011). In breast cancer models, resveratrol has been reported to influence DNA methylation in a protective manner (Medina-Aguilar et al., 2016). In honey bees, resveratrol has been found to increase lifespan under normal conditions, still, further studies are needed to corroborate these findings and hopefully gain potential insights into the mechanisms behind the reported lifespan increase (Rascon et al., 2012).

Caffeine, a stimulant of the central nervous system in humans, and mostly consumed in the form of coffee, tea, or cola, seems to confer cardiovascular and neuroprotective effects (O'Keefe et al., 2018; Zhou & Zhang, 2021). In humans, coffee consumption seems to have little effect on DNA methylation (Ek et al., 2017; Polinski et al., 2022). In honey bees, caffeine administration has been demonstrated to upregulate genes involved in neuronal development and glutamine metabolism, and improve cognitive performance in complex learning tasks (Kucharski & Maleszka, 2002; Si et al., 2005). Additionally, caffeine has been demonstrated to increase lifespan and global DNA methylation in honey bees (Strachecka et al., 2014; Yusaf, 2012). On the other hand, Strachecka and colleagues (2014) reported global DNA methylation levels between approximately 5-25%, where other studies using different methods have never reported global levels above 2% (Biergans et al., 2015; Foret et al., 2012; Lyko et al., 2010). Additionally, the authors reported a 10% increase in global DNA methylation levels in 32-day old control bees compared to 25-day old control bees. This corresponds to roughly 6 million cytosine methylated over the course of one week. This phenomenon has not been reported earlier.

Another bioactive food compound that does not need prior metabolization to exert its biological activities is curcumin. Curcumin is one of the main active curcuminoids isolated from the rhizome of the turmeric plant. Turmeric is also the common name of the spice from the same rhizome. Curcumin has been shown to infer health benefits by protecting against a range of different diseases (Gupta et al., 2013; Varatharajalu et al., 2016). However, exploring these leads further in controlled clinical setting is difficult due to the low bioavailability of curcumin (Burgos-Moron et al., 2010; Nelson et al., 2017). Ethanol is an effective solvent for curcumin, but care must be taken when designing experiments as ethanol disrupts the one-carbon metabolism, by inhibiting methionine synthase, thereby inducing folate deficiency (Suh et al., 2016).

In honey bees, curcumin has been shown to extend the lifespan in a Nosema (an intestinal parasite) infection setting (Strachecka et al., 2015). An increase in the antioxidant related enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase was also observed in the same study. The precise mechanism by which curcumin confers its positive effects is not yet known. Various clues have been unraveled in recent years, as curcumin can act as a topoisomerase II poison, causing affected cells to undergo apoptosis (Martin-Cordero et al., 2003). Curcumin has also been shown to inhibit DNA methyltransferases, histone acetyltransferases, and histone deacetylases in silico and in vitro (Balasubramanyam et al., 2004; Bora-Tatar et al., 2009; Kang et al., 2005). Curcumin's DNMT inhibitory effects are, however, still debated (Link et al., 2013; Liu et al., 2009; Medina-Franco et al., 2011). In honey bees, curcumin has been reported to delay an age-related increase in global DNA methylation with approximately 20 days (Strachecka et al., 2015). As in the caffeine study (Strachecka et al., 2014), Strachecka and colleagues (2015) reported global DNA methylation levels between approximately 5-25 % and an almost 10 % increase in global DNA methylation in seven days. These findings have not been reproduced outside of their lab so far.

An example of a class of bioactive food compounds that must be metabolized either by the host itself, or by the gut microbiota residing within the host, are the short chain fatty acids (SCFAs). These are fatty acids with fewer than six carbon atoms. Food sources include complex carbohydrates and fibers which are then metabolized by the gut microbiota to yield SCFAs (Cummings et al., 1987). Notable SCFAs include acetate, propionate, butyrate, valeric acid and isovaleric acid, where butyrate in its sodium salt form is one of the most studied SCFAs (Gerhauser, 2018). Butyrate, together with acetate and propionate have been shown to confer HDACi activity, with sodium butyrate proving to be the most potent (Stilling et al., 2014). Additionally, sodium butyrate has been shown to have numerous health beneficial effects. For example, in Alzheimer's disease models, sodium butyrate has been shown to slow disease progression (Silva et al., 2020). Additionally, sodium butyrate has been demonstrated to reduce inflammatory signaling in microglia in neurological diseases which an increased inflammatory state is part of the disease profile. With regards to immune function, sodium butyrate has been revealed to induce differentiation of T-regulatory cells via a combined HDACi and FFAR2 (A G-protein coupled receptor) activation activity.

Although strictly not a microbiota derived SCFA, valproic acid, a synthetic analogue of valeric acid has been used for decades in the treatment of epileptic seizures (Ghodke-Puranik

et al., 2013). Valproic acid, as with many other SCFAs, possesses HDACi activity (Phiel 2001). On the contrary, health and epigenetic effects of isovaleric acid remain largely unstudied, although there are studies linking altered gut microbiota and elevated isovaleric acid levels with depression (Szczesniak et al., 2016).

1.6 Honey bees in ethanol research

Honey bee foragers can encounter a range of different chemical compounds when they are out gathering nectar or pollen. Some of these compounds might be directly damaging to foragers. For instance, floral nectar can contain ethanol (de Vega et al., 2009). In humans, ethanol is one of the most dangerous substances to abuse in Europe and one of the leading causes of death in the US (Mokdad et al., 2004; Nutt et al., 2010; van Amsterdam et al., 2015). Most commonly, ethanol intake is associated with higher mortality risk (Stockwell et al., 2016). A systematic review by Stockwell and colleagues (2016) also failed to find any health benefits in groups with low or occasional alcohol consumption.

Rodent model organisms, such as mice and rats, have been used for decades in ethanol research. Unlike humans, rats and mice can develop conditioned taste aversion for ethanol, meaning that prolonged ethanol exposure is difficult to carry out with mice (Broadbent et al., 2002; Brunetti et al., 2002; Crabbe et al., 2019; Dyr et al., 2016; Lucas & McMillen, 2002). The inbred mice strains used in alcohol research also display large variations in ethanol tolerance (Rhodes et al., 2007; Risinger & Cunningham, 1995). Another potential issue with rodent models is the mode of ethanol administration. Mice and rats are preferentially intoxicated by ethanol vapors, bypassing any effect that the gut microbiome might have on ethanol metabolism (Holleran & Winder, 2017; Vendruscolo & Roberts, 2014). Nevertheless, insights gained from studies in mammals suggests that chronic ethanol intake reduces folate levels (Cravo et al., 1996; Halsted et al., 1971). As mentioned previously (See Section 1.5), folate deficiency leads to anomalous DNA methylation patterns (Skjærven et al., 2016; Skjærven et al., 2018). Ethanol also increases risk of cancer, probably trough DNA methylation mechanisms (Choi et al., 1999; Varela-Rey et al., 2013) . For instance, ethanol seems to downregulate expression of DNMTs, possibly because of a lack of SAM (due to folate deficiency), which again causes aberrant DNA methylation patterns.

Nonetheless, honey bees have been used for research on ethanol (Abramson et al., 2000; Scholz & Mustard, 2013). Honey bees have several advantages that set them apart from rodent models. They do not develop a conditioned taste aversion toward ethanol, meaning that

honey bees are probably not repelled by the taste of ethanol (Varnon et al., 2018). Not only are honeybees not repelled by ethanol, but they also seem to prefer ethanol-containing diets over other isocaloric diets (Mustard et al., 2019). After ingestion, ethanol levels in the honey bee hemolymph sharply increases, but appears to stabilize at around 2 hours (Bozic et al., 2007). Ethanol impacts honey bee survival, where negative effects have been found in concentrations down to 2.5% (Mustard et al., 2019). However, a single dose of a 35% ethanol solution did not significantly impact survival (Mura et al., 2020).

Furthermore, ethanol has demonstrated effects on honey bee behavior. For example, a high concentration of ethanol (20%) increases aggressiveness in Africanized honey bees (a more aggressive strain of honey bees compared to the western honey bee) (Abramson et al., 2004). Lower doses of ethanol (2.5- 5%) increase bouts of walking made by honey bees, but not at higher concentrations (Maze et al., 2006; Mixson et al., 2010). While again, higher concentrations of ethanol (50-75%), make honey bees spend more time upside down, stopped, and decreases bouts of walking (Maze et al., 2006). Additionally, ethanol increases tremble dance behavior at 5% but not at 1% (Bozic et al., 2006). Tremble dance behavior has been linked to stress, e. g. overcrowded or detrimental food sources (Jacobus Christiaan, 2003; Thom, 2003). Regarding flight behavior, ethanol influences wing kinematics differently depending on ethanol concentration (Ahmed et al., 2022). In aversive learning, ethanol induces analgesic effects, making honey bees less sensitive for shock with a linear increase in ethanol concentration (Giannoni-Guzman et al., 2014). All in all, ethanol effects on behavior seems to vary between studies were some studies report linear dose dependent effects, while other reports non-monotonic effects.

On a molecular level, there is a genetic component to ethanol sensitivity, enabling the selective breeding towards sensitive/resistant bees, and investigation of genes involved in ethanol sensitivity (Ammons & Hunt, 2008). Ethanol also increases levels of heat shock protein 70 (HSP70) in honey bee brain, yet, only at a 5% concentration, and not lower or higher (2.5% and 10% respectively) (Hranitz et al., 2010). In humans, HSP70 is involved in stress response (Morano, 2007; Tavaria et al., 1996).

2.1 Aims of the thesis

This thesis has four aims. The first aim is to perform a reversion experiment while thoroughly documenting the process to create a detailed protocol for the research community's use. While, additionally, documenting worker reversion and accelerated aging using molecular and behavioral techniques. The second aim is to contribute a mini review for researchers that are unfamiliar with DNA methylation in honey bees, and specifically discusses the enzymatically oxidized derivates of 5mC. This review will also promote the use of the honey bee as a model organism for researching gene body methylation in a social context. The third aim is to quantify the levels of 5mC, 5hmC, 5fC and 5caC in brain and abdomen tissues from the honey bee, while additionally quantifying 5mC and 5hmC in adult fruit flies, an organism where the existence of DNA methylation has historically been controversial. Using a screening approach, the fourth and final aim is to investigate effects of certain bioactive food compounds on lifespan and DNA methylation. Collectively, these aims address the overarching research goal of the thesis, which is to expand the knowledge of DNA methylation in honey bees, including 5hmC, 5fC and 5caC, in a nutritional and social context.

3.1 Methods and materials

3.2 Animal handling

The honey bees kept in this study ranged from a typical Norwegian beekeeping setting in an outdoor hive placement, as in Papers I and III, to a more artificial indoor cage system used in Paper IV.

In Papers I and III, the honey bees were obtained from the same experiment. This allowed us to use bees not in the direct analysis foci of Paper I. In animal research, this is an example of the 3Rs – replace, reduce, and refine – in which one always strives to minimize animal use when doing research (Russell & Burch, 1959; Russell et al., 2009).

Furthermore, the work on honey bees described in this thesis was carried out before regulations on the certified training of researchers and approval from the Norwegian Food Safety Authority came into force.

3.3 Outdoor bee handling

In Paper I, when setting up a reversion experiment, we chose the double cohort colony (DCC) strategy over the single cohort colony (SCC) strategy. An SCC means that all bees belong to the experimental cohort. In a DCC, only one group of bees belongs to the experimental cohort, with bees in the other cohort (usually selected with less stringency) acting as a reinforcing group. The SCC strategy has the benefit that it is easier to set up, as one does not need to mark emerging bees, and later on, marking returning foragers is easier, as all returning foragers are assumed to belong to the experimental group. However, the SCC strategy is more labor intensive, as these colonies require many donor hives and careful planning to synchronize the emergence of enough bees, or the SCCs would contain an insufficient amount of bees, leaving the hives prone to collapse, jeopardizing the experiment (Seeley, 1995).

Henceforth, we used a DCC strategy where unmarked bees with an undefined age span were added to cohorts of marked bees with a defined age span (Amdam, G. V. et al., 2005; Herb et al., 2012). This allowed the use of colonies with a greater number of bees, minimizing the risk of worker depletion, robbery, and other negative effects compared to a single cohort strategy.

3.4 Indoor laboratory housing of bees

The cage system used in Paper IV was an evolution of a cage designs previously used in our lab (Rojahn, 2013). We used wooden cages previously (Rangberg et al., 2015); however, the switch to plastic cages meant simplified cleaning and a higher reuse rate. For a photograph of

a typical cage, see Figure 2 below. The feeder design used in Paper IV consisted of drilling small holes into standard 15 ml falcon tubes. The holes had to be big enough to enable the bees to feed, but small enough to retain the liquid in the tubes by exploiting the weak negative pressure inside it. This was a challenging part of the cage design, which consisted of much trying and failing. During the work on Paper IV, we became aware of a paper comparing different cage and feeder designs (Huang et al., 2014). Unfortunately, our experiment progressed too far, so switching the cage design would be unfruitful. However, the feeder design from Huang and colleagues (2014) was adopted in later cage designs from our lab (Cervetti, 2020).

Figure 2: Overview of the early cage design used in this study. Adapted from Pedersen (2014).

3.5 LC-MS/MS

In Paper III, the main method used for the quantification of the different modified cytosines is liquid chromatography tandem mass spectrometry (LC-MS/MS). It is a method that combines the separation capabilities of high-performance liquid chromatography (HPLC) with the mass/charge ratio determination capabilities of the mass spectrometer.

The DNA samples used as input are first enzymatically hydrolyzed to single nucleosides as both oligomers and phosphate groups will interfere with detection later in the mass spectrometer [REF]. Deoxyribose is not removed to distinguish DNA derived bases from RNA derived bases, as traces of RNA are always present in preparations after DNA extraction.

Separation is based on the adsorption principle where the mobile phase, consisting of water and an organic solvent like methanol together with the mixture being investigated (the analyte), is passing through the stationary phase, usually consisting of chemically coated silica particles which reside inside the analytical column, at high pressure (Pitt, 2009). Different components of the mobile phase will have different interactions with the stationary phase, and thus affect the speed of which the different compounds pass through the analytical column. This makes it possible to physically separate the compounds in the analyte aiding in the detection of single compounds in combination with the tandem mass spectrometer. In order to extend analytical columns life, a guard column is often used upstream of the analytical column in order to remove contaminants and other problematic compounds that can damage the stationary phase of the analytical column. After passing through the analytical column and a detector, compounds of interest are then pumped through to the ionization source. The electrospray ionization (ESI) source is usually a metal capillary maintained at high voltage. When the liquid sample reaches the tip of the capillary, the samples are nebulized forming a spray of finely charged droplets. By adjusting the pH in the mobile phase one can influence the charges that the ions have after nebulization. This is done by adjusting the pH lower than the pKa of the nucleosides so the ions will more likely end up with a positive charge, which is more ideal for DNA bases. After nebulization, and by addition of heat and nitrogen, the droplets are evaporated, and the residual electric charge is transferred to the analytes. Ions are then transferred in increasing vacuum to the mass spectrometer.

The mass spectrometer used in this thesis is a triple quadrupole analyzer. It consists of four parallel metal rods, in which the ions can move along the axis of the quadrupole (FIGURE). The first quadrupole is set to monitor mode, only permitting ions with a specific mass-to-charge ratio (*m/z*) to pass to the next quadrupole. This is done by adjusting the voltages of the quadrupole, so that only the target ions achieve a stable resonance and can pass through the quadrupole, while rejected ions does not achieve a stable resonance and crash into the quadrupole. The specific values of m/z are predetermined by running ultra-pure standards of the targeted analytes, in this case 5-hm(dC), 5-f(dC), 5-ca(dC), dA,dG, and T in advance. In the second quadrupole, the ion collides with an inert gas (usually N_2 or Argon) further fragmenting the ion before being passed onto the third quadrupole. The third quadrupole is also set to monitor mode, permitting only ions with a specific *m/z* to pass onto the detector. This is an example of a tandem mass spectrometer were third quadrupole acts to increase the sensitivity and specificity for detecting targeted analytes. During nebulization at the

electrospray ionization, it is possible that multiple ions with the same m/z are formed from different analytes. However, after the second fragmentation at the second quadrupole, there is a very small chance of two analytes again forming ions with the same *m/z*. This is because ESI is considered a "soft" fragmentation method, not leading to many fragments, therefore, this tandem setup with an additional fragmentation step at the second quadrupole is often necessary for the precise detection of target analytes.

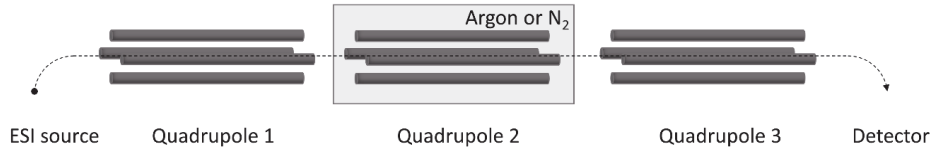

Figure 3: Simplified schematics of a triple quadrupole tandem mass spectrometer. Adapted from Pitt (2009).

Compared to the enzyme-linked immunosorbent assay (ELISA) method for quantifying modified cytosines, the LC-MS/MS method has numerous benefits and some drawbacks. LC-MS/MS is considered more sensitive than ELISA, as the sensitivity of LC-MS/MS depends on the amount of input DNA. Therefore, for genomes with a very low abundance of certain modified nucleotides, the amount of input DNA can be increased to lower the detection limits, while in an ELISA, there is a maximum amount of DNA that can be bound to the well surfaces of a microtiter plate, which together with the antibody sensitivity determines the detection limit. The cost of reagents for running the LC-MS/MS is cheaper than ELISA. The reagents needed besides sample materials are organic solvents, and HPLC-grade purifications of the nucleotides assayed used as standards. HPLC-grade chemicals are usually expensive; however, only minute amounts are needed for each LC-MS/MS run. The addition of several standards in the LC/SM/MS run makes it possible to detect multiple modified cytosines from the same samples, which is not possible using commercial ELISA kits.

Nevertheless, the instrument itself is very expensive and thus out of reach for many research laboratories. Proper maintenance and care of the LC-MS/MS system also require dedicated training and usually dedicated personnel. Although the LC-MS/MS can be run without human intervention, the ELISA is more suited for high-throughput analysis utilizing the 96-well plate format.

A major drawback of both ELISA and the LC-MS/MS method is that although they can quantify modified nucleotides, they cannot inform us about the genomic spatial context in

which these nucleotides reside. To determine this, one must use more costly DNA sequencing methods in which the sequence of all unmodified bases is determined with (usually just) a single type of cytosine modification of interest.

3.6 ELISA

In Paper IV, the ELISA method was used to quantify global DNA methylation levels. There are several methods to assess these, each with its own drawbacks and benefits. ELISA is not the cheapest method when it comes to the cost of consumables; however, it does not require very expensive equipment and is thus within the financial reach of most research laboratories. ELISA is also fairly easy to set up and can thus be performed by students with moderate pipetting experience. It can also be automated in a high-throughput fashion through automated plate washers and readers.

The ELISA method used in Paper IV is an indirect type of ELISA in which the interrogated DNA is made single-stranded and then coated to the well surface of a 96-well plate. An anti-5mC monoclonal antibody is then added to the wells, which binds 5-methylcytosine. Furthermore, a horseradish peroxidase (HRP) conjugated antibody is added to the wells that bind the anti-5mC primary antibody. This makes this an indirect form of ELISA, since the HRP is not conjugated to the primary antibody but rather to a secondary antibody binding to the primary antibody. The role of HRP is to catalyze a chemical reaction that develops a color change that can be quantified using a spectrophotometer. HRP can oxidize many different substrates in combination with hydrogen peroxide as an oxidizing agent.

The main drawbacks of using ELISA-based methods for detecting global DNA methylation are the quality of the primary antibody used and, for absolute quantification, the quality of the standard curve. There are many different antibodies raised against 5mC available commercially. One of the most popular versions is a monoclonal antibody based on the 33D3 clone with over 127 publications, according to one manufacturer (https://www.abcam.com/5-methylcytosine-5-mc-antibody-33d3-ab10805.html#description_references, retrieved 07.01.2022). However, the origins of the monoclonal antibody used in the 5-mC DNA ELISA Kit from Zymo Research in Paper IV are unknown (Togashi, 2013). The construction of the standard curve is critical for the accurate absolute quantification of 5mC in unknown samples. Thus, pipetting errors will influence the robustness of the standard curve and deteriorate any qualitative assumptions made. Therefore, the standard curve setup is quite sensitive to pipetting errors.

3.7 Sequencing of 5-mC

DNA sequencing methods excel over LC-MS/MS and ELISA-based methods by providing the context in which the modifications appear. Unlike global methylation methods, DNA-sequencing methods are more costly for reagents, instruments, and sample preparation. As sequencing methods generate more data, data analysis is much more demanding, usually requiring some expertise in bioinformatics. As a curiosity, the R code needed to analyze and generate Supplemental Figure 6 in Paper IV, the ELISA data, was a little under 100 lines. However, the R code needed to analyze the whole genome bisulfite sequencing dataset in the same paper was over 450 lines (not including the code written in bash).

A key component driving up costs is the size of the genome of the species in question. The larger the genome, the more costly it is to sequence a species at the same sequencing depth. In this regard, the honey bee has a relatively small genome at about 250 MB (Elsik et al., 2014) compared to other model organisms, such as mice, at around 2.7 GB (GRC m39 available from ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/001/635/GCA_000001635.9_GRCm39) and zebrafish, at around 1.4 GB (GRCz11 available from ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/035/GCF_000002035.6_GRCz11/). This means that 10 million sequencing reads should statistically cover the honey bee genome 10-fold higher than the mouse genome.

Coverage is important when dealing with sequencing datasets, as the accuracy of sequencing methods is never 100%. In daily speech, an accuracy of 98% sounds impressive; however, when translated to sequencing experiments in the honey bee, this means that approximately five million bases will be wrongfully called by the sequencing instrument. A researcher would overcome this uncertainty by making sure the average base is sequenced the minimum number of times required to be able to answer his or her research hypothesis.

When dealing with whole genome bisulfite sequencing (WGBS) experiments, having high enough coverage is important. WGBS is today considered the gold standard for DNA methylation sequencing. The main principle of WGBS is to subject the DNA to bisulfite, which converts all cytosines to thymines; however, 5-methylcytosines and 5h-hydroxymethylcytosines are protected from this conversion. By comparing a sequenced library to a reference sequence (most usually the latest official assembly of the species in question), the researcher can determine with up to a single base accuracy which cytosines are

methylated. Thus, the sequence complexity is reduced, reducing the number of reads successfully mapped to the reference sequence.

Furthermore, bisulfite treatment often degrades DNA, which can be partly compensated by increasing the input material. Taken together, these two drawbacks of bisulfite sequencing will decrease coverage if not addressed and thus increase costs. As mentioned earlier, the bisulfite-aided conversion of cytosine to thymine is never 100% complete. This means that, just as with the accuracy of the general sequencing method, a lower conversion rate will lead to wrongfully assigned methylated bases, and thus genomic regions might appear more methylated than they are. However, samples with poor conversion efficiency cannot be rescued by other means, so samples under a specific conversion rate are usually dropped altogether from further analyses. There is no universally defined consensus on the conversion rate cut-off; however, most literature report values somewhere between 97–99% (Chen et al., 2014; Song et al., 2013).

To avoid some of the drawbacks of the WGBS method, the reduced representation bisulfite sequencing method was devised, a method that has gained popularity and is still being refined (Gu et al., 2011). RRBS works by using a restriction enzyme that has a recognition site that includes a CpG dinucleotide (Typically MspI). These cleaved fragments, which cover most of the genomes' CpGs, are then used as substrates for adapter ligation. In this way, researchers can interrogate most genome's CpGs at a fraction of the cost compared to WGBS. However, RRBS has drawbacks. Depending on the restriction enzyme used, not every CpG is cleaved and thus not sequenced. RRBS is a computationally more demanding method, as not all statistical and bioinformatical packages can be used with RRBS directly (Klein & Hebestreit, 2015). In addition, in species where RRBS has not been performed before, in-silico digestion of candidate restriction enzymes should be performed beforehand to evaluate the potential outcomes and degree of the included CpGs. Depending on the level of bioinformatic experience, this may or may not be a trivial task, as easy-to-use software for this does not currently exist.

A historically popular method was methylated DNA immunoprecipitation sequencing (Sørensen & Collas, 2009) (MeDIP-seq). It relied on enriching DNA with the use of a 5-methylcytosine-specific antibody and subsequent sequencing of enriched fragments. Although the MeDIP-seq method did not suffer from the drawbacks of WGBS, such as degraded DNA, reduced sequence complexity, and incomplete conversion, the MeDIP-seq method did suffer from drawbacks common to antibody-based methods (see section 3.6 ELISA above), which

made it difficult to estimate the degree of methylation at a single CpG level. The input DNA requirements were also quite high (Sørensen & Collas, 2009). These shortcomings and the fact that WGBS is constantly being improved have made WGBS a more popular choice over MeDIP-seq (Yong et al., 2016).

At the start of this work, many ready-made and low-input kits sold commercially today were not available on the market. Using kits that had only been tested with organisms with much higher methylation levels than the honey bee could be a hit-or-miss strategy. Luckily, a rather general and detailed protocol with numerous quality-control steps during library preparation was available (Urich et al., 2015). This protocol, dubbed MethylC, is a widely published variant of WGBS that has been fined tuned through years of experience and used on both plants and animals. The protocol published in *Nature Protocols* is quite detailed and can be used as a bench protocol almost immediately.

To validate that we had a functional sequencing library before shipping and sequencing, we used a quantitative PCR (qPCR) method to estimate the number of adapters containing fragments that would amplify during sequencing (Kapa Biosystems, 2014). This PCR method is based on quantifying the targets against standards with known concentrations, thereby constructing a standard curve. By using a newly released Microsoft Excel function, we could also quickly estimate the number of additional PCR cycles needed to amplify the library without overamplifying it. Overamplification greatly reduces the complexity of the completed libraries and is usually filtered out during data processing. This allowed us to be confident that we had a working sequencing library before committing to overseas shipment and sequencing.

Once sequencing is finished and raw files in the form of FASTQ files are obtained from the sequencing facility, the files must go through pre-processing steps before any assumptions on differential methylated regions between samples can be made. These steps usually include quality control, adapter trimming, mapping with software designed to handle bisulfite sequencing samples (e.g., Bismark), and often some post-mapping cleanup software as well. Many different software packages exist for these steps, and the differences between them are quite trivial. Thus, the choice between them usually boils down to experience, and where this is lacking, as in our case, to collegial experience.

Once the sequencing data had been quality controlled and processed to the point where "cytosine coverage" files were generated by the Bismark mapping software, a choice of which statistical software to use for analysis had to be taken.

Many popular statistical packages are written in R, a statistical programming language, and hosted at the Bioconductor repository (Huber et al., 2015; R Core Team, 2019).

Two of the more popular packages were tested (Shafi et al., 2017). MethylKit was found to be unable to handle lower coverage samples very well and was severely lacking in visualization and plot functions (Akalin et al., 2012). Data visualizations had to be performed with other software or by creating plot functions from scratch. MethylKit also had problems dealing with all the small, unplaced scaffolds in the honey bee genome, which in the Amel 4.5 assembly accounts for over 5,000 scaffolds (Elsik et al., 2014). Sequence reads mapping to those scaffolds had to be removed before analysis. BSmooth was also tested; however, that package had no options for correcting for multiple testing, although it had appealing visualization features (Hansen et al., 2012).

In the end, we used a recently published custom pipeline that fulfilled our needs for software that could handle the lower coverage samples, species with generally low methylation levels, corrections for multiple testing, and that had appealing visualization features (Herb et al., 2018). The custom pipeline makes extensive use of the built-in packages of R, but it is also dependent on BSmooth for importing "cytosine coverage" files and comb-p for combining spatially correlated p-values (Pedersen et al., 2012). The open nature of this pipeline makes it easy to investigate what is going on "under the hood" and to further customize and optimize scripts according to one's dataset.

4.1 Summary of papers

Compared to mammals, honey bee epigenetics is still a research field in its infancy. At the start of this thesis work, most prior publications had investigated 5mC in the brain only. We wanted to go beyond our comfort zone with a research hypothesis that would also include 5hmC, 5fC, and 5caC in tissues other than the brain. Previous work by group members on the molecular and physiological underpinnings of aging and social structure (and especially the reversible switching between worker subcastes), and the effects of bioactive food compounds, opened avenues for deeper exploration of the epigenetic involvement of that work.

4.1.1. Paper I: Obtaining Specimens with Slowed, Accelerated and Reversed Aging in the Honey Bee Model

Published in *Journal of Visualized Experiments* in 2013

For Paper I, we identified a knowledge gap regarding a detailed protocol for generating differently aged worker phenotypes. Of course, protocols were available before the onset of this work (Amdam, G. V. et al., 2005; Huang & Robinson, 1996; Robinson et al., 1992); however, they were perhaps not detailed enough that research groups without any previous experience would dare undertake setting up this kind of experiment, as it demanded numerous resources with a relatively low chance of success for the first trial. We used a shared spreadsheet to plan the staggered hive manipulations and to maintain an overview of the accompanying color codes used. The use of pen-like markers made paint application quicker and more precise than applying paint with brushes (Nilsen et al., 2011). This opened up the possibility of multiple thoracic markings, as markings on the abdomen had a higher chance of disappearing.

On average, we marked 603 foragers from each hive (excluding the first foragers). Of those foragers, on average 24 workers per hive were confirmed to revert when concluding the experiment, while on average, only 10 workers continued with foraging tasks. This meant that the expected yield of reverted workers, which is often the focal group in these types of experiments, was very low; thus, special attention was needed to ensure a robust yield of reverted workers. While sampling reverted workers, we recorded whether the bees were double marked (one mark for cohort and one for the onset of foraging), situated on a brood comb, having their heads down in wells with open brood, and wing wear. This was essential for discriminating between true reverted workers and foragers not engaged in nursing tasks.

In addition to these direct phenotypic observations, we confirmed the reversion of worker tasks by monitoring brood combs with open brood capped by worker bees during the experiment.

Furthermore, we investigated the levels of lipofuscin, a cellular biomarker of senescence (Di Guardo, 2015; Terman & Brunk, 2004), in the honey bee brains and confirmed accelerated aging in workers with extensive foraging experience compared to age-matched nurse bees or chronologically younger nurses or foragers (*P*F17d vs. $F_{1d/N1d/N17d} < 0.001$, Fisher's LSD). This suggests that foraging activity is the main driver of lipofuscin accumulation, rather than just chronological age.

4.1.2 Paper II: Cytosine modifications in the honey bee (*Apis mellifera*) worker genome

Published in *Frontiers in Genetics* in 2015

For Paper II, we identified a knowledge gap in the literature where most reviews discussing DNA methylation in honey bees and social insects did so only in the context of 5mC, with little to no focus on 5hmC, 5fC, and 5caC. As numerous reviews on 5mC in a social context in honey bees exist (Flores et al., 2013; Gabor Miklos & Maleszka, 2011; Herb, 2014; Li-Byarlay, 2016; Lyko & Maleszka, 2011; Maleszka, 2016), we sat out to write a review that would serve as a short introduction to students and researchers new to the field of cytosine DNA methylation and its enzymatically oxidized derivatives in honey bees.

In summary, social insects have a larger epigenetic repertoire than other established invertebrate model organisms, such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. The very small amount of DNA 5mC found in the fruit fly is, for example, mostly confined to repetitive sequences. The existence of 5mC is also controversial, as *Drosophila melanogaster* lacks DNMT1 and DNMT3, which are the maintenance and the *de novo* DNA methylation transferases in mammals (Law & Jacobsen, 2010). The honey bee genome harbors the full range of enzymes involved in DNA methylation and demethylation, as is known in mammals; however, the function of these enzymes is still enigmatic, as the distribution and function of its products are vastly different from mammals (Cingolani et al., 2013; Flores et al., 2012; Lyko et al., 2010). Nevertheless, the gene body-specific DNA methylation and its diverse social behavioral range make the honey bee an excellent model system for studying the interplay between intragenic DNA methylation and social behavior.

Precise mapping of 5hmC, 5fC, and 5caC (for the latter two, if even present in the honey bee genome) at gene body CpGs along with physical mapping of TET and TDG binding sites could perhaps also give clues about the function of active demethylation in the honey bee.

As this was a review, no scientific methods were used. However, Figure 1 was created from scratch using the GNU Image Manipulator Program (GIMP) available from the Microsoft Store in Microsoft Windows.

4.1.3 Paper III: DNA base modifications in honey bee and fruit fly genomes suggest an active demethylation machinery with species- and tissue-specific turnover rates

Published in *Biochemistry and Biophysics Reports* in 2016

For Paper III, we investigated some of our conclusions about knowledge gaps in Paper II experimentally. Specifically, we wanted to investigate how abundant the different oxidized forms of 5mC were in honey bee worker abdominal and brain tissues. As we had access to a highly sensitive LC-MS/MS setup, we wanted to use *Drosophila melanogaster* as a reference species for its low abundance of enzymatically modified DNA bases (Capuano et al., 2014).

To represent a diversity of worker sub-castes, we sampled three worker types: nurse bees, foragers with short foraging experience, and foragers with long foraging experience (Seeley, 1995). To minimize the effects of chronological age, all three groups were of similar chronological age.

As mentioned in Section 3.5 LC-MS/MS, the level of detection for the LC-MS/MS method is dependent on the amount of input DNA. To maximize the extracted DNA from each individual, we used the phenol-chloroform and isoamyl alcohol extraction protocol, by many considered dated for its use of toxic chemicals. Nevertheless, the protocol remains prevalent due to its perceived high yields and being popularized by Sambrook and Russell (2001).

We detected 5mC and 5hmC in honey bee brain tissue and abdominal tissue, with statistically significant higher levels of 5hmC in the fat body than in the brain. Our results thus offer the first insights into the amounts of these two molecules in the fat body, an organ functionally homologous to the mammalian liver and white adipose tissues.

As expected, the levels of 5mC and 5hmC were low in honey bees compared to mammals. While mammalian levels of 5mC and 5hmC (in % of C) range from 5.3–2.6% and 0.01–0.68%, respectively, honey bee levels range from 0.15–0.34% and 0.0004–0.0006% for 5mC and 5hmC, respectively. To make more meaningful comparisons across species and tissues,

we reevaluated comparable reported values from the literature and comprised them in a simple table format.

Furthermore, we quantified the unmodified canonical bases in the honey bee, fruit fly genome, and select mammalian tissues and cells to investigate if a low GC content could explain the low levels of modified cytosines in the honey bee genome. However, even when accounting for lower GC content in honey bees, the levels of 5mC and 5hmC were still 10–1,000 fold lower respectively.

Additionally, we detected 5mC and 5hmC in adult fruit flies, a species in which the existence of DNA methylation has been controversial, as the fruit fly lacks several enzymes that are necessary for DNA methylation in mammals (Boffelli et al., 2014; Dunwell & Pfeifer, 2014; Krauss & Reuter, 2011; Urieli-Shoval et al., 1982).

Finally, we did not detect 5fC and 5caC in any of our honey bee samples, suggesting that these modifications, if present, are below the detection limits of 10 and 0.03 per 10^6 unmodified DNA bases, respectively. For 5fC, this translates to less than 15 hemi-modified sites per genome. In summary, our sensitive LC-MS/MS assay detected 5mC and 5hMC in honey bees and fruit flies, but failed to detect 5fC and 5caC in honey bees.

4.1.4 Paper IV: Screening bioactive food compounds in honey bees suggests curcumin blocks alcohol-induced damage to longevity and DNA methylation

Published in *Scientific Reports* in 2021

For Paper IV, we wanted to build upon previous work in our lab that indicated that some bioactive food components might influence and prolong honey bee survival.

Several bioactive food components are suspected to regulate the epigenome via histone modifications (e.g., Balasubramanyam et al. (2004); Davie (2003); Kang et al. (2005)). As considerable crosstalk between histone modifications and DNA methylation has been demonstrated (Du et al., 2015; Nan et al., 1998; Ooi et al., 2007), we wanted to screen bioactive food compounds first for survival differences, excluding any consumption differences, then for global methylation differences, and finally end up with a compound(s) suitable for WGBS. In addition, we wanted to investigate whether a bioactive food compound can counteract the negative effects of ethanol (Mustard et al., 2019).

To minimize physiological age differences, we used the winter bee phenotype (Fluri, 1990; Mattila et al., 2001; Münch et al., 2013). These bees were housed in a laboratory setting

(Dickel et al., 2018; Huang et al., 2014). Bees were fed a food blend containing one of the following compounds: sodium butyrate, curcumin, ethanol, isovaleric acid, valproic acid, folic acid, and cyanocobalamin, at three different concentrations, while survival and consumption were monitored. A second experiment with the same conditions as the first was set up, but terminated after 10 days of treatment to generate bees for DNA methylation investigation using ELISA and subsequent WGBS on promising candidates.

ELISA screening yielded no significant results; thus, candidates selected for WGBS were determined based on dose-response observations. The WGBS was performed using a previously detailed protocol and a custom statistical pipeline adapted to the unique characteristics of the honey bee methylome (Herb et al., 2018; Urich et al., 2015).

We observed survival differences for treatments containing isovaleric acid and valproic acid at all tested concentrations. We also observed survival differences for one treatment of cyanocobalamin (2 µg/ml), one treatment of folic acid (5 µg/ml), and one co-treatment of curcumin and ethanol (100 µg/ml curcumin and ethanol). However, we detected significantly less consumption for the two treatments of isovaleric acid (1 mg/ml and 10 mg/ml), meaning that the observed reduced survival for these concentrations could not be ruled out (Fisher-LSD, $p_{1mg/ml\ /\ 10mg/ml} < 0.05$). As expected, we observed reduced survival for bees treated with ethanol (1% [v/v]). Remarkably, survival was increased in bees cotreated with both ethanol and curcumin (100 µg/ml). We did not detect survival differences for any of the tested concentrations of sodium butyrate (0.01 mg/ml, 0.1 mg/ml, and 1 mg/ml) and cyanocobalamin at 0.02 µg/ml and 0.2 µg/ml, folic acid at 50 µg/ml and 500 µg/ml, and curcumin at 1 µgg/ml and 10 µg/ml.

As mentioned previously, we did not detect any significant global DNA methylation differences between treatments (one-tailed Mann-Whitney test; $p > 0.05$). To select a candidate for WGBS, we chose treatments with an observed non-significant dose response.

For WGBS, we selected bees fed with ethanol (1% [v/v]) and bees co-fed with ethanol together with curcumin (1 % [v/v] and 100 µg, respectively). We detected seven differentially methylated regions (DMRs) in total. Four of these DMRs were in the ethanol-only treatment, where three of those again were hypermethylated and the last was hypomethylated (compared to controls). In the curcumin and ethanol cotreatment group, we detected three DMRs, all hypomethylated. These three DMRs overlap with the three hypermethylated DMRs in the ethanol-only treated group, just in the opposite direction (hypomethylated). These overlapping

DMRs are situated in the gene bodies of loci GB49219, GB47217, and GB43904. The *Drosophila melanogaster* homologue of GB49219 is known as Gudu and is necessary for male fertility in flies (Cheng et al., 2013). GB47217 is a calcitonin receptor involved in aggressive behavior in honey bees (Herb et al., 2018). GB43904 is part of a complex that binds to tubulin and mediates tubulin transport in cilium-containing cells (Bhogaraju et al., 2013; Duran et al., 2016). Further research is necessary to investigate the significance of these few but treatment-specific DNA methylation changes.

In conclusion, our data indicate that bioactive food compounds modulate honey bee lifespan. Taken together, the survival and WGBS data from the ethanol and curcumin treatments suggest that curcumin can block ethanol-induced damage to longevity and DNA methylation (Figure 4). Further research seems warranted to explore the exciting possibilities of curcumin acting against the harmful effects of ethanol.

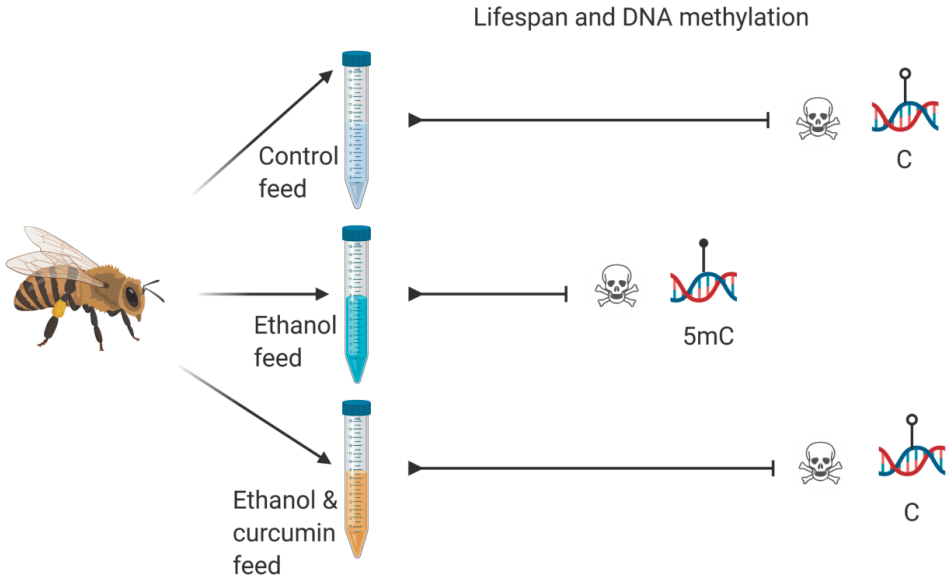

Figure 4: Graphical abstract of main findings in Paper IV concerning ethanol and curcumin

5.1 Discussion

This research has been ongoing for over a decade (2011–2022). For instance, Paper I was published in 2013, with Papers II, III, and IV following in 2015, 2016, and 2021, respectively. This means that the first papers have been available to the scientific community for some time. Therefore, it is possible to discuss the impact these papers have had on science by examining how they were cited and by comments made on social media (mainly Twitter). Further, my thesis exemplifies that work crafted with a specific reader in mind can be used differently and unexpectedly, and that the research community ultimately decides which parts (if any) of a paper are interesting. Thus, Papers I-III will be discussed in the context of attention received from the scientific society through citations and Twitter feedback. Paper IV is a more recently published paper and has not gained as many citations and social media attention; therefore, it will be discussed more traditionally. Concluding the discussion is a separate section with a general discussion.

5.1.1 Paper I: Obtaining Specimens with Slowed, Accelerated and Reversed Aging in the Honey Bee Model

The main contribution of Paper I is not the results it generates, but rather the method it details and refines. The rationale for the work was that a detailed protocol for the generation of differently aged worker phenotypes was missing in the literature. What is more, Paper I delivers results on lipofuscin levels, which are elevated in workers with extensive foraging experience compared to chronologically age-matched nurse bees or chronologically younger nurses or forager bees. Yet, the increase in lipofuscin with chronological age has previously been reported in honey bees (Hsieh & Hsu, 2011b; Hsu & Chan, 2013). Nonetheless, this lipofuscin increase seems to be most appreciated by the scientific community, as this point from Paper I has been cited more than the actual use of the reversion method (Ali et al., 2019; Depeux, 2020; Lucas & Keller, 2018). The results section of Paper I could have easily been expanded with additional experiments, as only a minor portion of the samples was used for the lipofuscin assays and the experiments in Paper III. However, most worker samples were reserved for planned experiments in separate manuscripts and were not available for my work.

5.1.2 Paper II: Cytosine modifications in the honey bee (*Apis mellifera*) worker genome

After the acceptance and online publication of Paper II, a critique against Figure 1 concerning the simplification of DNA methylation patterns over gene structures in select species was posted on social media. This criticism was understood to be directed at the oversimplification

of this figure, making it inaccurate. Paper II is not supposed to be an in-depth review of honey bee worker DNA methylation from a genomics and chromatin biology perspective, but an easy introduction to the field of honey bee DNA methylation; thus, several simplifications were needed to emphasize the main points of the paper. Judging by its citations, Paper II has mostly been cited by researchers not directly and exclusively working with honey bees (Alves et al., 2021; Athanásio et al., 2018; Norouzitallab et al., 2018; Pratx et al., 2018).

The field of insect and honey bee DNA methylation has evolved since Paper II was published. More specifically, the dynamic nature of DNA methylation has come into question (Libbrecht et al., 2016). The contribution of paternal sequence polymorphisms and epialleles is not discussed at all in Paper II, which a researcher new to the field of honey bee epigenetics and insect epigenetics needs to know (Kucharski & Maleszka, 2020; Wedd et al., 2015; Yagound et al., 2019; Yagound et al., 2020). Therefore, Paper II no longer serves as a primer on its own for those researchers.

5.1.3 Paper III: DNA base modifications in honey bee and fruit fly genomes suggest an active demethylation machinery with species-and tissue-specific turnover rates

In this thesis, Paper III is perhaps the most frequently cited for its results (e.g., Deobagkar (2018); du Preez et al. (2020); Duncan et al. (2022); Lai et al. (2019); Nica et al. (2017); Tsurumi and Li (2020)). This is probably in part due to Table 1 in Paper III, as this table puts our results into context with other comparable literature. Additionally, this table makes it easier for less-experienced readers to get an overview of comparable LC-MS/MS results, as reported values have been transformed to a common denominator. Another important contribution that Paper III makes is the warning against using conditions that do not allow for the clear separation of an interference peak from dG and true 5-f(dC). Using such conditions with inappropriate columns will lead to artificially inflated 5fC levels in the samples. Columns used in other publications were tested for this effect, and some were unable to separate the interference peak from the true 5fC peak. However, this finding was outside the scope of Paper III and was not pursued any further.

5.1.4 Paper IV: Screening bioactive food compounds in honey bees suggests curcumin blocks alcohol-induced damage to longevity and DNA methylation

Possible health beneficial effects of the bioactive food compound curcumin, investigated in Paper IV, have been studied extensively, with over 250 publications to date (https://pubmed.ncbi.nlm.nih.gov/?term=curcumin+health+benefits retrieved 24.04.2022).

The therapeutic effects of curcumin are reportedly so diverse that one might start wondering if there is anything that curcumin cannot cure (Goel et al., 2008; Gupta et al., 2013). The main research around curcumin seems to be concentrated on cancer efforts. However, there does not seem to be a specific cancer type that is more investigated than others (https://clinicaltrials.gov/ct2/results?cond=&term=curcumin retrieved 16.06.22). Taken together, one easily gets the feeling that curcumin research is still in its infancy, with researchers desperately pursuing every positive result they get, and that curcumin research is mostly guided by external factors (funding and personal interest). The large disparity in therapeutic fields where curcumin has been applied also means that there is no therapeutic field in which curcumin has a clear and large effect against that illness. The lack of clear and large effects is multifaceted, but a recurring theme in the literature is the low bioavailability and chemical stability of curcumin (Anand et al., 2007; Burgos-Moron et al., 2010; Nelson et al., 2017; Siviero et al., 2015). Oral dosing of curcumin in humans can be extremely high (e.g., 12 g), with still disappointingly low levels in the blood (Lao et al., 2006).

The low bioavailability through oral dosing is mainly attributed to the poor absorption of gut epithelial cells (Siviero et al., 2015). Curcumin degrades easily, both when stored (e.g., in powder form) and in vivo (Anand et al., 2007; Burgos-Moron et al., 2010; Nelson et al., 2017; Siviero et al., 2015). This makes it difficult to compare studies, as the physical and chemical properties of curcumin diverge from each experiment, possibly even in the same study. In addition, reports of storage conditions and handling are usually unsatisfactory for such a brittle compound. Paper IV is unfortunately no exception. Curcumin is a hydrophobic compound that dissolves poorly in water. However, according to the manufacturer's website, solubility is satisfactory in both DMSO and ethanol (https://www.sigmaaldrich.com/NO/en/product/sigma/c1386 retrieved 16.06.22).

Ethanol is also one of the most damaging substances abused by humans in Europe (Nutt et al., 2010; van Amsterdam et al., 2015). Its acute side effects and many of its long-term effects have probably been known for as long as humans have abused alcohol. Recently, attention has been given to the non-liver cancer-causing effects of ethanol (Rumgay et al., 2021; Varela-Rey et al., 2013). There is also ongoing political work to add (additional) warning labels to alcohol in the EU and Norway (European Commission, 2022; Rossow & Grøtting, 2022). The honey bee is an established ethanol model (Abramson et al., 2000). Studies have shown that bees willingly consume alcohol, suggesting that the "drunken ape" theory about the origins of human ethanol consumption might have deeper evolutionary origins or evolved independently

in humans and bees (Campbell et al., 2022; Mustard et al., 2019). Honey bees have surprisingly been found to harbor complex behavior not thought to be present in animals so evolutionarily distant from humans (Shpigler et al., 2017).

Concerning ethanol effects on lifespan, Paper IV builds upon previous works and demonstrates a negative effect with an even lower dose rate (1% compared to 2.5% (Mustard et al., 2019)). This suggests that there is no lower "safe dose" of ethanol. Ethanol's effects on DNA methylation in insects seem not to have gained any attention so far. In humans, ethanol can affect DNA methylation precursor pathways, which leads to hypomethylation, genome instability, and ultimately cancer (for reviews, see Rumgay et al. (2021); Varela-Rey et al. (2013)). Thus, ethanol is expected to affect a methylome in a mostly stochastic manner. Indeed, at first glance, the DMR-connected genes in Paper IV seem functionally scattered with limited interactions according to their *Drosophila melanogaster* homologues (Figure 5). However, half of the genes (GB49219 and GB43904) seem to be involved in male fertility. This is surprising, as only female bees were investigated. Taken together, this can indicate that the effects are strongly influenced by paternal epialleles (Kucharski & Maleszka, 2020; Wedd et al., 2015; Yagound et al., 2019; Yagound et al., 2020).

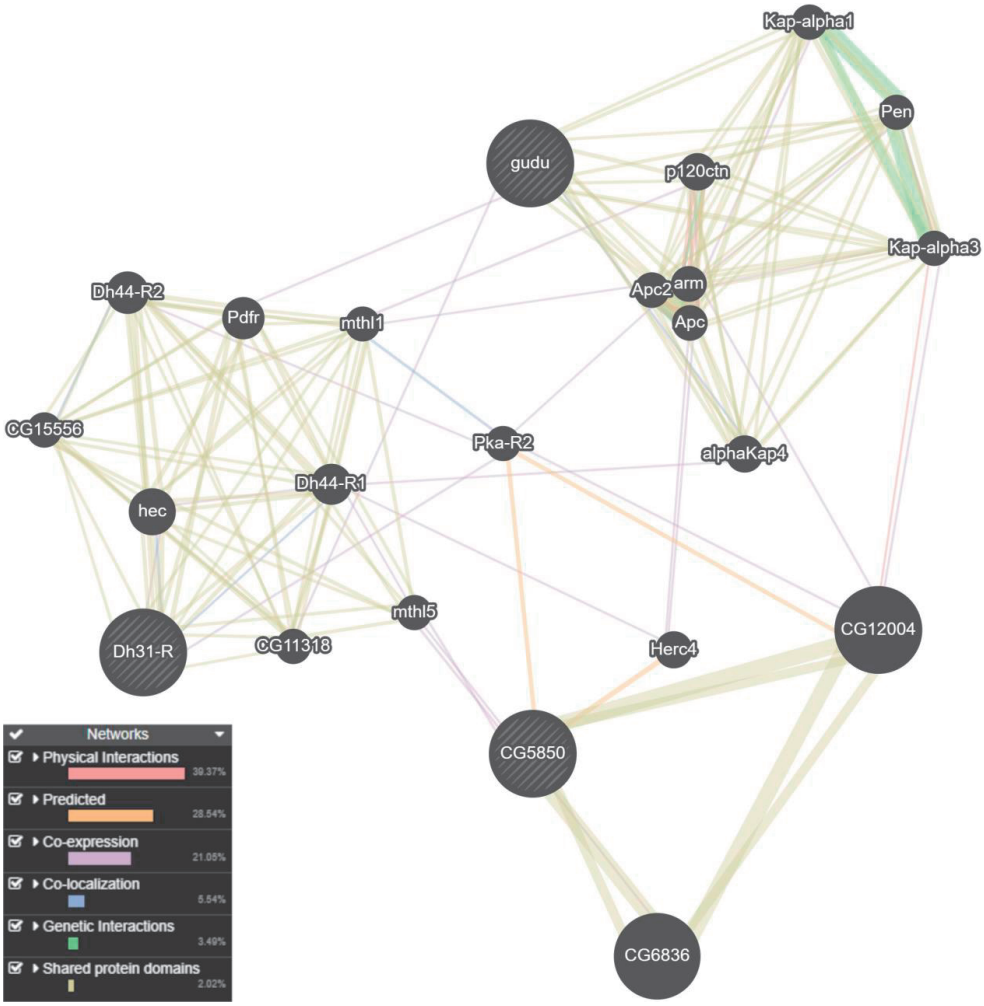

Figure 5: Gene network of unique genes underlying the DMRs in Paper IV, based on their Drosophila melanogaster *homologues. Percentages in the legend indicate the weight of each feature in calculating the model. GB43904 did not have a* D. melanogaster *homologue and was thus excluded from further analysis. The network was constructed using geneMANIA (Warde-Farley et al., 2010).*

In Paper IV, we used naturally multiple-mated queens, thus leaving little control of the paternal genomic and epigenomic contributions. However, the genetic variations introduced by multiple matings are more akin to natural situations. By having better control of the genetic background of the honey bees, we could better control for any paternal effects and probably also detect more subtle DMRs between the different treatments.

As mentioned previously, ethanol is expected to stochastically affect the methylome. Curcumin, on the other hand, is supposed to have a much narrower effect on the methylome than ethanol (Balasubramanyam et al., 2004; Liu et al., 2005). Therefore, it is again surprising

that curcumin affects almost the same DMRs (three out of four) as ethanol, but in the reverse direction. This leads further credence to a deep paternal influence on the epigenome. In vertebrates, co-administration studies with ethanol and curcumin have demonstrated protective effects against liver damage (Lu et al., 2015; Song et al., 2022; Wang et al., 2020; Xiong et al., 2015). These protective effects seem to involve pathways connected to the nuclear factor E2-related factor 2 (Nrf2). Overall, the low numbers of DMRs in Paper IV suggest that DNA methylation is not the major pathway where ethanol and curcumin exert their lifespan-changing effects.

Curcumin and ethanol were not the only compounds with lifespan-altering effects in Paper IV. The lifespan data from this screening study might be interesting for more comprehensive future studies on one or more of the compounds. This has already been suggested by other researchers regarding valproic acid (Santos et al., 2022). As Paper IV is the most recently published paper from this thesis, only one additional citation regarding curcumin's possible mode of action by blocking alcohol-induced damage to longevity and DNA methylation has been made on 10.06.2022 (Berkel & Cacan, 2021). However, Paper IV has attracted more attention on social media than the other papers in my thesis, with a focus on our finding that low-dose ethanol consumption harms lifespan.

5.2 General discussion

My thesis is an addition to the current understanding that honey bee epigenetics is something distinctive, and not just an intermediate peculiarity in a gradient from animals with no DNA methylation, like *C. elegans*, through animals with very restricted methylation like *D. melanogaster*, and culminating with animals with more or less unrestricted DNA methylation like in mammals. This is especially true with regards to the genes involved in DNA methylation generation and active removal, as their function seems to be somewhat changed in different species. In this case, the table in paper II, listing the different genomic copies across different species might be a bit misleading, as the enzymes are homologous, but not identical, so identical function cannot always be expected. As the title of Paper III emphasizes, were we investigated the products/substrates of TET and TDG, that these enzymes operate with species specific turnover rates.

With regards to the reversion protocol in Paper I, we might have established expectations that are too demanding to meet. As far as I know, there has been no citations of Paper I used as a reversion protocol, and only one publication using a similar reversion protocol after the

publication of Paper I (Guan et al., 2013). One reason for this could be that Paper I is not open access and thus not freely available to all potential readers. Paper I weas published in 2013, years before Plan S and open access publishing became mandatory from most funding agencies. Another reason for this could also be that the protocol is too difficult to repeat or that the scientific community has lost interest in questions requiring a reversion experiment.

Paper IV is also an attempt to refocus the research community from always expecting large number of reported DMRs. These long lists of DMRs can derive from pipelines prone to inflate the number of reported DMRs but also from experiments where true large differences exist. As the cost of sequencing and data analysis goes down, and visualization pipelines mature, more and more researchers will probably be tempted to use sequencing for experiments where large differences between groups are not expected. Thus, reports with fewer statistically significant DMRs are to be expected in the future. Although it is unlikely that the DMRs in Paper IV contribute significantly to the lifespan effects of ethanol and curcumin, as discussed in Section 5.1.4, it does not imply that future studies reporting lower number of DMRs are without biological meaning.

The recognition of obligatory epialleles in honey bees, epialleles that have not arisen due to stochastic differences, and the importance of underlying sequence polymorphism have implications for the interpretation of some previous studies in honey bee DNA methylation. As mentioned in Section 5.1.4, control of genetic background is essential. Inadequate control of genetic background can lead to unnecessary noise. This noise might mask true experimental DMRs and could be a reason for the low number of reported DMRs in Paper IV. The WGBS experiment performed in Paper IV, along with future WGBS experiments, can benefit from greatly increasing sequencing depth, along with more stringent tissue or cell type specific interrogation, to uncover the dynamics of potential obligatory epialleles. The recognition of obligatory epialleles also needs to be supplemented Paper II, if it is to serve as a primer into honey bee DNA methylation for researchers with little to no prior knowledge of this field.

6.1 Conclusions

The work presented in this thesis provides a wider understanding of DNA methylation and its oxidative derivatives in honey bee workers. It also exposes the effects of ethanol and curcumin on the survival and DNA methylome of honey bees. Additionally, a comprehensive protocol for obtaining workers with different aging characteristics is introduced.

My thesis investigates the effects of bioactive food compounds on survival and DNA methylation in a sequential linear screening paradigm. By selecting two promising compounds for further investigation, ethanol consumption was found to significantly shorten survival and induce locus-specific DNA methylation changes, which were blocked when curcumin was co-administered together with ethanol. These locus-specific DNA methylation changes were present in genes involved in fertility, temperature regulation, and cilium function. The work presented here increases the use of the honey bee as an ethanol model by introducing curcumin as an intervention agent.

In addition, this work expands on the current knowledge of 5mC in honey bee workers by discussing the enzymatically oxidized derivates 5hmC, 5fC, and 5caC in a general and social context. Moreover, the inferred properties of these modified cytosines and the enzymes responsible for their generation and removal in honey bees are compared to relevant model organisms. The oxidated derivatives, 5fC and 5caC, which have not been described before in honey bee were also determined to be (if at all present) below the detection limit of our very sensitive LC-MS/MS assay, which could detect 5mC in adult *Drosophila melanogaster*, a specie in which the existence of 5mC has been heavily disputed (Boffelli et al., 2014; Dunwell & Pfeifer, 2014; Lyko et al., 2000; Raddatz et al., 2013; Schaefer & Lyko, 2010).

Furthermore, my thesis delivers a detailed protocol for obtaining honey bee workers with slowed, accelerated, and reversed aging. Previous protocols are not as detailed as this one, making it easier for less experienced researchers to conduct such an experiment (Amdam, G. V. et al., 2005; Kuszewska & Woyciechowski, 2013; Robinson et al., 1992). In addition to a written protocol, a video explaining the setup accompanies the online version of this paper. Hopefully, Paper I will lead to the generation of robust datasets exploring the background of dynamic aging properties in honey bee workers.

In summary, by using honey bees, this thesis expands the literature on DNA methylation and bioactive food compounds.

7.1 Future perspectives

A statement often repeated by the reviewers of the papers within my thesis is that DNA methylation in insects is still an enigma. Although bioinformatic analyses confirm that the honey genome encodes many of the enzymes responsible for 5mC metabolism in mammals, the distribution of 5mC and its enzymatically oxidized derivatives suggest that these enzymes function differently in social insects compared to mammals (Libbrecht et al., 2016; The Honeybee Genome Sequencing Consortium, 2006). So far, most scientific literature investigating DNA methylation has focused on the early parts of the methylation cycle (Figure 1), namely 5-methylcytosine. This is probably due to it being the most abundant enzymatically modified cytosine species; thus, the toolset is most developed for this particular modified cytosine. However, focusing on the latter parts of the demethylation cycle could be rewarding when it comes to insects. As noted in Paper II, aphids seem to lack TDG, the enzyme responsible for completing the active demethylation cycle by ultimately removing 5fC and 5caC and replacing them with unmodified cytosine.

Additionally, the presence of 5fC and 5caC in aphids is unknown. Thus, using LC-MS/MS to investigate levels of 5fC and 5caC, together with bioinformatic studies and perhaps gene expression on TDG, could provide insights into the mechanisms of active demethylation in aphids. Additionally, as in Paper III, the failure to detect 5fC and 5caC in honey bees warrants further studies with highly sensitive assays to substantiate the seemingly non-existing levels of these modified cytosines in honey bees. These assays could be complemented by gene expression analyses to examine the expression levels of TDG. Taken together, honey bees and aphids can provide valuable insight into insect DNA methylation when researchers move their focus to the latter parts of the methylation cycle of such insects.

As previously mentioned, curcumin suffers from low bioavailability in mammals (Anand et al., 2007; Burgos-Moron et al., 2010; Siviero et al., 2015). This challenge is due to the low absorption of curcumin by gut epithelial cells, which leads to the low systemic bioavailability of curcumin. Although not a direct part of this work, we observed yellow-colored intestines in bees fed with curcumin and ethanol. However, these anecdotal observations should be investigated further by examining the intestinal contents and hemolymph of bees fed with curcumin (and co-fed ethanol) to explore whether ethanol influences the absorption and turnover of curcumin in honey bee hemolymphs. The effects of curcumin on invertebrates remain largely unstudied.

This thesis adds another dataset to the growing list of honey bee WGBS datasets publicly available (Cardoso-Junior et al., 2021; Cingolani et al., 2013; Drewell et al., 2014; Feng et al., 2010; Foret et al., 2012; Galbraith et al., 2015; Harris et al., 2019; He et al., 2017; Herb et al., 2012; Herb et al., 2018; Li-Byarlay et al., 2013; Li et al., 2017; Lyko et al., 2010; Remnant et al., 2016; Xu et al., 2019; Yagound et al., 2019; Yagound et al., 2020; Yi et al., 2020; Zemach et al., 2010). In addition, the honey bee reference genome has improved significantly with the latest assembly (Wallberg et al., 2019). Taken together, remapping public datasets represents an opportunity to conduct a meta-analysis and, for example, investigate specific temporal, caste, or tissue methylation differences that can be difficult to assess using just a single dataset. The possibility of conducting SNP analyses on WGBS data would allow an investigator to explore the robustness and genetic variation behind potential DMRs, as sequence polymorphisms and the inheritance of epialleles seem to have a larger impact on social insects than previously believed (Kucharski & Maleszka, 2020; Wedd et al., 2015; Yagound et al., 2019; Yagound et al., 2020). Such a study is of low risk, as the datasets are already published.

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Paper I

Video Article

Obtaining Specimens with Slowed, Accelerated and Reversed Aging in the Honey Bee Model

Daniel Münch[1], Nicholas Baker[2], Erik M.K. Rasmussen[1], Ashish K. Shah[1], Claus D. Kreibich[1], Lars E. Heidem[1], Gro V. Amdam[1,2]

[1]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences

[2]School of Life Sciences, Arizona State University

Correspondence to: Daniel Münch at daniel.munch@umb.no

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Abstract

Societies of highly social animals feature vast lifespan differences between closely related individuals. Among social insects, the honey bee is the best established model to study how plasticity in lifespan and aging is explained by social factors.

The worker caste of honey bees includes nurse bees, which tend the brood, and forager bees, which collect nectar and pollen. Previous work has shown that brain functions and flight performance senesce more rapidly in foragers than in nurses. However, brain functions can recover, when foragers revert back to nursing tasks. Such patterns of accelerated and reversed functional senescence are linked to changed metabolic resource levels, to alterations in protein abundance and to immune function. Vitellogenin, a yolk protein with adapted functions in hormonal control and cellular defense, may serve as a major regulatory element in a network that controls the different aging dynamics in workers.

Here we describe how the emergence of nurses and foragers can be monitored, and manipulated, including the reversal from typically short-lived foragers into longer-lived nurses. Our representative results show how individuals with similar chronological age differentiate into foragers and nurse bees under experimental conditions. We exemplify how behavioral reversal from foragers back to nurses can be validated. Last, we show how different cellular senescence can be assessed by measuring the accumulation of lipofuscin, a universal biomarker of senescence.

For studying mechanisms that may link social influences and aging plasticity, this protocol provides a standardized tool set to acquire relevant sample material, and to improve data comparability among future studies.

Video Link

The video component of this article can be found at http://www.jove.com/video/50550/

Introduction

The complex colony structures of highly social animals are maintained through the interaction of a reproductive caste, and a helper caste of typically non-reproducing workers with different social task behaviors. In the different workers, specific physiological adaptations enable distinct sib care behaviors, and are also linked to extreme lifespan differences. Honey bees and mole rats represent the best-developed animal models to study how sociality is linked to patterns of accelerated, negligible or reversed aging[1-3].

In honey bee colonies, a single egg-laying queen is assisted by thousands of workers that tend the brood, forage for food, and engage in guarding, thermoregulation or hygienic behaviors[4]. Among these workers are the extremely short-lived foragers, nurse bees with intermediate, and winter (*diutinus*) bees with longest lifespans. Individuals, however, are not permanently bound to a certain worker-type, but display a flexible behavioral ontogeny: they change from one social task behavior to another ("temporal castes"). Callow bees can change to brood tending nurse bees, which eventually may change to outside foraging. However, callow nest bees can also transform into longest-lived winter bees, and short-lived foragers can even revert into typically longer-lived nurses. Workers with extreme (winter bees) and intermediate (nurse bees) lifespan have well-developed food production and storage organs with copious resources - as opposed to short-lived foragers (reviewed in[1,5]). However, that the regulation of individual lifespan goes beyond simple changes in an individual's resource balance is suggested by research on a yolk protein, which has diverse adapted functions in the non-reproducing worker caste, such as jelly production[6], hormonal control[7], immune[8] and anti-oxidant defense[9].

Patterns of functional decline (senescence) mirror lifespan disparities among workers, as established for olfactory, and also for other brain or motor functions[10-13]. Specifically, the significant decline in learning function after only two weeks of foraging matches a similar mortality progression in foragers[14], as opposed to the lack of detectable decline (negligible senescence) in long-lived winter bees[15].

To identify the molecular fingerprints of flexible aging we adapted established experimental paradigms that allow for monitoring and manipulating aging-type transitions[8,16,17]. Experiment 1 details how to obtain samples in which the effects of chronological age and worker-type specific social behaviors on aging can be separated. Experiment 2 describes the reversal of foragers with accelerated into nurse bees with slowed aging dynamics. Experiment 3 provides an approach for probing effects of cellular senescence by anatomical quantification of an established biomarker for cellular aging (lipofuscin)[18].

Protocol

1. Decoupling Senescence from Chronological Age

This section describes the setup of double cohort colonies, which consist of a cohort of identified individuals that share the same chronological age ("single age cohort") and a cohort of nest bees. Same aged individuals of the single age cohort will eventually separate into different worker-types with different aging dynamics - these are nurse bees with slowed and forager bees with accelerated functional decline. All procedures are described for one experimental colony. We advise, however, to perform experiments for at least two colony replicates so that colony effects can be controlled for (two-replicate-design).

1. *Preparing hive boxes for the double cohort colonies:* Prepare one regular hive box that receives two food combs with honey, another food comb with pollen, and two empty combs. Make sure to locate a mated queen as well as a donor colony with more than 3,000 nest bees. Both will be introduced later (1.3).
2. *Obtaining and marking individuals with similar chronological age:* Collect combs with sealed brood that is about to emerge. For one replicate expect to collect combs with a total of 3,000-5,000 capped brood cells. For each replicate use a balanced amount of brood from at least three different hive sources to avoid skewed distributions of maternal genotypes (hive origin).
    1. Place brood combs in an incubator set to 34 °C with 60-70% relative humidity. Make sure to store combs in such a way that emerging brood cannot escape.
    2. Let bees emerge for two days and mark these bees with a small paint tag on the thorax (*e.g.* Uni POSCA, Mitsubishi Pencil Co. Ltd.). The paint mark will allow identifying the bees of the single age cohort (day of emergence), and to distinguish them from other replicate colonies.

3. *Setting up a double cohort colony, which includes the cohort of identified, single age bees:* On the day young bees have been marked, collect about 2,500-3,000 nest bees from a donor colony (compare section 1. in Discussion), and add these unmarked bees to the hive box that was prepared before (see step 1.1). The latter individuals will constitute the unidentified nest bee cohort.
    1. Add the queen, which will be initially confined to a queen cage (commercially available). Seal the cage with edible candy (*e.g.* Apifonda, Südzucker AG, Mannheim/Ochsenfurt, Germany) to make worker bees slowly release the queen.
    2. Add the newly emerged and marked bees, which will constitute the single age cohort. These bees are the only marked individuals, and are the focus group for all the following steps.

4. *Monitoring foraging onset and marking foragers:* To assess onset and dynamics of the nurse-to-forager transition in the single age cohort, monitor the demographical development of foraging activity every other day for each colony. Begin counting five days after colonies were set up (**Figure 1**).
    1. Count the total number of bees returning from foraging flights (entrance counts) within 3 x 20 min observation periods at fixed times. Make sure to not count bees during periods of orientation flights (see Discussion).
    2. When entrance counts indicate considerable foraging activity to commence (>100 entrance counts/day), begin marking foragers. To do so, foragers of the single age cohort (single marked individuals) receive a second paint mark upon returning from their first foraging flights. This paint mark will specify the day of foraging onset, and will allow to later identify the foraging age for each forager.
    3. Repeat daily markings until a sufficient number of bees has been marked. For estimating a sufficient number of marked foragers, expect a retrieval rate of no more than 5-10% after these bees had been aged, typically after 14 days of foraging.

5. *Sampling:* Since all initially marked bees have a similar chronological age, age-matched groups of nurses and old foragers can be collected simultaneously, when foragers have foraged for ≥14 days.
    1. Single marked nurse bees are collected within the hive, and are identified by nursing behavior (feeding and cleaning of larvae with heads put down in open brood cells).
    2. Double marked foragers are also collected within the hive before daily foraging activity begins.
    3. Collect bees in cages (tubes, boxes) that provide sufficient ventilation, and keep dark until further processing. Alternatively, for transcriptomic, epigenetic or proteomic studies, directly snap freeze bees in liquid nitrogen. Collect balanced numbers of individuals from all test groups and replicate colonies.

2. Reversal of Workers with Rapid to Workers with Slowed Aging by Changing the Hive's Demography

This section details how the reversal from workers with accelerated aging (foragers) to workers with slowed aging (nurse bees) is performed. Such behavioral reversal is induced, when foragers experience a lack of nurse bees, which normally engage in brood care. The reversion procedure will separate a single colony replicate into two hives: one hive with the nurse bee fraction ("nurse-derived"), and another one with the forager fraction ("forager-derived"). After successful reversal, possible symptoms of plastic and reversed aging can be studied in the single age cohort with reverted workers, continuing foragers, continuing nurse bees and newly recruited foragers. As before, identified bees of the single age cohort, not the cohort of unidentified nest bees, constitute the experimental focus group.

1. *Preparation:* Replicate hives with nurses (single marked) and foragers (double marked) are made available as described in the previous section. Make sure to not begin reversion with less than 500 marked foragers per replicate colony to ensure sufficient retrieval after the reversion has been completed.
    1. For safe identification of test groups after reversal it is crucial that the entire forager population in the original hive has been marked off before reversion. The following procedure is described for one replicate.
    2. The day before reversion, prepare one additional hive box for the forager-derived hive (see step 1.1). Locate two queens and two brood combs from donor hives. Before transfer to the experimental colonies brush away all adult bees from these combs. One caged queen (see step 1.3) and one brood comb will replace queen and brood combs in the original hive box. The other set of queen and brood comb will be used the next day for the new hive box. Similar allocation of new brood combs and alien queens for both, the original and the new hive is advised to make sure that separated foragers and nurses will equally experience changed hive cues ("hive smell").

2. *Reversion:* In the morning, just before the reversion, add the caged queen and the brood comb to the new box that will receive the forager-derived fraction. Wait until the peak foraging time begins. Then move the original colony with marked foragers and nurse bees at least 100 m away from the original location.
    1. At the original location, set up the new box for the forager-derived hive with brood and queen only.
    2. Foragers will leave the dislocated original hive box, and head back to the original location. Allow foragers to return to the original location for 2 hr in order to achieve next to complete separation of the forager population from nest bees.
    3. Then, to terminate separation, close off the original, now "nurse-derived" hive, and transfer it to an apiary at least 3 km away.

3. *Hive maintenance and monitoring for successful social task reversal:* Check the experimental hives regularly for healthy, open brood.
    1. During the first days after colony manipulation replace unattended and dead brood to reduce potential pathogen load.
    2. To validate successful reversal within the forager-derived hive, take pictures of brood combs before introducing these, and again when combs are replaced or when the reversion experiment is completed (**Figure 2**). Areas with previously uncapped brood and with open, live brood are reliable markers of nursing activity in forager-derived colonies.

4. *Sampling:* Physiological effects that accompany social reversal can be detected 3-8 days after foragers and nurses were separated.
    1. We advise sampling all test groups, *i.e.* reverted workers and continuing foragers (forager-derived hive), as well as continuing nurses and newly-recruited foragers (nurse-derived hive) 8 days after reversal is initiated.
    2. Collect samples as described in step 1.5.

3. Analyzing Worker-type Specific Cellular Senescence Patterns by Quantification of Lipofuscin

Lipofuscin is a universal biomarker of cellular senescence. As an intrinsic accumulation product, lipofuscin's specific autofluorescence ($emission_{max}$ = 530-650 nm) can be used for detection[18].

1. *Dissection and fixation:* Chill bees on ice until motionless; remove and dissect out the desired tissue sample.
    1. Transfer into fixative (4% paraformaldehyde in phosphate-buffered saline, PBS; pH 7.2) for overnight incubation at 4 °C.
    2. Wash samples 3 times in PBS.

2. *Tissue processing and mounting:* Cut tissue samples into sections with no more than 40 µm thickness, for example by using a vibrating blade microtome, *e.g.* Leica VT 1000S (Leica Biosystems, Nussloch, Germany).
    1. For clearing, incubate tissue sections overnight in 30% glycerol (PBS), and another 2 hr in 50% glycerol (PBS).
    2. Mount sections on microscopic slides in 50% glycerol (PBS). For long-term storage seal cover slips with nail polish.

3. *Image acquisition:* To detect lipofuscin, we suggest using a laser scanning confocal microscope that provides laser lines with λ = 514, 561nm or similar for excitation, and with the detector bandwidth set to 570-650 nm.
    1. For better identification of lipofuscin, include a second channel, and do a simultaneous scan at shorter wavelength spectra (excitation = 405 nm; emission = 410-450 nm). The longer wavelength channel will reveal both, granular lipofuscin, but also unspecific "background" due to autofluorescent trachea and other non-granular structures. The second, shorter wavelength channel will only reveal unspecific autofluorescence, but not lipofuscin. Thus, lipofuscin identification can be facilitated by comparing the signals in both channels, with only one of them revealing the granules with lipofuscin specific fluorescence.
    2. For acquisition of high-resolution images use an objective with 40X magnification or higher, and preferably a numerical aperture of 1.25 or higher. Scan image stacks with dimensions of about 100 x 100 x 10 $µm^3$. Every individual and tissue sample has to be represented by multiple image stacks.
    3. To reduce intra-individual and inter-individual variation caused by technical variation, always keep laser power and detector sensitivity constant.
    4. To reduce bias by day-to day technical variations scan equal sample numbers of all test groups in each of the several scanning sessions.

4. Image processing: Use software packages with modules that allow for advanced processing of microscopic image stacks, for example ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).
    1. Generate a 2D maximum projection for each of the 3D image stacks.
    2. Apply a Gauss filter with a modest kernel size in order to attenuate high frequency noise, and to preserve structures with dimensions of lipofuscin granules.
    3. Merge both color channels to facilitate identification of lipofuscin (see step 3.2).

5. *Image analyses:* Make sure that the subject performing the quantification steps will be blind to test group identity.
    1. For all images, first choose a region of interest (ROI) that covers the relevant structures, and has similar dimensions as the ROIs from other images.
    2. Then select the desired number of lipofuscin granules that represent each ROI. When selecting lipofuscin granules within a ROI, the application of the following rules will reduce subjective bias.
        1. Choose a consistent location for selecting the first granule. This can be, for example, the leftmost edge of a ROI, and the granule that is closest to the edge will always be the first selection.
        2. One after another, granules are chosen that are closest to the previous selection (next neighbor).
        3. When choosing the next neighbor, move only into one direction, for example only search right from the previous selection. This rule prevents that the selection is dominated by occasional clusters of densely packed granules.
        4. When selection is completed, assess the size of each lipofuscin particle by outlining and measuring the respective granule area. Use appropriate statistical tests to compare individuals of the different test groups.

Representative Results

Protocol sections 1 and 2 detail how test groups can be obtained to study attributes of accelerated, slowed and reversed aging in colonies with a single age cohort. To monitor worker-type differentiation that accompanies the normal ontogeny we assessed forager counts ("entrance counts") for 6 colonies (**Figure 1**, compare section 1). The graphs show that considerable change from nurse to the forager state is typically not observed before individuals are more than 10 days old. Marked variability in forager counts was observed with regards to the timing of foraging onset among different colonies, and as a marked day-to-day variation within each colony. Apart from colony specific demographic factors, such as different brood load, much variability is explained by changing weather conditions (time points marked in red in **Figure 1**). Close monitoring of foraging dynamics therefore is advised to optimize marking and collection efforts during the experiment.

The reversed ontogeny (section 2) from foraging back to nursing tasks can be validated by inspecting brood combs that are introduced into the forager-derived colonies (see steps 2.2 and 2.3). For three replicates **Figures 2A, C, and E** show brood combs before introduction into forager-derived colonies. **Figures 2B, D, and F** show the respective combs after removal. Patches of newly capped brood, healthy larvae, and increased pollen storage around brood cells indicate that former foragers now had successfully performed typical nest, including nursing tasks.

Lipofuscin (section 3) is a highly conserved symptom of cellular senescence, and can be readily assessed for post-experimental analyses in the various bee tissues. **Figure 3** contrasts lipofuscin accumulation, measured as granule size (**Figure 3E**), in the hypopharyngeal glands of age matched nurse and forager bees. The difference in chronological age between the two young and the two old groups was ≥17 days, with only one group (foragers) spending these ≥17 days with outside flight and food collection activities. Representative microscopic images (**Figures 3A-D**) show increased lipofuscin accumulation only for the group of older foragers after more than 17 days of foraging (**Figure 3D**), not for older nurse bees of similar chronological age (37-43 days; **Figure 3B**). A two-factorial ANOVA with the fixed main factors worker-type (foragers, nurses) and age difference (Δage ≥17 days) revealed significant effects for worker-type, age difference and the interaction between both factors (F_{type} = 33.67, P<0.001; $F_{\Delta age}$ = 21.93, P<0.001; $F_{type\ x\ \Delta age}$ = *22.07*, P<0.001). However, post-hoc tests showed significant effects only when contrasting older foragers (≥17 days of foraging) to younger foragers, or to both nurse groups (PF17d vs. $F_{1d/N1d/N17d}$<0.001, Fisher's LSD; **Figure 3E**). No difference was detected among the latter three groups, including chronologically young and old nurse groups (all tests with P >0.5, Fisher's LSD; **Figure 3E**). This suggests that lipofuscin accumulation depends on forager specific activities (foraging age), rather than being function of chronological age only per se.

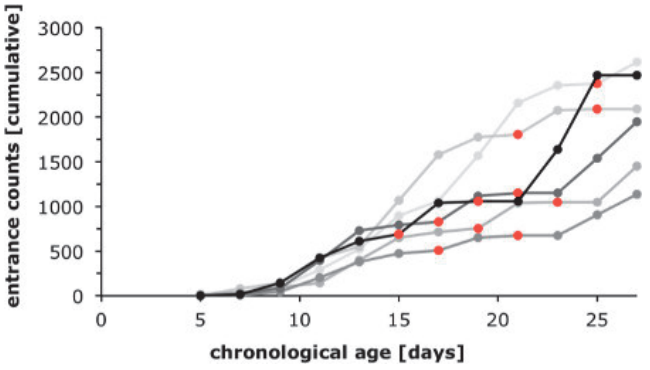

Figure 1. Worker-type differentiation during normal ontogeny. The graph displays entrance counts of foragers returning from foraging flights counted for 6 different colonies beginning 5 days after they were established (for details compare Protocol section 1.4). Considerable transition from nest to foraging activities was first observed when marked individuals of the single age cohort were about 10 days old. Varying slopes for the cumulative entrance counts indicate that the dynamics of the nurse bee to forager transition differ between colonies, and are affected by climatic factors. For example, on days with rain and less than two hours of foraging, the increase in entrance counts typically flattened out (data points in red).

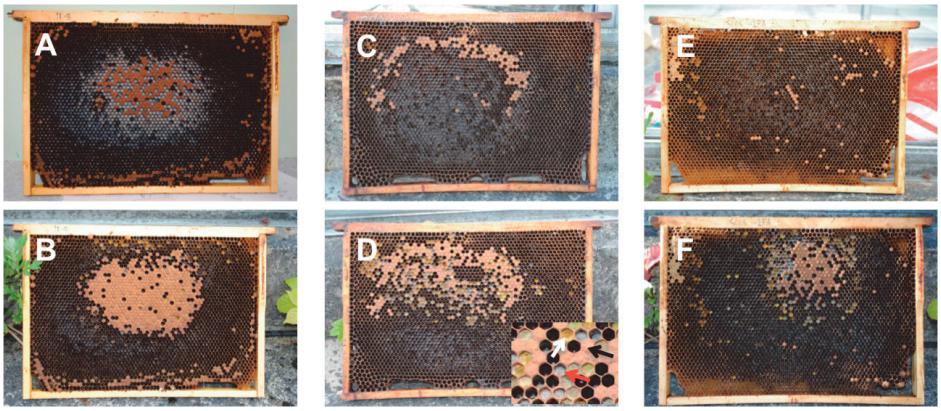

Figure 2. Validating behavioral reversion. To test if foragers have successfully reverted to nursing tasks, we compared brood combs before they were introduced into forager-derived hives, and after they were removed from these hives. Representative images show brood combs before introduction **(A, C, E)** and after removal **(B, D, F)** from three different forager-derived hives, respectively. Brood care by previous forager bees is indicated by an increasing number of cells with capped brood **(B, D, F**; black arrow, inset in **D)**, sustained survival of larvae in open cells (red arrow) and increased storage of pollen close to brood cells (white arrow). Note that forager-derived-colonies initially are typically less efficient in tending the brood than nurse-derived colonies. This can lead to higher larval mortality in the forager-derived-colonies. Pictures in **B, D, F** were taken 5, 4 and 7 days after brood combs were introduced into forager-derived colonies.

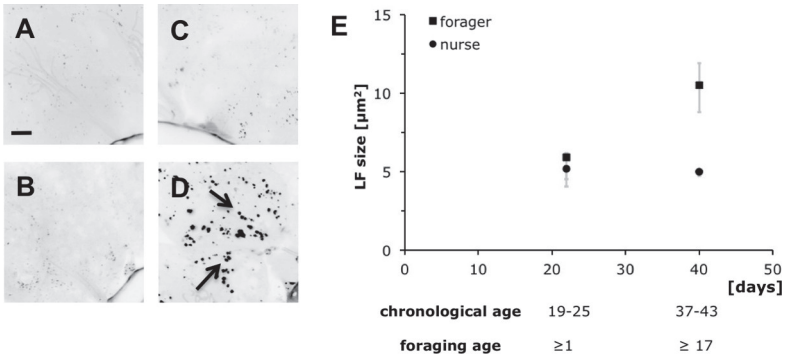

Figure 3. Accumulation of lipofuscin, a biomarker of cellular senescence, can indicate worker-type specific tissue deterioration. Representative microscopic images of hypopharyngeal glands in young **(A)** and old nurse bees **(B)**, as well as in age-matched forager bees with ≥1 day **(C)**, respectively ≥17 days foraging experience (scale bar in A = 20 µm). Lipofuscin accumulation was measured as granule size, and is given as medians and quartiles for *N* = 5 individuals for each age and worker-type **(E)**. Foraging for 17 days resulted in significant lipofuscin accumulation, while the same period did not lead to lipofuscin changes in nurse bees (for statistics see Results).

Discussion

We here adopt previously described approaches[8,16,17,19,20], and integrate them into a single workflow that will facilitate studying flexible aging in honey bees. Our aim is to provide scientists that are novice to this field with a standardized tool set to obtain relevant sample material, and to improve experimental reproducibility among different research teams. While our procedures are simplified and do not require special equipment as in earlier descriptions (compare for example[8]), some measures of precaution are advised and are collected below.

Decoupling senescence from chronological age. A most critical aspect is to avoid false identification of forager bees during initial confirmation of foraging behaviors (2nd marking). Therefore, when foragers are to be monitored ("entrance counts") or marked, do strictly avoid daily periods with orientation flights. During these periods many pre-foraging stage bees will depart from or enter the hive. These bees do not display typical physiological characteristics of mature foragers, but build up a spatial map of the hive surroundings by readily identifiable circular flight patterns[21].

While most bees change to foraging with the age of 2 weeks and older, sporadic foraging is observed already at very young ages (**Figure 1**). Extremely precocious foragers typically develop directly from callow nest bees without having passed through the nurse stage. To not include

individuals with such an aberrant ontogeny (compare[22] and references therein), individuals that begin foraging with the age of 10 days or less are not considered for further analyses.

To further avoid overrepresentation of precocious foragers, we do not make use of classical "single cohort colonies" that consist only of the single age cohort[17,23]. Instead, when setting up colonies we add random nest bees ("nest bee cohort") to the marked single age cohort (see steps 1.1 and 1.3). Since random nest bees are typically older, they can reduce the pressure on very young bees to develop into extremely precocious foragers[17]. Such double cohort colonies, therefore, may better resemble a natural hive demography with individuals that slowly progress from nursing to foraging.

When long-term worker specific adaptations are to be studied, collect all test groups outside foraging hours. This is advised to reduce bias by more acute metabolic adjustments due to recent locomotor activities, for example exhausting flight.

Reversal of workers with rapid to slowed aging by changing the hive's demography. After foragers had flown back to the original location it is essential to move away the nurse-derived hive (>3 km). This is to avoid that pre-foraging stage bees are recruited and guided to the old location by other bees, respectively through pheromone communication[24].

To further prevent any nurse or other pre-foraging individuals from entering the forager-derived hive, we advise keeping with the following rules: (I) Terminate the separation procedure before daily orientation flights begin. (II) Only attempt reversal on days when strong foraging activity is observed. (III) During and after the initial translocation of the original hive, avoid unnecessary agitation of bees, in particular do not open the hive.

In principle, more artificial setups that confine foragers in a nurse-deprived environment also may lead to reversion. However, such setups only have limited informative value as the forager-derived fraction experiences other stressful environments, thus precluding a direct comparison with control groups from nurse-derived colonies.

Confirming different senescence patterns by quantification of lipofuscin, a biomarker of cellular senescence. Here we exemplified lipofuscin assessment with images and statistical data of hypopharyngeal glands because lipofuscin is most easily detectable in this tissue. This, we believe, is important to help the inexperienced observer setting up the correct protocols for microscopic detection. However, unlike other tissues, hypopharyngeal glands do display significant apoptosis and necrosis during nurse to forager transition[25]. Such processes may interact with accumulation of the senescence marker, even though we did not detect increased levels of lipofuscin in young foragers that recently had changed from nursing tasks (**Figures 3C, E**). However, to assess senescence measures in other bee tissues, the microscopy-based methods described here can be easily adapted.

Alternatively, flow-cytometric approaches are less time consuming[26]. Microscopy-based analyses have the advantage that cellular aging symptoms can be assessed for different regions or even for cells within a single organs[27]. For studies in brain and other complex organs with spatial heterogeneity in cellular aging[28], we therefore recommend the microscopy based approach.

Disclosures

We have nothing to disclose.

Acknowledgements

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Materials list

Materials List for:

Obtaining Specimens with Slowed, Accelerated and Reversed Aging in the Honey Bee Model

Daniel Münch[1], Nicholas Baker[2], Erik M.K. Rasmussen[1], Ashish K. Shah[1], Claus D. Kreibich[1], Lars E. Heidem[1], Gro V. Amdam[1,2]

[1]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences

[2]School of Life Sciences, Arizona State University

Correspondence to: Daniel Münch at daniel.munch@umb.no

URL: https://www.jove.com/video/50550
DOI: doi:10.3791/50550

Materials

Name	Company	Catalog Number	Comments
Apifonda	Südzucker AG, Mannheim/ Ochsenfurt, Germany		
paraformaldehyde	Sigma-Aldrich	158127	
phosphate-buffered saline	Sigma-Aldrich	P4417	
Glycerol	Merck	1.04094.1000	

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Cytosine modifications in the honey bee (*Apis mellifera*) worker genome

***Erik M. K. Rasmussen*[1]* and *Gro V. Amdam*[1,2]**

[1] *Department of Chemistry, Biotechnology and Food Science, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Aas, Norway*
[2] *School of Life Sciences, Arizona State University, Tempe, AZ, USA*

Edited by:
Greg J. Hunt, Purdue University, USA

Reviewed by:
Bernardo Lemos, Harvard University, USA
Amy Lynn Toth, Iowa State University, USA

***Correspondence:**
Erik M. K. Rasmussen, Department of Chemistry, Biotechnology and Food Science, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Christian Magnus Falsens vei 1, N-1432 Aas, Norway
e-mail: erik.rasmussen@nmbu.no

Epigenetic changes enable genomes to respond to changes in the environment, such as altered nutrition, activity, or social setting. Epigenetic modifications, thereby, provide a source of phenotypic plasticity in many species. The honey bee (*Apis mellifera*) uses nutritionally sensitive epigenetic control mechanisms in the development of the royal caste (queens) and the workers. The workers are functionally sterile females that can take on a range of distinct physiological and/or behavioral phenotypes in response to environmental changes. Honey bees have a wide repertoire of epigenetic mechanisms which, as in mammals, include cytosine methylation, hydroxymethylated cytosines, together with the enzymatic machinery responsible for these cytosine modifications. Current data suggests that honey bees provide an excellent system for studying the "social repertoire" of the epigenome. In this review, we elucidate what is known so far about the honey bee epigenome and its mechanisms. Our discussion includes what may distinguish honey bees from other model animals, how the epigenome can influence worker behavioral task separation, and how future studies can answer central questions about the role of the epigenome in social behavior.

Keywords: honey bee, methylation, demethylation, 5-hydroxymethylcytosine, social behavior

INTRODUCTION

Since the first honey bee methylome was sequenced in 2010, our understanding of the functional implications of DNA methylation in the honey bee has begun to unfold (Lyko et al., 2010). 5-methylcytosine (5mC) is believed to be involved in alternative splicing, caste differentiation and worker behavioral task separation (Lyko et al., 2010; Flores et al., 2012; Herb et al., 2012). Recently, several other cytosine modifications were discovered in mammalian genomes (Kriaucionis and Heintz, 2009; He et al., 2011; Ito et al., 2011). These modifications are believed to have separate functions from 5mC as they are distributed differently in the genome, and specific reader proteins for one of these modifications exist (Spruijt et al., 2013). Although studies to investigate cytosine modifications other than 5mC in bees have been performed, little is known about their functions and distributions (Cingolani et al., 2013; Wojciechowski et al., 2014). Here we review cytosine modifications and the enzymatic machinery responsible for their generation in different model organisms.

HONEY BEES

Nutritional cues lead female honey bee larvae into one of two developmental trajectories. The larvae either develop into a queen or into a worker (Winston, 1991). Queens are larger, highly fecund and long-lived (years), while the smaller workers are functionally sterile and shorter lived (weeks, months). Workers show a flexible physiological and behavioral progression that typically starts with care behavior toward siblings (nursing) and culminates in food collection (foraging) weeks later. Nursing is associated with enhanced somatic maintenance and slower aging than foraging (Münch and Amdam, 2010). Yet, foragers can return to nursing tasks, and this behavioral reversion can put age-associated cognitive decline in reverse as well (Baker et al., 2012).

Honey bees, in other words, display a wide range of phenotypes that include complex social caste development and behavior, behavioral shifts, and plasticity of aging. Epigenetic mechanisms are already found to likely play major roles in queen-worker development as well as in worker behavioral progression and reversion (Kucharski et al., 2008; Spannhoff et al., 2011; Herb et al., 2012). These findings put the honey bee forward as a very interesting study organism to investigate the interplay between the social milieu and the epigenome. The use of the honey bee for complex epigenetic research is, furthermore, not diminished by the mainstream models fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*), since they do not have the full complement of the mammalian epigenetic machinery (**Table 1**).

EPIGENETIC MACHINERY

DNA methyltransferases (DNMTs) are enzymes that add a methyl group to the 5′ carbon of the DNA base cytosine from the donor *S*-Adenosyl methionine (Law and Jacobsen, 2010). DNMT1 is the "maintenance" DNMT that copies the methylation pattern to the newly synthesized strand during DNA replication. DNMT3 is the *de novo* methyltransferase that can methylate specific loci independently of replication. DNMT2 is primarily an RNA methyltransferase that methylates t-RNA^{Asp} (Goll et al., 2006), however, DNA activity has been shown *in vivo* in the fruit fly (Phalke et al., 2009). The *de novo* and the maintenance DNMTs

Table 1 | Genomic copies of enzymes implicated in DNA methylation and demethylation and presence of epigenetically modified cytosines in select metazoan groups.

Organism	DNMT1	DNMT3	DNMT2	TET	TDG	5mC in CpG	5hmC	5fC	5caC
Nematode							?	?	?
Fly			•	•	•		?	?	?
Aphid	••	•	•	•		•	?	?	?
Jewel Wasp	•••	•	•	•	•	•	?	?	?
Bee	••	•	•	•	•	•	•	?	?
Mammals	•	•••	•	•••	•	•	•	•	•

Sources: (Kriaucionis and Heintz, 2009; Law and Jacobsen, 2010; Lyko et al., 2010; Walsh et al., 2010; Ito et al., 2011; Cingolani et al., 2013; Beeler et al., 2014) and assembled genomes available at http://blast.ncbi.nlm.nih.gov.

are found in a range of species including honey bees, mammals, aphids, and jewel wasps (**Table 1**). They are catalytically active in the honey bee (Wang et al., 2006), while fruit fly and nematode only contain a single copy of DNMT2. Nevertheless, 5mC originating from DNA has been reported in the fruit fly in both embryos and adult flies (Lyko et al., 2000), suggesting that DNMT2 has some DNA methylation activity *in vivo*. The impact of 5mC in the fruit fly genome is still debated, however (Phalke et al., 2010; Schaefer and Lyko, 2010).

In mammals, the ten eleven translocation (TET) enzyme is responsible for further oxidizing 5mC to 5-hydroxymethylcytosine (5hmC) that again can be oxidized to 5-formylcytosine (5fC), and ultimately 5-carboxylcytosine (5caC) (Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011). 5fC and 5caC are recognized by the thymine DNA glycosylase (TGD), which is a part of the base excision repair pathway of the mammalian cell (Maiti and Drohat, 2011). The TET enzyme together with TDG are probably central to the mammalian active demethylation pathway (Pastor et al., 2013). Mammalian genomes harbor multiple TET enzyme genes, while bees, fruit flies, aphids, and jewel wasps only have one (**Table 1**). The RNA expression levels of the different mammalian TET enzymes vary greatly between developmental stages and cell types. The honey bee TET catalytic domain is catalytically active *in vitro,* and active transcription of the honey bee TET gene has been shown to vary in different stages of development as well as in different adult tissues (Wojciechowski et al., 2014). Interestingly, some species (including fruit fly) that contain only DNMT2 have well conserved TET orthologs, but their activity and function have not been deciphered (Dunwell et al., 2013).

The honey bee genome encodes several core histone modifying enzymes, which are also part of the epigenetic machinery of the honey bees (The Honeybee Genome Sequencing, 2006). However, the impact of and the mechanisms behind histone modifications are beyond the scope of this review.

5-METHYLCYTOSINE

The distribution and relative abundance of 5mC vary significantly between mammals, honey bee and fruit fly (**Figure 1**). 5mC is primarily located in a CpG dinucleotide context within repeat sequences and in proximity of promoter areas in mammals (Law and Jacobsen, 2010), whereas in bees methylated CpGs are primarily located within genes (Lyko et al., 2010). However, 5mC can exist in a non-CpG dinucleotide context in both mammals and honey bees (Lister et al., 2009; Cingolani et al., 2013). In addition, the honey bee genome is much more sparsely methylated than mammalian genomes, thus reducing overall complexity and simplifying data analyses for studies conducted in bees. In the fruit fly genome, 5-mC is located within a non-CpG dinucleotide context and seems to be distributed randomly within the genome at an abundance 3- to 100-fold less when compared to honey bees and mammals (Mandrioli and Borsatti, 2006; Phalke et al., 2009). *C. elegans*, on the other hand, does not contain 5mC in its genome (Simpson et al., 1986).

The effect of 5mC on transcription varies between metazoans and genomic context. In mammalian promoters, 5mC is principally a repressive mark, silencing transcription (Bird, 2002). On the other hand, 5mC within gene bodies in mammals, honey bees, and the fruit fly, does not influence transcription levels to the same extent (Mandrioli and Borsatti, 2006; Flores et al., 2012). In honey bees, 5mC within gene bodies rather plays a role in the generation of alternative splice variants on the genome-wide level (Flores et al., 2012; Foret et al., 2012; Li-Byarlay et al., 2013). This role is not clearly defined in mammalian cells, as the role of 5mC in gene bodies differs between cell types and depends on whether 5mC is in a CpG context or not (Lister et al., 2009). These findings make honey bees an attractive system for studies on how 5mC influences the generation of alternative transcripts.

5-methylcytosine is found in multiple cell types, tissues, and life stages in both honey bees and mammals (Ikeda et al., 2011; Ziller et al., 2013). In *D. melanogaster*, 5mC is mostly found during early embryonic stages (Lyko et al., 2000). Although adult 5mC has been reported in fruit fly, the content is too low to be robustly detected by bisulfite sequencing, the gold standard in base resolution 5mC interrogation techniques, making further studies difficult with many established methods depending on bisulfite conversion (Capuano et al., 2014).

5-HYDROXYMETHYLCYTOSINE

The TET oxidative products of 5mC recently became a center of attention in mammalian epigenetic research. Many questions about TET and 5hmC dynamics have been answered in embryonic stem cells (Pastor et al., 2013), although 5hmC has been detected in different tissues at different life stages (Kriaucionis and Heintz,

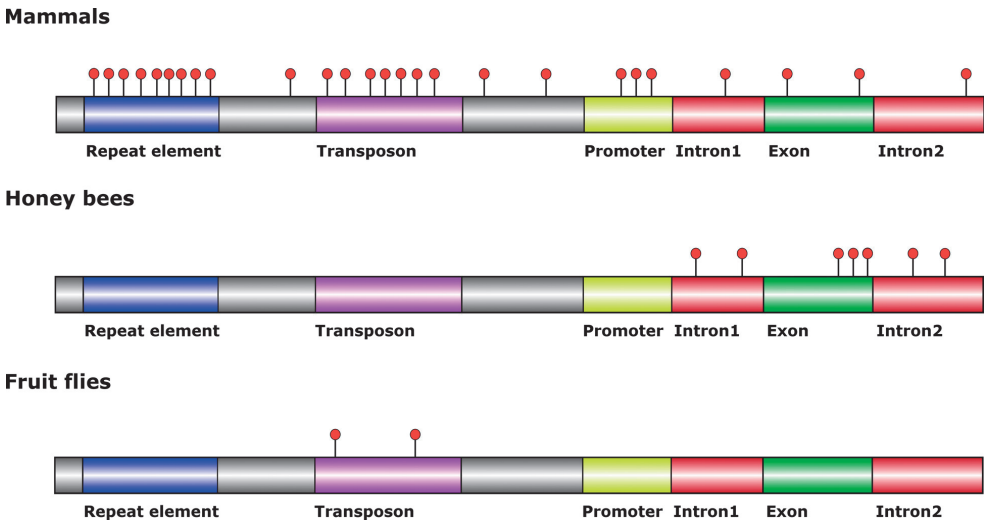

FIGURE 1 | General features of the 5-methylcytosine distribution in DNA from mammals, honey bees and fruit flies. Red circles indicate 5-mC. Mammalian genomes are typically methylated in transposon and repeat elements, and at some promoter regions. Intergenic DNA methylation occurs albeit at lower levels. Honey bee genomes are typically methylated in exons close to the exon-intron borders. Non-CpG methylation occurs in introns. Methylation outside of transposons has not been mapped in fruit fly genomes.

2009; Ivanov et al., 2013). The abundance of 5hmC compared to 5mC is much lower ranging from 2- to 100-fold times less depending on tissue (Kriaucionis and Heintz, 2009; Song et al., 2012). The distribution of 5hmC does not seem to be directly linked to 5mC, as 5hmC is found more often in promoter areas and enhancers, and much less in repetitive elements (Pastor et al., 2011; Stroud et al., 2011; Yu et al., 2012). In addition, proteins capable of specifically binding 5hmC have been discovered, fueling the theory that 5hmC exists as separate epigenetic mark and not simply just as an intermediate in an active demethylation pathway (Frauer et al., 2011; Méllen et al., 2012; Spruijt et al., 2013). In honey bees, 5hmC has been characterized in multiple tissues, and its abundance seems to be highest in germ cells and the brain (7–10% of 5mC and about 4% of 5mC, respectively), following the trend in mammalian cell types (Wojciechowski et al., 2014). Only one study has attempted to map 5hmC in honey bees at a single nucleotide resolution (Cingolani et al., 2013). This same study, surprisingly, mapped the majority of 5hmC in head tissue to non-CpG intronic sequences. Further studies seems warranted to precisely quantify and map 5hmC in bees, especially in non-brain tissue, which has received less interest so far. To date, 5hmC together with 5fC and 5caC have not been identified in the fruit fly, aphid, jewel wasp, and *C. elegans* genomes. However, since *C. elegans* has no 5mC precursor or TET homolog, the existence of 5hmC, 5fC, and 5caC seems highly unlikely.

5-FORMYLCYTOSINE AND 5-CARBOXYLCYTOSINE

The recently identified nucleotides 5fC and 5caC have, so far, not accumulated the same level of information as their precursors 5mC and 5hmC. This situation is in part due to extremely low abundance, especially for 5caC, making robust detection difficult (in mammals 5caC is 10- to 1000-fold less abundant than 5hmC). Moreover, the molecular toolbox for investigating 5fC and 5caC is not as developed as it is for 5hmC (Song and He, 2013). Bisulfite sequencing for example, only discriminates between "methylated" and "unmethylated" cytosines, so that 5mC and 5hmC are identified as "methylated" and 5fC and 5caC as "unmethylated" (Pastor et al., 2013). Such data are therefore difficult to use as guidelines in narrowing down possible locations of 5fC and 5caC.

The extremely low abundance of 5caC suggest that this nucleotide is merely an intermediate step in complete demethylation (Song and He, 2013). Although 5fC is a more prominent epigenetic mark than 5caC, its function is still not fully understood. It is possible that 5fC might regulate transcription through stalling of RNA pol II (Kellinger et al., 2012), but further research is needed to elucidate the role of 5fC and 5caC in both vertebrates and invertebrates. In honey bees, 5fC and 5caC have not been investigated yet, though their precursors and catalytic enzyme have been reported (Lyko et al., 2010; Wojciechowski et al., 2014).

FUTURE WORK: EPIGENETICS AND WORKER BEHAVIOR

Epigenetic mechanisms have been linked to the queen-worker differentiation of honey bees (Kucharski et al., 2008), as well as to worker behavioral progression and reversion (Herb et al., 2012). Herb et al. (2012) bisulfite sequenced brains of age-matched nurses, foragers, and reverted workers (previous foragers now involved in care behavior). Their data revealed differentially

methylated regions (DMRs) between the behavioral groups indicating that DNA methylation can play a role in regulation of social behavior. These DMRs are associated with genes involved in development, nuclear pore formation, and ATP binding. RNA sequencing revealed that these same DMRs were connected to alternative splicing events. It is also very likely that the "behaviorally sensitive" DMRs of honey bees are hydroxymethylated at some point during either transition from nurse to forager, or reversion from forager to nurse. Since the study was conducted in adult brain tissue, which has no neurogenesis (Fahrbach et al., 1995), dilution by replication would be unlikely or would only display minor effects. This situation makes these DMRs excellent candidates for investigating if 5hmC is associated with worker behavioral transitions, and if these hydroxymethylated regions are differentially hydroxymethylated between nurses, foragers, and reverted worker bees. Such a study could be the first to establish a putative link between hydroxymethylation and behavior.

Future studies should also dissect the role of TET in worker transitions from nurse to foragers, and back. Other candidate tissues than brain should include the fat body. This tissue is functionally homologous to liver and white adipose tissue and undergoes major remodeling during honey bee behavioral change (Chan et al., 2011). Functional implications of an RNA interference-mediated TET knockdown should provide insight into TET function. Studies can be conducted in honey bee larvae to investigate if TET knockdowns are capable of both queen and worker development. Similarly, consequences for behavioral plasticity can be studied in adult honey bee workers and perhaps link TET and its products with behavior for the first time.

Finally, a possible link between 5hmC and alternative splicing can be investigated by combining 5hmC sequencing at single nucleotide resolution with RNA sequencing of honey bee tissue samples. 5mC is reportedly implicated in the generation of alternative transcripts in the bee, but using methods not able to distinguish 5mC from 5hmC (Flores et al., 2012; Herb et al., 2012). Therefore, further studies that can map 5hmC alongside RNA sequencing data seems warranted, and could potentially give 5hmC a novel function in gene regulation.

CONCLUSION

The honey bee offers a system where the interplay between DNA methylation and social behavior can be studied in great detail. Published studies of the honey bee epigenome are dominated by questions surrounding queen and worker development, while the epigenetic dynamics of worker behavioral castes have only more recently gained attention. The readily identifiable social behaviors of worker honey bees make setting up precise, large scale experiments feasible (Münch et al., 2013). Better knowledge about honey bee epigenetics also has a dual purpose; increasing the understanding of epigenetic machineries in general, and gaining specific information about gene regulatory mechanisms in an economically important beneficial insect.

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DNA base modifications in honey bee and fruit fly genomes suggest an active demethylation machinery with species- and tissue-specific turnover rates

Erik M.K. Rasmussen [a,*], Cathrine B. Vågbø [b], Daniel Münch [a], Hans E. Krokan [b], Arne Klungland [c], Gro V. Amdam [a,d], John Arne Dahl [c]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, NO-1432 Aas, Norway
[b] Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway
[c] Department of Microbiology, Division of diagnostics and intervention, Institute of Clinical Medicine, Oslo University Hospital, Rikshospitalet, NO-0027 Oslo, Norway
[d] School of Life Sciences, Arizona State University, Tempe, AZ, USA

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ABSTRACT

Well-known epigenetic DNA modifications in mammals include the addition of a methyl group and a hydroxyl group to cytosine, resulting in 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) respectively. In contrast, the abundance and the functional implications of these modifications in invertebrate model organisms such as the honey bee (*Apis mellifera*) and the fruit fly (*Drosophila melanogaster*) are not well understood. Here we show that both adult honey bees and fruit flies contain 5mC and also 5hmC. Using a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) technique, we quantified 5mC and 5hmC in different tissues of adult honey bee worker castes and in adult fruit flies. A comparison of our data with reports from human and mouse shed light on notable differences in 5mC and 5hmC levels between tissues and species.

1. Introduction

DNA methylation along with regulation of chromatin packaging and recruitment of transcription factors by post-translational modifications of core histone proteins are well-studied epigenetic features. Cytosine methylation is responsible for the on and off switching of numerous genes, regulation of splice variants, and silencing of transposable elements [1]. Methylation patterns can be stable throughout an individual's lifetime, or dynamically shifting in response to different environmental and socio-environmental cues [2,3].

In mammals, the *de novo* methylation of cytosine is catalyzed by a member of the family of DNA methyltransferases (DNMT3) and occurs primarily within CpG dinucleotides at the 5th carbon of cytosine. The removal of the methyl group can be passive due to reduced activity of the "maintenance" DNA methyltransferase DNMT1 following DNA replication, or active through enzymatic demethylation independent of DNA replication. The discovery of the TET (Ten–eleven translocation methylcytosine dioxygenase) family of enzymes and their role in oxidizing the methyl group to a hydroxymethyl group [4], has provided clues to our understanding of the active demethylation pathway, and its regulation. Transcription factors and other protein factors have been shown to specifically bind 5-hydroxymethylcytosine (5hmC) [5,6], thus giving rise to the speculation that 5hmC might serve a specific biological role. In recent years, it was established that 5hmC could be further oxidized to 5fC and 5caC also by the TET dioxygenases. The latter two of these modified bases could potentially be recognized and excised by the Thymine DNA Glycosylase (TDG) family of enzymes, leaving an abasic site that can be repaired by the Base Excision Repair (BER) pathway [7].

In mammals, promoter methylation is linked to silencing of transcripts [8]. Gene body methylation is present throughout all eukaryotic kingdoms and seems to be highly conserved [9]. However, gene body methylation seems to be associated with active transcription in some species, suggesting that DNA methylation might play a different role in promoter regions and in intragenic regions [10]. Hymenoptera genomes, including that of the honey bee (*Apis mellifera*) are almost exclusively methylated within gene bodies, and are therefore very suitable models to study the effect of DNA methylation on exon expression and splice variants [11]. In mammalian tissues hydroxymethylated bases are usually present at a 1:5–1:100 ratio relative to the 5mC precursor [12,13]. Formylated

* Corresponding author.
E-mail address: erik.rasmussen@nmbu.no (E.M.K. Rasmussen).

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and carboxylated cytosines are present in even lower amounts, and are in some mammalian tissues only present in fmol amounts, close to the levels of oxidized bases resulting from DNA damage [13]. The modified bases produced by the TET dioxygenase homologs have not gained much attention yet in insects.

In honey bees, different phenotypes with specific social roles can emerge from a single genotype, which makes them an attractive model for epigenetic studies. For example, during larval stages female bees can develop into queens or workers, the female helper caste [14]. In addition, adult worker bees progress through a series of different social tasks that are linked to particular physiological and behavioral specializations. These include differences in social feeding patterns, with nurses producing brood food (royal jelly) and foragers collecting nectar and pollen outside the hive. The particular social task behaviors are enabled by physiological changes in tissues such as the brain and the fat body (functionally homologous to liver and white adipose tissue) [15–17]. Hallmark features of the social phenotypes have been studied most extensively in these two organs [18–20]. For example, brain DNA methylation patterns between nurses and foragers differ. However, they become more similar again, if foragers are induced to return back to nursing tasks [3]. Also the fat body proteome is extensively remodeled during the individuals' transition from nursing to foraging [21], yet the fat body has so far received less attention in epigenetic research.

Unlike fruit flies (*Drosophila melanogaster*), honey bees contain a broader set of DNA methyltransferases [22]. Knockdown of DNMT3 in honey bee workers via RNA interference results in queen-like individuals [23]. Previous work suggests that honey bees have the capacity to undergo active demethylation, but it is unclear how this is achieved on a molecular level [3]. The fruit fly has been extensively probed for DNA methylation in different tissues and developmental stages were early studies gave support for methylated DNA in early embryonic stages [24]. Recently, DNA methylation in adult fruit flies was detected using liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods with detection limits two orders of magnitude lower than traditional methods such as bisulfite sequencing [25].

Although the enzymes that are responsible for DNA methylation and oxidation of 5mC are present in several insects, their catalytic specificity and mechanistic role remains somewhat elusive. In mammals, DNMT3A exists in an auto-inhibitory state whose activation involves histone H3 and DNMT3L (a catalytically inactive paralog) [26]. Some insects as the silk worm (*Bombyx mori*) lack the *de novo* DNA methyltransferase, buts still have a distinct 5mC signature [27,28]. Knockdown of the only known methyltransferase with some degree of activity towards DNA, MT2 in the fruit fly does not abolish genomic 5mC generation, indicating that this specie might have a undisclosed mechanism of maintaining DNA methylation [29,30]. In honey bees, DNMT1b is also involved in memory formation, and the TET homolog seems very abundant compared to mammals [31,32].

Here we aimed to identify if demethylation intermediates can be detected in the brain and fat body of different worker types in honey bees. Using the fruit fly as a reference species for its low abundance of modified DNA bases, we quantified 5mC, 5hmC, 5fC and 5caC using a highly sensitive LC/MS/MS technique. We detected 5mC and 5hmC in both species, albeit at markedly higher levels in honey bees compared to fruit flies. As the GC content of an organism's genome is of relevance when comparing the amount of modified cytosines between species, we assessed the unmodified bases adenine, thymine, cytosine and guanine in honey bee tissues as well as in fruit flies along with tissues from mice, rat and human cells. We confirmed the previously reported low GC content in the honey bee genome. However, this alone cannot explain the relatively low abundance of cytosine modifications and especially the drastically reduced 5hmC to 5mC ratio.

2. Materials and methods

2.1. Obtaining honey bee specimens:

To represent different honey bee worker types (*Apis mellifera carnica* Pollman), we obtained one group of nurses as well as two groups of foragers with short (young foragers) and long foraging experience (old foragers), as described before [33]. To identify effects of chronological age, and separate these from possible age effects, the three worker types were of similar chronological age. To this end, worker bees were marked on the day of adult emergence to track their chronological age before adding them to two replicate host colonies. Foragers returning from their first foraging flight were marked with a second paint mark to identify foraging onset, and subsequently the number of days they had foraged. This approach enabled us to collect age-matched groups of nurses, young and old foragers on the same day. Foraging durations for the young and old foragers were 5–8 days and at least 12 days, respectively. All honey bees were collected and snap frozen in liquid N_2 in the early morning hours before daily foraging began.

2.2. Honey bee tissue preparation and DNA extraction:

Brains were dissected out from the head capsule in Phosphate Buffered Saline (PBS). The stinger along with the gut were pulled out of the abdomen and discarded. The remaining abdomen carcass was used for further analyses of the fat body, as described before [21]. Tissues (i.e. either brains or fat bodies) from a total of 6 worker bees were pooled for each biological replicate. DNA was extracted using a phenol:chloroform:isoamylalcohol extraction as previously described but modified for honey bee tissue samples [34]. Briefly, brains were homogenized in a lysis solution containing 0.4 mg Proteinase K (Sigma Aldrich P8044), 47.6% PBS (Sigma-Aldrich P4417), 47.6% Buffer AL (QIAGEN 19075) while abdomen carcasses with adhering fat body were homogenized in ATL buffer (QIAGEN 19076) containing 0.4 mg Proteinase K. Samples were then incubated at 56 °C with shaking at 400 rpm for 16 h in a Thermomixer (Eppendorf 5355 000.011). The lysate (500 μl) was extracted in phenol:chloroform:isoamylalcohol (25:24:1) before genomic DNA was precipitated by adding 1/10 vol equivalents of 3 sodium acetate (pH 5.3), 10 μl of linear acrylamide (ThermoFisher Scientific AM9520) and 2.5 vol of ethanol, washed twice in 70% ethanol, air dried, and dissolved in dH_2O.

2.3. Fruit fly tissue preparation and DNA extraction.

Drosophila melanogaster (Oregon-R lab strain) were anesthetized under CO_2, and female adults of all ages were fixed and stored in 96% ethanol. Fruit flies were dissected in PBS by removal of the gut. Twenty fruit flies were pooled for each replicate. DNA was extracted using the DNeasy Blood & tissue kit (QIAGEN 69506) as per manufacturer's instruction.

2.4. DNA hydrolysis and liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Genomic DNA was enzymatically hydrolyzed to deoxynucleosides essentially as described before [35], by adding 3 vol of methanol and centrifuged (16,000 g, 30 min, 4 °C). The supernatants were dried and dissolved in 50 μl 5% methanol in water (v/v) for LC/MS/MS analysis of the deoxynucleosides 5-hm(dC), 5-f(dC), and 5-ca(dC). A portion of each sample was diluted for the

quantification of 5-m(dC) and unmodified deoxynucleosides dA, dC, dG, and T. Chromatographic separation was performed on a Shimadzu Prominence HPLC system with an Ascentis Express C18 150 × 2.1 mm ID (2.7 μm) column equipped with an Ascentis Express C18 5 × 2.1 mm ID (2.7 μm) guard column (Sigma-Aldrich). Zorbax SB-C18 2.1 × 150 mm i.d. (3.5 μm) column equipped with an Eclipse XDB-C8 2.1 × 12.5 mm i.d. (5 μm) guard column (Agilent Technologies). The mobile phase consisted of water and methanol (both added 0.1% formic acid), for 5-m(dC), 5-hm(dC), 5-f(dC), and 5-ca(dC) starting with a 5-min gradient of 5–60% methanol, followed by 6 min re-equilibration with 5% methanol, and for unmodified nucleosides maintained isocratically with 15% methanol. Mass spectrometry detection was performed using an MDS Sciex API5000 triple quadrupole (Applied Biosystems) operating in positive electrospray ionization mode, monitoring the mass transitions 258.1/142.1 (5-hm(dC)), 256.1/140.1 (5-f(dC)), 272.1/156.1 (5-ca(dC)), 242.1/126.1 (5-m(dC)), 252.1/136.1 (dA), 228.1/112.1 (dC), 268.1/152.1 (dG), and 243.1/127.1 (T). Due to an interference from unmodified dG, the detection limit for 5-f(dC) was substantially higher than for 5-ca(dC) (10 and 0.03 per 10-6 unmodified DNA bases, respectively). This increased detection limit hindered the clear separation of the interference peak from dG and true 5-f(dC) in order to quantify 5-f(dC).

3. Results

We first asked if cytosine modifications, which are typical demethylation intermediates in vertebrates, are also present in the honey bee worker genome, exemplified in different worker types and ages. Mass spectrometry based methods are highly sensitive and specific, and have been previously used for detecting modified cytosines in various tissues in different mammals [36]. Using LC/MS/MS we analyzed cytosine modifications in the brain and fat body of adult honey bee workers (all female), and adult female fruit flies (Fig. 1A). We identified 5mC and 5hmC in both honey bees and fruit flies (Fig. 1B), while 5fC and 5caC could not be detected using this method, meaning that the level of 5fC and 5caC is below 10 (5fC) and 0.03 (5caC) per 10^6 unmodified DNA bases (see Section 2 for details). In the investigated honey bee tissues we could not detect a significant difference of 5mC levels between brain and abdomen with adhering fat body tissue (ANOVA p-value = 0.07; Fig. 2A). However, we observe a trend towards higher 5mC levels in the fat body compared to the brain. Our results on 5mC content in the brain are consistent with previous studies using bisulfite sequencing [37]. We also quantified 5hmC in honey bee brain and abdominal tissue (Fig. 2B). Importantly, and in contrast to 5hmC level differences between mammalian tissues (measured as 5hmC % of cytosine), we found significantly higher levels of 5hmC in the abdomen (with adhering fat body) relative to the brain (ANOVA p-value 0.009; Fig. 2B).

Levels of 5mC and 5hmC are not significantly different between the three tested worker bee groups (ANOVA, p-value: 0.18 and 0.98 respectively) indicating that the global level of these modifications does not change as worker honey bees undertake different social roles (Table S1).

We then assessed to what extent the low levels of cytosine modifications in the honey bee could be explained through the

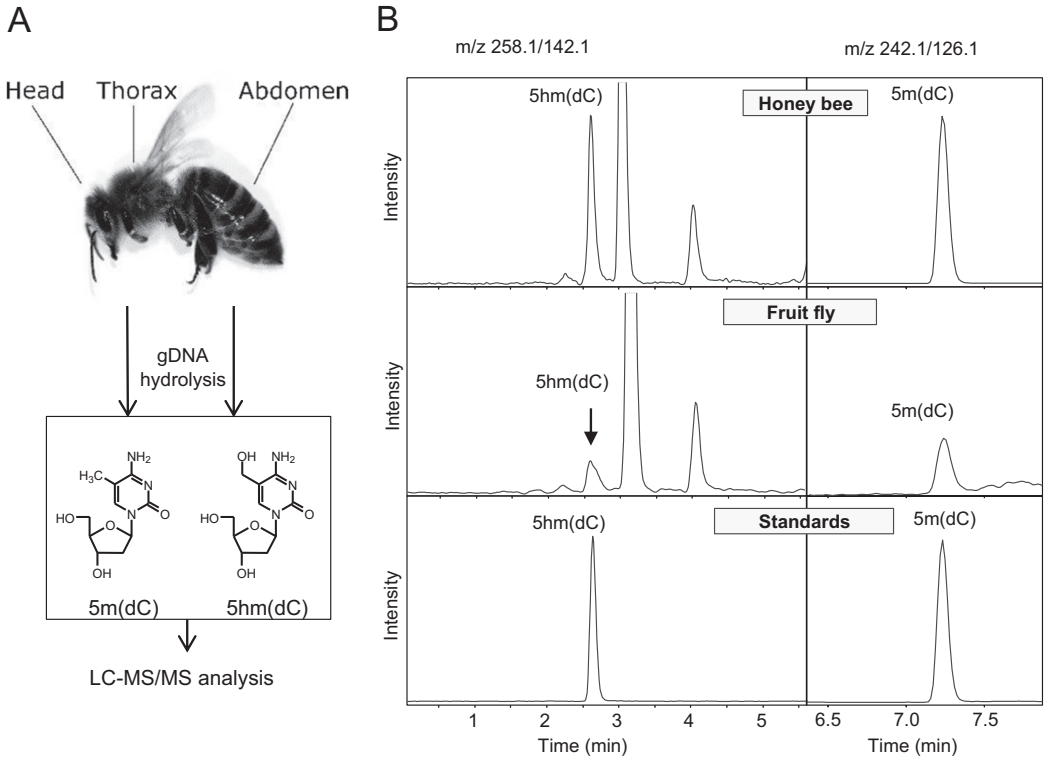

Fig. 1. Detection and quantification of cytosine modifications in honey bee and fruit fly. (A) Overview of the experimental setup. (B) Representative LC/MS/MS chromatograms showing 5hmC (left column) and 5mC (right column) in honey bees (top) and fruit fly (middle). Pure nucleoside standards are shown at bottom.

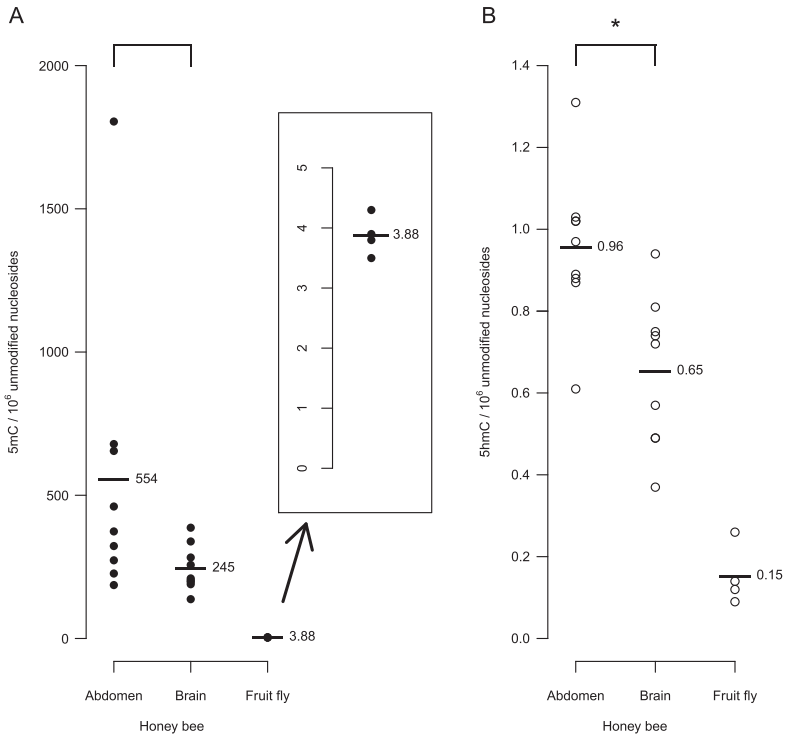

Fig. 2. Modified cytosine levels in honey bees and fruit flies. (A) 5mC levels in honey bee tissues, with fruit fly 5mC data shown as an insert. (B) 5hmC levels in honey bee tissues and fruit fly. Values are mean values. Asterisk denotes significance ($p < 0.05$, ANOVA) between comparable groups.

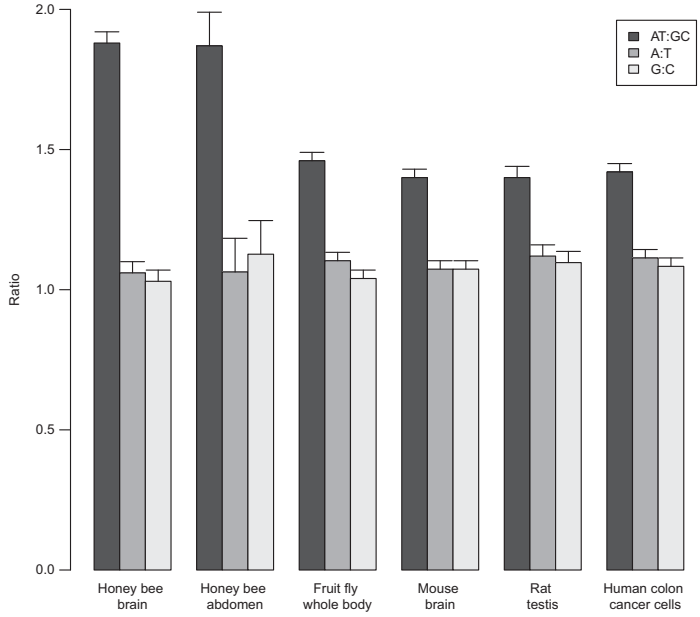

Fig. 3. Unmodified DNA base levels in different taxa. Relative levels of unmodified adenosine, thymine, guanine and cytosine in honey bee brain, honey bee abdomen, fruit fly, mouse brain, rat testis and human cancer colon cells. Error bars=SD.

low GC content of the honey bee genome. To this end, we investigated the proportion of unmodified cytosine, guanine, thymine and adenine in tissues from honey bees, fruit flies, rat, mice and a human cell line. Overall, the vertebrate and dipteran genomes have a higher GC:AT ratio (0.70) than the honey bee (0.53) (Fig. 3). Our results indicate that the honey bee genome has a GC content of about 33%, which is consistent with a previous study using whole genome sequencing [38]. In comparison, the GC content of human and mouse is 41% and 42% respectively [39]. Our data suggest that the low GC content of the honey bee samples can only partly explain the comparatively low abundance of 5mC and 5hmC.

Furthermore, we detected and quantified 5mC levels in adult fruit flies (Fig. 2A) which are markedly lower than in honey bee tissues. However, we found a lower level of 5mC in fruit fly as compared to a recently published study using a similar method as ours (Table S2) [25]. To our surprise, we could also detect 5hmC in the fruit fly genome albeit at 6.4 and 4.3 times lower levels as compared to honey bee abdomen and brain, respectively (Fig. 2B).

4. Discussion

Here we report the presence of genomic 5mC and 5hmC in the brain and abdominal tissue (primarily fat body) from honey bees, and in the adult fruit fly. Both modifications have been reported previously as present in genomic DNA of the honey bee brain, but a sensitive global quantification has been lacking. Importantly, we provide the first insight into the levels of 5mC and 5hmC in the honey bee fat body. Our results shed light on notable differences in 5hmC levels between tissues and species.

In honey bees, we detected higher absolute amounts of 5mC and 5hmC in abdomen as compared to brain tissue. We did not observe such high levels of 5hmC in the honey bee brain as a previously conducted study using antibody detection [40]. Reasons for this discrepancy might include methodological differences between the anti-CMS dot blot used previously and our LC-MS/MS method. Generally LC-MS/MS based methods are considered more sensitive than immuno-based methods [41]. However, our data does fit with the data reported by Wojciechowski et al. [32]. In mammals, global levels of 5mC and 5hmC differ considerably between cell types and tissues (Table 1). 5hmC levels (compared to cytosine) are highest in mammalian brain derived tissues, and lowest in cultured cells (Table S2). Our results indicate that the honey bee does not follow this pattern as 5hmC levels (compared to cytosine) are higher in abdomen derived tissues than in the brain.

The GC content of honey bees is lower than that of fruit flies, humans, mice and rats. However, even when taking this into account, honey bees have ~10 fold lower abundance of 5mC relative to cytosine (Table 1). Moreover, the abundance of 5hmC relative to cytosine is ~100–1000 fold lower in honey bee workers. In other words, differences in GC abundance between the honey bee and mammals only accounts for a minor fraction of the large differences in DNA base modification levels. The context in which genomic 5mC occurs also differs distinctly between honey bees and mammals (compare Introduction) [42]. Taken together this suggests different roles or significance for phenotype determination through cytosine modifications in the honey bee genome as compared to that in mammalian genomes. Future comparative studies may offer a novel perspective to better understand a possible mammal specific role of 5hmC in the brain.

The global level of 5hmC in fruit fly is the lowest of all compared species (Table 1), however, its relative level compared to 5mC surpasses the honey bee and is on par with some mammalian tissues. Previous studies using bisulfite sequencing, which is unable to distinguish 5hmC and 5mC, might therefore have overestimated 5mC in the fruit fly. On the other hand, most bisulfite sequencing studies have been performed in early embryo stages, and therefore a robust characterization of the adult methylome is

Table 1
Relative abundance of 5mC and 5hmC in humans, mice, honey bees and fruit flies.

Species	Tissue/cells	5mC % of C	5hmC % of C	5hmC % of 5mC	References
Homo sapiens	Brain			18.60%	Khare et al. [45]
	Liver	5.30%	0.60%	11.34%	Ivanov et al. [46]
	HEK293	2.60%	0.01%	0.35%	Ito et al. [36]
Mus musculus	Purkinje neurons			40%	Kriaucionis and Heintz [12]
	Granule neurons			~13%	Kriaucionis and Heintz [12]
	Cerebellum	4.39%[a]	0.33%[a]	7.52%	Münzel et al. [47]
	Cerebral cortex	4.50%[a]	0.65%[a]	14.44%	Münzel et al. [47]
	Brainstem	4.50%[a]	0.55%[a]	12.22%	Münzel et al. [47]
	Olfactory bulb	4.54%[a]	0.53%[a]	11.67%	Münzel et al. [47]
	Hippocampus	4.31%[a]	0.59%[a]	13.69%	Münzel et al. [47]
	Hypothalamus	3.33%[a]	0.68%[a]	20.42%	Münzel et al. [47]
	Retina	4.48%[a]	0.31%[a]	6.92%	Münzel et al. [47]
	Brain cortex	3.10%	0.67%	21.58%	Ito et al. [36]
	Kidney	2.70%	0.21%	7.81%	Ito et al. [36]
	Lung	2.75%	0.16%	5.93%	Ito et al. [36]
	Heart	2.70%	0.14%	5.33%	Ito et al. [36]
	Pancreas	2.50%	0.09%	3.74%	Ito et al. [36]
	Liver	2.75%	0.14%	5.24%	Ito et al. [36]
	Spleen	2.90%	0.06%	2.00%	Ito et al. [36]
	Thymus	3.05%	0.02%	0.79%	Ito et al. [36]
	Brain			13.60%	Khare et al. [45]
	Pancreas			2.80%	Khare et al. [45]
	ESC J1	4.5%[b]	0.15%	3.33%	Le et al. [48]
Apis mellifera	Abdomen	0.34%	0.0006%	0.17%	This study
	Brain	0.15%	0.0004%	0.27%	This study
	Worker larvae head	0.24%			Foret et al. [49]
Drosophila melanogaster	Female adult fly	0.002%	0.0001%	3.87%	This study
	Adult fly	0.034%			Capuano et al. [25]

[a] Percentages are of guanine.
[b] Percentages are of total pool of cytosine.

lacking [29]. Since the fruit fly genome harbors a well-conserved TET homolog, functional studies using fruit flies with disrupted TET expression (RNA interference-mediated knockdown or TET null mutants) could answer a lot of questions around the TET gene and its function in the fruit fly [43]. As a note of caution, it is conceivable that the 5hmC levels we detected are due to non-specific activity of the TET homolog, as the fruit fly TET homolog has recently been implicated in regulation of adenine methylation in DNA [44]. The buildup of 5mC in drosophila to detectable levels might be explained by the TET enzyme's preferred substrate being modified adenine instead of cytosine.

Future work on 5hmC in these insect species should focus on coupling RNA expression data together with very sensitive deep sequencing of 5hmC in order to elucidate the role of 5hmC in regulation of transcription and generation of alternate transcripts. In addition, investigating potential reader proteins of 5hmC can shed further light on the role and fate of this particular modified DNA base.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/ 10.1016/j.bbrep.2016.02.011.

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Supplementary material

Specie	Phenotype	Tissue	5mC / 10^6 unmodified nucleosides	5hmC / 10^6 unmodified nucleosides	Replicate
Apis mellifera	Nurse	Brain	387	0,94	1
	Nurse	Brain	339	0,49	2
	Nurse	Brain	203	0,75	3
	Young Forager	Brain	198	0,37	1
	Young Forager	Brain	283	0,81	2
	Young Forager	Brain	137,5	0,72	3
	Old Forager	Brain	210	0,49	1
	Old Forager	Brain	257	0,74	2
	Old Forager	Brain	189,7	0,57	3
	Nurse	Abdomen	1805	0,97	1
	Nurse	Abdomen	679	0,88	2
	Nurse	Abdomen	273,2	0,87	3
	Young Forager	Abdomen	374	1,02	1
	Young Forager	Abdomen	461	0,89	2
	Young Forager	Abdomen	227,1	1,03	3
	Old Forager	Abdomen	323	1,02	1
	Old Forager	Abdomen	655	1,31	2
	Old Forager	Abdomen	186,8	0,61	3
Drosophila melanogaster	Female	Whole body	3,8	0,14	
	Female	Whole body	3,9	0,12	
	Female	Whole body	4,3	0,26	
	Female	Whole body	3,5	0,09	

Species	Genome size (bp)	Tissue/Cells	5mC pr genome	5hmC pr genome	5mC% of cytosine	5hmC% of cytosine	5mC% of CpG	5hmC% of CpG	5hmC% of 5mC	References
Homo sapiens		IMR90	4,50E+07		4,25 %					Lister et al., 2009
		Placenta	2,06E+07		3.14% *					Dwi Putra et al., 2014
		Sperm					70-75 %			Molaro et al., 2011
		ICM					42,0 %			Guo et al., 2014
		Post Impl Embryos					78,1 %			Guo et al., 2014
		Oocyte					53,8 %			Okae et al., 2014
		Blastocyst					25,7 %			Okae et al., 2014
		H9 hESC					80,7 %			GEO accession number: GSM706059
		Neonatal blood cells					76,2 %			Okae et al., 2014
	3 221 487 035	2 cell embryo						0,03 %		Wang et al., 2014
		HEK293			2,60 %	0,009 %			0,35 %	Ito et al., 2011
		HEK293FT		0.009 % **		0,043 %				Song et al., 2011
		HeLa		0.007 % **		0,033 %				Song et al., 2011
		CDT4+T Newborn					80,50 %			Heyn et al., 2012
		CDT4+T 103-year-old					73,00 %			Heyn et al., 2012
		CDT4+T 26-year old					77,80 %			Heyn et al., 2012
		Brain							18,6 %	Khare et al., 2012
		Liver			5,30 %	0,60 %			11,34 %	Ivanov et al., 2013
		Leukocytes			4,00 %					Kok et al., 2007
Mus musculus		Cerebrum		3,40E+06		0.54 % ***				Zhang et al., 2012
		Cerebellum		2,39E+06		0.38 % ***				Zhang et al., 2012
		Cerebellum			4.39 % *	0.33 % *			7,52 %	Münzel et al., 2010
		ESC J1	2,84E+07	9,45E+05	4.5 % ****	0.15 % ****			3,33 %	Le et al., 2011
		ESC		~4-6E+06						Huang & Rao, 2012
		Brain Cortex			3,10 %	0,67 %			21,58 %	Ito et al., 2011
		Kidney			2,70 %	0,21 %			7,81 %	Ito et al., 2011
		Lung			2,75 %	0,16 %			5,93 %	Ito et al., 2011
		Heart			2,70 %	0,14 %			5,33 %	Ito et al., 2011
		Pancreas			2,50 %	0,09 %			3,74 %	Ito et al., 2011
		Liver			2,75 %	0,14 %			5,24 %	Ito et al., 2011
		Spleen			2,90 %	0,06 %			2,00 %	Ito et al., 2011
		Thymus			3,05 %	0,02 %			0,79 %	Ito et al., 2011
		Cerebral cortex			4.5 % *	0.65 % *			14,44 %	Münzel et al., 2010
		Brainstem			4.5 % *	0.55 % *			12,22 %	Münzel et al., 2010
		Olfactory bulb			4.54 % *	0.53 % *			11,67 %	Münzel et al., 2010
		Retina			4.48 % *	0.31% *			6,92 %	Münzel et al., 2010
		Hippocampus			4.31 % *	0.59 % *			13,69 %	Münzel et al., 2010
	2 800 055 571	Hypothalamus			3.33 % *	0.68 % *			20,42 %	Münzel et al., 2010
		Multiple tissues			4.30 % *					Globisch et al., 2010
		Spinal cord				0.47 % *				Globisch et al., 2010
		kidney, nasal epithelium, bladder, heart, skeletal muscle, and lung				0.15-0.17 % *				Globisch et al., 2010
		liver, spleen, and the endocrine glands				0.03-0.06 % *				Globisch et al., 2010
		Purkinje neurons		0,60 %		2,86 %			40 %	Kriaucionis & Heintz, 2009
		Granule neurons		0,20 %		0,95 %			~13 %	Kriaucionis & Heintz, 2009
		ES		0,03 %		0,15 %				Tahiliani et al., 2009
		Brain							13,60 %	Khare et al., 2012
		Pancreas							2,80 %	Khare et al., 2012
		mESC		2,06E+06				2,03E+06		Yu et al., 2012
		Somatic tissues					78,30 %			Hon et al., 2013
		E14 mESC		1,22E+07				1,17E+07		Sun et al., 2013
		Oocytes					40,00 %			Kobayashi et al., 2012
		Sperm					89,40 %			Kobayashi et al., 2012
		Liver at ZR13			3,00 %					Xia et al.,2015
Apis mellifera		Abdomen	1,39E+05	2,40E+02	0,34 %	0,0006 %			0,17 %	This study
		Brain	6,13E+04	1,63E+02	0,15 %	0,0004 %	0,50 %		0,27 %	This study and Lyko et al., 2010 (for CpG data)
	250 287 000	Brain		1,44E+04		0,0003 %				Wojciechowski et al., 2014
		Male Whole bee					0,93 %			Feng et al., 2010
		Worker larvae head	1,00E+05		0,24 %					Foret et al., 2012
Drosophila melanogaster		Female adult fly	5,41E+02	2,09E+01	0,002 %	0,0001 %	NA		3,87 %	This study
		Adult fly	2,00E+04		0,034 %					Capuano et al., 2014
	139 485 381	Adult tissues		<20						Zhang et al., 2015
		Embryo (2-16 h)		<20						Zhang et al., 2015

* Percentages are of Guanine
** Percentages are of total nucleotides of the genome
*** Percentages are of a pool of cytosine, 5-hydroxymethyltytosine and 5-methylcytosine
****Percentages are of total pool of cytosine

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Screening bioactive food compounds in honey bees suggests curcumin blocks alcohol-induced damage to longevity and DNA methylation

Erik M. K. Rasmussen[1,6]✉, Kristine L. Seier[1,7], Ingrid K. Pedersen[1,8], Claus Kreibich[2], Gro V. Amdam[2,3], Daniel Münch[2,4]✉ & John Arne Dahl[5]✉

Various bioactive food compounds may confer health and longevity benefits, possibly through altering or preserving the epigenome. While bioactive food compounds are widely being marketed for human consumption as 'improving health and longevity' by counteracting harmful effects of poor nutrition and lifestyle, claimed effects are often not adequately documented. Using the honey bee (*Apis mellifera*) as a model species, we here employed a multi-step screening approach to investigate seven compounds for effects on lifespan and DNA methylation using ELISA and whole genome bisulfite sequencing (WGBS). A positive longevity effect was detected for valproic acid, isovaleric acid, and cyanocobalamin. For curcumin, we found that lifespan shortening caused by ethanol intake, was restored when curcumin and ethanol were co-administered. Furthermore, we identified region specific DNA methylation changes as a result of ethanol intake. Ethanol specific changes in DNA methylation were fully or partially blocked in honey bees receiving ethanol and curcumin together. Ethanol-affected and curcumin-blocked differentially methylated regions covered genes involved in fertility, temperature regulation and tubulin transport. Our results demonstrate fundamental negative effects of low dose ethanol consumption on lifespan and associated DNA methylation changes and present a proof-of-principle on how longevity and DNA methylation changes can be negated by the bioactive food component curcumin. Our findings provide a fundament for further studies of curcumin in invertebrates.

Once thought to be a stable epigenetic mark, it is now widely accepted that DNA methylation can be dynamic. Recent advances in uncovering enzymatically oxidative derivates of 5-methylcytosine have led to a rising interest in the field of functional epigenetics and especially dynamic DNA methylation[1–3]. The recognition of the dynamic nature of epigenetic marks provides a foundation for assessing the effect the environment and specific compounds have on the epigenome of living organisms.

Micronutrients and other bioactive food components hold potential as epigenome effecting drugs[4–6]. The diversity of food supplements that is marketed towards humans for potential health benefits includes vitamins, plant extracts and synthetic analogues to natural compounds. While health effects are apparent for some supplements, claimed benefits are unclear for others despite traditional beliefs[7–9]. In addition, modes of actions are often elusive when concerning potential epigenetic effects. The assessment and understanding of epigenetics in metabolic studies is still in its infancy. Folic acid (vitamin B9) however, is an exception of a food supplement

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Aas, Norway. [2]Faculty of Ecology and Natural Resource Management, Norwegian University of Life Sciences, 1433 Aas, Norway. [3]School of Life Sciences, Arizona State University, Tempe, AZ, USA. [4]Animalia – Norwegian Meat and Poultry Research Centre, 0513 Oslo, Norway. [5]Department of Microbiology, Oslo University Hospital, Rikshospitalet, 0027 Oslo, Norway. [6]Present address: Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 1433 Aas, Norway. [7]Present address: Vestre Viken Hospital Trust, 3004 Drammen, Norway. [8]Present address: Institute for Energy Technology, 2007 Kjeller, Norway. ✉email: erik.rasmussen@nmbu.no; daniel.munch@animalia.no; j.a.dahl@medisin.uio.no

compound with demonstrated inherent linkage to epigenomic mechanisms, as it is already established as prophylactic drug during pregnancy to protect the fetus against neural tube defects[10]. Sodium butyrate (mostly produced in the intestine from fermentation of dietary fiber, but also present in some dairy products) was one of the first bioactive food components shown to interfere with the posttranslational modifications on the N-terminal tails of core histones by acting as an inhibitor of certain histone modifying enzymes[11–13].

The synthetic compound valproic acid, an analogue of valeric acid derived from the plant *Valeriana officinalis* has for decades been used in treatment for epileptic seizures (for a review see[14]). Hints about the mode of action was provided in the early 2000s when Phiel and coworkers (2001)[15] discovered that valproic acid could inhibit histone deacetylases. Plant extracts are usually a mixture of different compounds, and improper storage may lead to formation of breakdown products like in the case of *Valeriana officinalis*, isovaleric acid[16,17]. The effects of isovaleric acid on the epigenome remains largely unstudied.

Curcumin, the main compound isolated from the rhizome of the turmeric plant, has been implicated in histone hypoacetylation[18,19]. Considerable crosstalk between post-translational modifications of histones and DNA methylation has been demonstrated[20–22]. In addition, Curcumin has been shown to confer some level of protection to the liver against damages from alcohol abuse in mice[23]. However, low bioavailability of curcumin and usage of poor solvents (i.e. water) plague many of the earlier studies[24,25]. These are challenges that must be considered when conducting in vivo studies.

Ethanol, one of the most harmful substances abused by humans in Europe and a leading cause of death in the US, is also a versatile solvent of many organic compounds, including bioactive food components[26,27]. However, rodent model organisms like mice display large strain variations in ethanol tolerance and development of conditioned taste aversion[28–31].

Scoring of survival in a model organism can be a powerful screening assay to directly determine advantageous or deleterious effects of plant-derived compounds fed at a range of concentrations. Promising hits can be further studied by whole genome bisulfite sequencing (WGBS) to assess the whole genome for DNA methylation changes associated with consumption of a compound. This strategy can uncover compounds that confer health and longevity benefits and provide insight into molecular mechanisms and pathways related to these benefits. Arguably, the honey bee is a cost effective and, due the short life span, an efficient model organism for such studies.

The honey bee is a demonstrated model for dynamic DNA methylation[32–34]. The honey bee hive is a highly complex structure with functionally sterile worker bees making up the bulk of the bees, communicating and performing specialized task to support the hive[35]. Importantly, compared to Dipterans like *Drosophila melanogaster*, honey bees appear to have a functionally broader DNA methylation system[32,36]. Herb and co-authors (2012)[33] have shown that a social perturbation of the DNA methylome in honey bees is possible, reversing previously established DNA methylation patterns when older worker bees revert to tasks usually performed by younger workers.

In addition to its semi-domesticated hive habitat, honey bees can be housed inside the laboratory with relatively little effort or use of lab space[37]. This allows tight control of food intake, and usage of many individuals facilitates screening of different compounds at a range of concentrations. Many insects are attracted to ethanol[38,39], and the honey bee is no exception[40,41]. The honey bee has been used to study the effects of ethanol with special emphasis on behavior, learning and memory[42]. Ethanol has been shown to alter the honey bees' locomotor behavior making bees more likely to spend their time upside down[43]. Also, honey bees fed ethanol showed impaired learning and memory recall performance[44]. However, the effects of ethanol on the honey bee DNA methylome remains to be investigated.

In this study we use a three-step screening approach were seven compounds, at a range of concentrations, were first screened for effects on longevity. All compounds and concentrations were assessed for any deviation in consumption, and only compounds that did not affect food consumption were included in further analysis. These compounds were then screened for global methylation changes. The most promising compound was selected for WGBS and in-depth analyses of how DNA methylation may be affected by substance exposure. A positive survival effect was detected for valproic acid, isovaleric acid, and cyanocobalamin. We show that ethanol has a detrimental effect on worker bee lifespan and induces changes in DNA methylation patterns. For curcumin, we found that lifespan shortening caused by ethanol intake, was restored when curcumin and ethanol were co-administered. Furthermore, we identified region specific DNA methylation changes as a result of ethanol intake. Ethanol specific changes in DNA methylation were fully or partially blocked in honey bees receiving ethanol and curcumin together. We present a proof-of-principle on curcumin restoring alcohol-associated shortened lifespan and DNA methylation changes in an important model organism. Our findings provide a fundament for further studies of curcumin in invertebrates and offer an avenue to explore regarding possible prevention of health issues related to alcohol consumption.

Material and methods

Animals. Honey bees (*Apis mellifera carnica* Pollmann) were kept at the Norwegian University of Life Sciences' apiary at Aas, Norway. Honey bee studies on survival and feed consumption were carried out during fall of 2013, specimen for DNA methylation studies were collected during winter 2014 (see below).

To establish similar life history traits among all tested specimens and to minimize physiological age differences, we exclusively used one worker type: winter bees. To achieve this, we used established tools to manipulate the hive's social demography and worker bee tasks[45]. In brief, the characteristic feature of winter (*diutinus*) bee colonies is the absence of brood, which triggers physiological changes in workers, e.g., by diverting resources into increased nutrient storage, winter worker types typically show higher resilience and longer survival regardless of time of emergence[46–50]. To assure collection of winter worker type, all colonies for sampling in January were confirmed to be broodless prior to collection. To obtain broodless hives and winter worker types already in fall

(September–October), we removed all brood combs with eggs and larvae at least 2 weeks prior collection, and queens were prevented from egg-laying through confining them in queen cages[47].

Survival assays and assessment of feed consumption. Sample collection in fall, was essentially carried out as follows: honey bees were collected from two individual donor colonies ('colony replicate') with naturally mated queens in two rounds, each separated by two to three days ('day replicate'). From each donor colony, four cages with approximately 50 bees in each cage were collected, resulting in a total of 16 cages and approximately 800 bees for each round.

For the entire subsequent test period, bees remained in the plastic cages, which had the following dimensions: width/depth/height: 19 × 18x23 cm and were lined with patches of mesh for ventilation. After collection, cages were transferred into a climate chamber, set to temperature of 26 °C with approximately 60% relative humidity (e.g.[37,51]. For acclimation and to control for possible effects of sample collection on initial mortality rates, all cages received the control diet (see below) for the first two days of captivity. Bees that died during this initial period were removed and not considered in survival statistics.

For subsequent survival and food consumption testing, bees had ad libitum access to a water feeder and another feeder containing the respective treatment diet. Treatment identity (factor: dietary supplementation) was balanced with respect to day and colony replicates, and was otherwise randomly assigned to the individual cages. Each day, dead bees were removed and the number of dead individuals per cage was recorded. Similarly, food and water feeders were changed, and the consumed volume was recorded daily. After 41 days the experiment was terminated, and any remaining bees were censored.

The average daily consumption per bee for the first 10 treatment days was calculated per cage in order to investigate if any lifespan differences could be attributed to differences in consumption.

We have tested for possible effects of dietary supplementation with isovaleric and valproic acid, sodium butyrate, cyanocobalamin, folic acid, ethanol, and curcumin. All treatment diets were made based on the same stock feed solution, which also served as the control diet. The stock feed solution contained 50% Bifor® (Nordic Sugar; 37% sucrose, 19% glucose, 19% fructose in water), 2% Grace's amino acid mix for insect cell culture (Sigma-Aldrich cat no G0148) or RPMI 1640 (Sigma-Aldrich cat no R7131), 1% lipid mix for insect cell culture (Sigma-Aldrich cat no L5146) and 47% dH_2O. In treatment diets the dH_2O fraction of the stock feed was partly substituted by the respective compound (details in Supplementary Table 1). All tested compounds except ethanol were given at 3 concentrations (considered low, medium, high concentration based on literature) with a 10 × concentration increment[52–57]. Each treatment (factor: concentration) was given to four replicate cages with 50 bees each, making a total of 3*4 = 12 cages plus 16 (for isovaleric acid, valproic acid, folic acid, and cyanocobalamin) or four (for sodium butyrate, ethanol, and curcumin) control fed cages (50 bees each) per tested compound.

Preparation of bees for molecular testing. Honey bees were collected from two separate donor hives with naturally mated queens on January 15th, 2014. In total, 18 cages with approximately 50 bees each were collected from both hives resulting in approximately 900 bees.

All honey bees were fed a control diet with the same constituents as the survival assay experiment for the first 2 days of captivity. After 2 days, food diets were exchanged with the same treatment diets used in the survival assay experiment. For a detailed makeup of diets used see Supplementary table 1. Each treatment diet was given to two cages to reduce the chance of bias due to cage effects.

Temperature, humidity, number of dead honey bees, food and water consumption was recorded daily. Fresh food and water were also given daily along with removal of any dead bees. After 10 days of treatment diet feed, all remining honey bees were flash frozen in liquid nitrogen and stored at – 80° C until further processing.

DNA extraction. DNA was extracted essentially as described previously[58]. The stinger along with the gut was pulled out of the separated abdomen and discarded. The remaining abdominal carcass with the fat body tissue was used for further analyses. Briefly, abdomen carcasses with adhering fat body were homogenized in ATL buffer (QIAGEN 19076) containing 0.4 mg Proteinase K (Sigma Aldrich P8044). Samples were then incubated at 56 °C with shaking at 400 rpm for 16 h in a Thermomixer (Eppendorf 5355 000.011). The lysate (500 µl) was extracted once in phenol:chloroform:isoamylalcohol (25:24:1) before the aqueous phase was RNAse treated by incubating the sample with 5 µl of RNAse A (20 mg/ml) at 37 °C with shaking at 550 rpm for 30 min. The aqueous phase was extracted again using phenol:chloroform:isoamylalcohol (25:24:1) before genomic DNA was precipitated by adding 1/10 volume equivalents of 3 M sodium acetate (pH 5.3), 10 µl of linear acrylamide (ThermoFisher Scientific AM9520) and 2.5 vol of ethanol, washed twice in 70% ethanol, air dried, and dissolved in nuclease free water.

Assessment of global methylation state using 5mC specific ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed using the Zymo Research 5-mC DNA ELISA kit according to the manufacturer's instructions. Briefly, 100 ng of DNA was diluted with 5-mC coating buffer to a volume of 100 µl. The DNA was denatured at 98 °C for 5 min and immediately incubated on ice for 10 min. Denatured DNA was transferred to wells and incubated in darkness at 37 °C for 1 h, allowing the DNA to settle and adhere to the wells. After incubation, the buffer was discarded, the plate was washed three times with 5-mC ELISA buffer, and kept with the 5-mC ELISA buffer at 37 °C for 30 min in darkness. After incubation, the buffer was discarded, and primary and secondary Horseradish Peroxidase conjugated antibodies were added to each well for labeling of 5mC (1:2000 and 1:1000 concentrations respectively). The plate was incubated at 37 °C for 1 h in darkness. After incubation, the plate was washed three times with 5-mC ELISA buffer, HRP-developer was added and allowed to develop for

45 min before absorbance at 405 nm was measured with a microplate reader. The percentage of 5mC present in the samples were calculated according to the manufacturer's description.

Whole genome bisulfite sequencing library preparation. Sequencing libraries were constructed essentially as described before[59]. Briefly, 2 µg of genomic DNA spiked with 10 ng of λ-phage DNA which is devoid of DNA methylation was fragmented using a Sartorius Labsonic M sonicator, with cycle settings at 0.5 and power/amplitude set to 30%. Average fragment length was assessed on an Agilent Bioanalyzer DNA High Sense chip. Upperand lower size cut-offs were performed using Agencourt AMPure XP beads at a volume of 0.6 × and 1.4 × to DNA solution respectively (selecting for fragments in the ~ 100 to ~ 600 bp range). Sticky ends were repaired using Epicentre End-it DNA end repair kit according to the manufacturer's recommendations. Adenosine tails were added to blunt end fragments using New England Biolabs (NEB) Klenow Fragment (3′ --> 5′ exo-) and NEBNext® dA-Tailing Reaction Buffer according to the manufacturer's recommendations. The reactions were cleaned up using Agencourt AMPure XP beads at a volume of 1.4 × to DNA solution (selecting for fragments < 100 bp). Methylated adapters were ligated to eluted DNA using NEB T4 DNA ligase according to manufacturer's recommendations. Ligation reactions were purified twice using Agencourt AMPure XP beads at a volume of 1.0 × to DNA solution. Eluted DNA was bisulfite converted using Human Genetic Signatures MethylEasy™ Xceed kit according to the manufacturer's recommendations. A low cycle PCR (between four to eight cycles) was performed to increase sequencing depth. To estimate the number of cycles needed, PCR reactions were quantified using KAPA Library Quantification Kit using the manufacturer's recommendations. Completed PCR products were cleaned up using Agencourt AMPure XP beads at a volume of 1.0 × to DNA solution. Libraries were pooled at equimolar ratios and sequenced on the HiSeq 2500 (Illumina) in paired-end (100 bp) read mode with 100 run cycles.

Identification of differentially methylated regions.. Preliminary quality control was performed using FastQC (available from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low quality bases were removed using Trimmomatic[60]. The bisulfite conversion efficiency was calculated by mapping the samples to the lambda phage genome. We set the conversion rate cut-off at 98%, thus samples with a bisulfite conversion rate < 98.0% were not analyzed further. Trimmed reads were aligned to the Amel 4.5 reference genome[61] using Bismark[62] with Bowtie 1[63]. Mapped reads were then deduplicated to remove PCR bias. Cytosine report files where then imported into R (version 3.5.3) using functions from bsseq[64,65]. The identification of differentially methylated regions was performed essentially as before[34]. Briefly, only cytosines with an average coverage of ≥ 6 in all samples and ≥ 10% methylation levels in three or more samples were defined as methylated. Percentage measurements derived from ratios of reads containing methylated and unmethylated cytosines in the CpG context where then arcsine transformed. Suggestive CpGs were identified using Student's t-test. Correction for multiple testing was performed using the qvalue package (available from https://github.com/StoreyLab/qvalue). Regions of individually methylated CpGs were designated by the bumphunter package[66]. The raw p values for each methylated CpG in each region were combined using the comb-p software package and corrected for multiple testing[67].

Data analyses of survival and consumption assays. Survival data was analyzed, and plots were generated using the statistical software packages survival and survminer in R (version 3.5.3)[68], available from https://cran.r-project.org/package=survminer[65]. To assess overall effects among multiple treatment groups we used the log-rank test for trends. For pairwise comparison between treatment concentration and respective control we calculated log-rank statistics.

Corrections for multiple testing were done using the False Discovery Rate/Benjamini Hochberg Approach[69]. Consumption data was analyzed using the STATISTICA 13 software package (Statistica, Dell Inc, Tulsa, USA). We used one-way ANOVA analysis to assess overall effects among multiple treatment groups. We used the Fisher LSD as a post-hoc test to determine which concentration(s) differed from the control(s) if any.

Ethics statement. The work on honey bees described in this study was carried out in 2013 and 2014 when certified training of researchers and an approval from the Norwegian Food Safety Authority was not required to carry out research with honey bees.

Results and discussion

Screening of food bioactive compounds reveals effects on survival in honey bees. We selected seven food bioactive compounds for our study that are relevant regarding human exposure through food, dietary supplements, pharmaceuticals and abuse. We first asked if intake of the seven chosen compounds could affect the lifespan of honey bees. A three-step screening approach was applied, were 7 substances, at a range of concentrations, were first screened for effects on longevity. All substances and concentrations were assessed for any deviation in consumption, and only substances that did not affect food consumption were included in further analysis. These substances were then screened by ELISA for global methylation changes. The most promising candidate was selected for WGBS and in-depth analyses of how DNA methylation may be affected by substance exposure. For an overview of the flow of screening methodologies see Fig. 1. Honey bees are short lived and easy to keep in the laboratory. Henceforth, this enabled us to use just under 200 bees per tested compound concentration, a number of individuals that would be out of reach for most labs using rodent model organisms. Kaplan–Meier survival analyses on overall data (Log-rank test for trends) revealed significant treatment effects on survival for isovaleric acid ($N_{control/0.10\ mg/ml/1.0\ mg/ml/10.0\ mg/ml} = 795/188/197/182$; $p < 0.005$), valproic acid ($N_{control/0.1\ mg/ml/1.0\ mg/ml/10.0\ mg/ml} = 795/194/192/193$; $p < 0.005$), cyanocobalamin (Vitamer of B12)

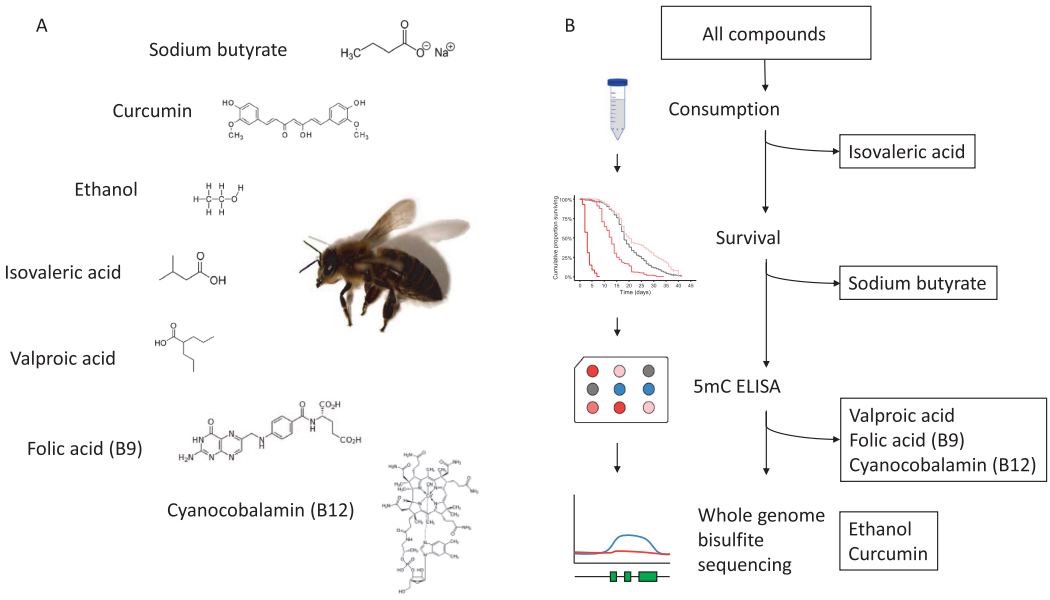

Figure 1. Overview of experimental setup. (**A**) Overview of the 7 initial compounds used. (**B**) Overview of flow of screening methodologies. A three-step screening approach was applied, were the 7 substances, at a range of concentrations, were first screened for effects on longevity. All substances and concentrations were assessed for any deviation in consumption, and only substances that did not affect food consumption were included in further analysis. These substances were then screened by ELISA for global methylation changes. The most promising candidate was selected for WGBS and in-depth analyses of how DNA methylation may be affected by substance exposure.

($N_{control/0.02\mu g/ml/0.2\mu g/ml/2\mu g/ml} = 795/182/185/193$; $p < 0.005$) and folic acid ($N_{control/5\mu g/ml/50\mu g/ml/500\mu g/ml} = 795/192/193/191$; $p < 0.005$), but not for sodium butyrate ($N_{control/0.01\ mg/ml/0.1\ mg/ml/1.0\ mg/ml} = 197/191/122/208$; $p > 0.005$). Pair-wise log-rank tests against controls with corrections for multiple testing were done to identify survival-effective concentrations for each compound (for an overview of all post-hoc tests see Fig. 2A). We report longer survival for honey bees fed isovaleric acid at concentrations of 0.1 and 1 mg/ml ($N_{control/0.10\ mg/ml/1.0\ mg/ml/} = 795/188/197$; $p_{0.1\ mg/ml/1\ mg/ml} < 0.05$), however, at 10 mg/ml we found survival to be shorter ($N_{control/10.0\ mg/ml} = 795/182$; $p_{10\ mg/ml} < 0.05$) (Supplemental Figure 1). For valproic acid fed honey bees we observed longer survival for 0.1 mg/ml ($N_{control/0.1\ mg/ml} = 795/194$, $p < 0.05$, Supplemental Figure 2), and found significantly shortened survival for higher concentrations ($N_{control/1.0\ mg/ml/10.0\ mg/ml} = 795/192/193$; $p_{1.0\ mg/ml/10.0\ mg/ml} < 0.05$). Valproic acid is a potent anticonvulsant drug used in the treatment of multiple neurological and psychological diseases in humans[14]. However, its use often needs to be carefully monitored as numerous side-effects can occur. Our results indicate that high sensitivity for valproic acid is also conserved in honey bees. Cyanocobalamin given at a concentration of 0.2 µg/ml significantly shortened survival ($N_{control/0.2\ \mu g/ml} = 795/185$, $p < 0.05$, Supplemental figure 3). For folic acid (Vitamin B9) fed honey bees an effect on survival, i.e. shortened survival, was only found for the lowest concentration (5 µg/ml) ($N_{control/5\ \mu g/ml} = 202/2192$; $p < 0.05$, Supplemental figure 4). Folic acid and cyanocobalamin have received little attention in insects[57]. However, there are indirect evidence that insects can obtain folate from gut bacteria[70]. One may envisage that the gut microbiome might be shifted in a harmful way by exposure to folic acid rich diets, possibly leading to reduced fitness and survival.

In accordance with what has previously been reported, we show detrimental effects of ethanol on honey bee survival ($N_{control/ethanol} = 198/208$; $p < 0.05$, Fig. 2B). Previous work reported a negative effect of ethanol on life span in honey bees for concentrations down to 2.5% (v/v)[40]. Using a longer-lived phenotype than Mustard et al. we observed reduced life span caused by an ethanol concentration as low as 1% (v/v), indicating that low concentration of ethanol is more harmful than previously assumed.

Remarkably though, when honey bees were fed a diet that contained a high concentration of curcumin (100 µg/ml) together with ethanol (1%, v/v), the life span was significantly increased as compared to ethanol intake only (Fig. 2B) ($N_{ethanol/ethanol\ \&\ curcumin\ 100\ \mu g/ml} = 208/204$; $p < 0.05$). In fact, with 100 µg/ml curcumin in the ethanol containing diet, the ethanol associated reduction in life span was largely restored to that observed in the control (Fig. 2B). However, honey bees fed with lower concentrations of curcumin (1 µg and 10 µg/ml) together with ethanol (1% (v/v)) did not show a significant increase in life span ($N_{ethanol/10\ \mu g/ml/1\ \mu g/ml} = 208/204/201$; $p > 0.05$). This demonstrates the need for a relatively high concentration of curcumin for the favorable effect of restoring lifespan shortening caused by ethanol intake. Curcumin has previously been reported to increase

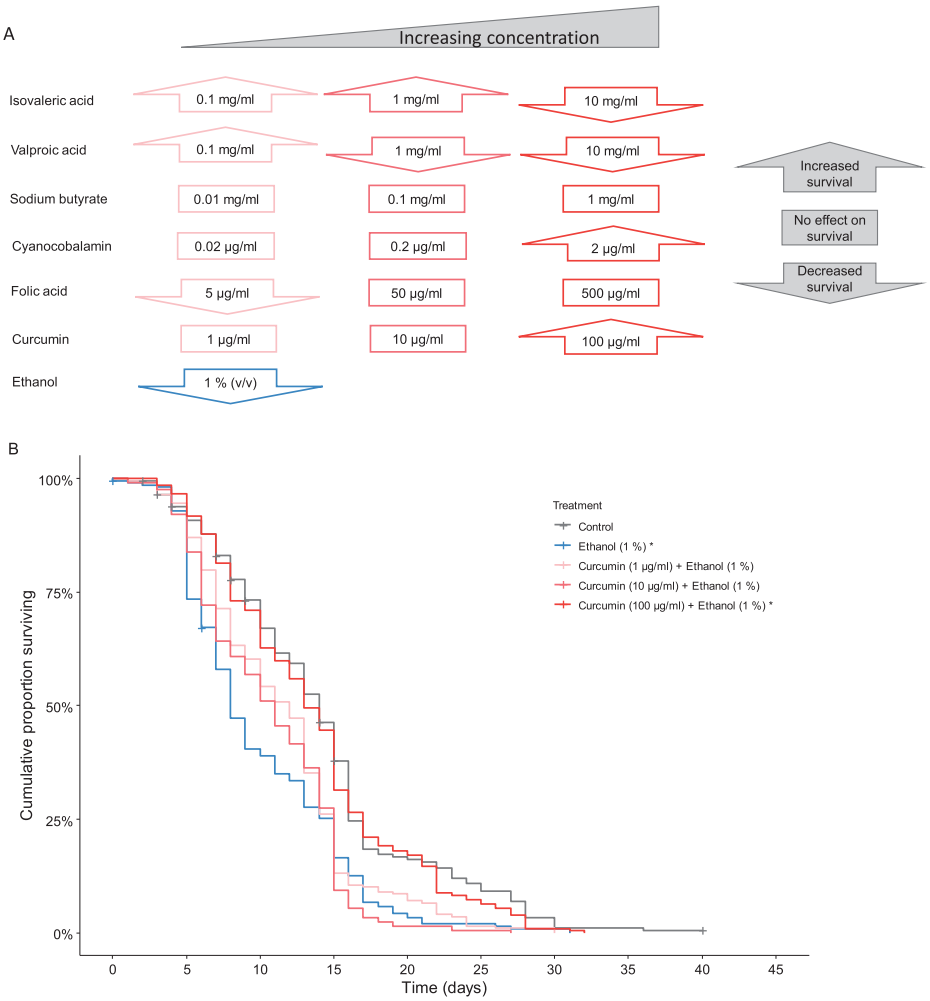

Figure 2. Effects of bioactive food compounds on honey bee survival. (**A**) Overview of all substances studied and the selected concentrations used in the feed. Upward pointing arrow signifies increased life span, rectangular box, no statistical effect on life span, downward pointing arrow signifies shorter life span ($p < 0.05$ Pair-wise Log-Rank test corrected using FDR). (**B**) Kaplan–Meier plot of survival curves of ethanol (1% (v/v)) alone and in combination with curcumin at 1, 10 and 100 µg/ml. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival compared to controls.

lifespan in a study of infections in honey bees[55]. Taken together with our results, one may speculate that curcumin could possibly act through a common mechanism, for example by increasing stress resilience in honey bees.

In a survival study based on modifications of the feed composition, one need to consider that differences in survival could potentially be affected by food palatability, or other more adverse effects on food intake. To control for this, we monitored food consumption. Significant food consumption differences were only found for isovaleric acid (One-way ANOVA, $N_{control/10\ mg/ml/1.0\ mg/ml/0.1\ mg/ml} = 4/4/4/4$; $F = 29.76$; $p < 0.001$). A post-hoc test revealed that bees given the 10 mg/ml and 1 mg/ml diet consumed significantly less food (Fisher-LSD, $p_{1\ mg/ml} < 0.05$, $p_{10\ mg/ml} < 0.001$). Therefore, we cannot rule out that the observed differences in survival for feed containing 10 mg/ml and 1 mg/ml isovaleric acid was due to lower food intake. There was however no effect on food consumption for feed containing 0.1 mg/ml isovaleric acid. We observed no effect on food consumption for any of the other studied food bioactive compounds, and the observed effects on life span can therefore likely be attributed to the intake of the studied compounds. As we had to change the amino acid source during the experiment due to supplier constraints, we also investigated if the source of amino acids had any effect on survival. Differences in survival were indeed observed (Log-rank test, $N_{Grace's\ aa\ mix/RPMI\ 1640} = 198/795$, $p < 0.0001$)

(Supplemental figure 5). However, we have not made any direct comparisons among compounds that use a different amino acid source for feed stock solutions.

Assessment of effects of food bioactive compounds on global DNA methylation. Next, we asked if differences in lifespan after treatment with the tested compounds could be linked to changes in global DNA methylation. In order to reduce complexity, only two concentrations from each compound (along with their respective controls) were selected for assessment by ELISA (Supplementary Table 1). Despite the relatively sparse methylated genome of the honey bee, ELISA based methods have successfully been applied to detect changes in global DNA methylation[71]. For our assessed conditions we did not observe any significant effect on the global DNA methylation level among any of the tested compounds (One-tailed Mann–Whitney test; $p > 0.05$). However, we did observe a trend towards hypermethylation when bees were fed ethanol alone. Notably, this trend was reversed in honey bees that had consumed the feed containing both ethanol (1% (v/v)) and the highest curcumin concentration (100 µg/ml) (Supplementary figure S6) (One-tailed Mann–Whitney test; $p = 0.07446$; $n_1 = 4$, $n_2 = 4$). Locus-specific changes in DNA methylation may still occur although they cannot be identified when analyzing DNA methylation changes on a global level with the ELISA methodology that only has modest sensitivity.

Exons are enriched for CpG methylation in honey bees. Encouraged by the observed trends in global DNA methylation changes in honey bees fed ethanol alone and co-fed ethanol and the highest curcumin concentration, we sat out to perform whole genome bisulfite sequencing (WGBS) in order to assess region specific DNA methylation changes throughout the honey bee genome. We carried out WGBS on DNA from abdominal extracts, which contain the fat body organ that is in part analogous to mammalian liver and white adipose tissue, thus important in energy metabolism, from a total of 13 honey bees, where five were fed 1% (v/v) ethanol ($n_{ethanol} = 5$), three with 1% (v/v) ethanol combined with 100 µg/ml curcumin ($n_{curcumin} = 3$), and five controls ($n_{control} = 5$). Too low bisulfite conversion rates hindered the inclusion of two more of the curcumin and ethanol co-fed individuals for DNA methylation analysis. Assessment of bisulfite conversion rates and additional quality controls were carried out for all samples (Section M&M and Supplementary table 2).

As an added quality measure, we investigated if exons would be enriched for methylated CpGs, as methylated CpGs have been reported to be confined to exons in most insect species (For review see[32]). A CpG dinucleotide was included for analysis if the average coverage was six times or more, and that at least three samples showed a methylation level of 10% or higher for this CpG. As expected, exons were enriched for methylated CpGs compared to whole mRNA sequence bins (Supplemental Figure 7). We then asked if any DNA methylation differences between treatments could be observed on a global scale using nucleotide resolution data. However, and as observed with the ELISA based method, no statistically significant differences could be found on the global level (One-tailed Mann–Whitney tests, $n_{control/ethanol/curcumin} = 5/5/3$, $p > 0.05$, Supplemental figure 8).

Curcumin blocks alcohol associated DNA methylation changes. Using a previously established pipeline for honey bee methylomics[34], we identified seven hits for genomic regions that were significantly differentially methylated (Fig. 3, Supplemental figures 9–11 and Supplemental table 2). Four regions were differentially methylated in ethanol exposed honey bees as compared to controls. Of these, three regions were hypermethylated and one hypomethylated. Three regions were differentially methylated (all hypomethylated) in curcumin and ethanol fed honey bees as compared to ethanol-only. (Supplementary Table 2). Upon further investigation, it became clear that three of the differentially methylated regions in curcumin and ethanol co-fed honey bees versus ethanol-only, overlapped with three of the differentially methylated regions observed in bees fed ethanol only. Ethanol induced hypermethylation in these three regions (Fig. 3A–C), whereas curcumin remarkably protected against ethanol-induced hypermethylation in all of these regions.

Curcumin administered together with ethanol resulted in DNA methylation levels similar to the levels observed in the control honey bees for three out of four ethanol affected regions (Fig. 3A–C and supplemental figures 9–11). These three ethanol-affected and curcumin-blocked differentially methylated regions are located in the gene bodies of the loci GB49219, GB47217 and GB43904, within linkage groups 7, 11 and 8, respectively. The Drosophila homologue of GB49219 is known as Gudu, which is necessary for male fertility[72]. Based on this it would be interesting to investigate if male bees (drones) would experience transiently reduced sperm quality after excessive alcohol consumption, like human males do[73,74]. GB47217 is a calcitonin receptor and is involved in temperature preference rhythm regulation and body temperature preference rhythms during active phases in Drosophila melanogaster and mice respectively[75]. Hypomethylation of the calcitonin receptor is involved in aggression behavior in honey bees[34]. Our results show that ethanol induces hypermethylation of the calcitonin receptor, thus it is unlikely that ethanol would invoke a state of aggression in honey bees, speculatively rather resulting in more sedate behavior that could possibly have a negative effect on life span. GB43904 is part of the IFT-B complex that binds to tubulin and mediates tubulin transport within the cilium[76,77].

In the one region that ethanol induced hypomethylation, curcumin co-consumption failed to fully protect against this hypomethylation, however we observed a trend of partial protection (Fig. 3D). This region is spanning part of the gene body of locus GB52826, a transmembrane protein that seems to be ubiquitously expressed in human tissues and associated with thyroid cancers[78,79].

In summary, all the loci that were affected by DNA hypermethylation as a result of ethanol consumption showed full or partial protection when curcumin was consumed together with ethanol. Ethanol-affected and curcumin-blocked differentially methylated regions covered genes involved in fertility, temperature regulation and tubulin transport. Interestingly, none of these genes have previously been reported to play a role in the effect of curcumin in mammals[80]. Our results indicate few curcumin-ethanol interactions on the level of methylation

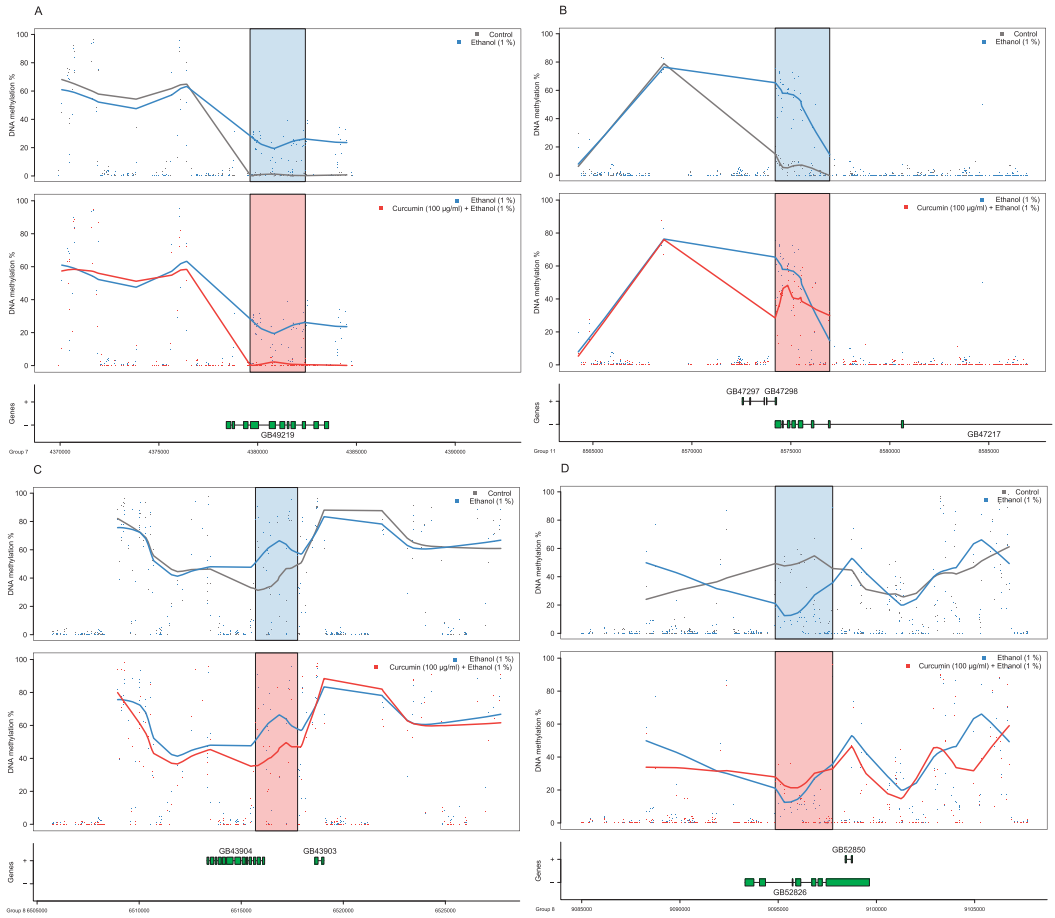

Figure 3. (**A**–**D**) Differentially methylated regions where the top panel depicts the situation in control (grey) versus ethanol (blue) fed honey bees, and the blue shaded area indicates the significant DMR. 10 kbp upstream and downstream (if no gaps exist in the Amel 4.5 genome build) are plotted for reference (white background). The middle panel depicts the comparison between ethanol-only (blue) and the honey bee group fed ethanol and curcumin (red). The lower panels depict the gene structure by exons (boxes) and introns (lines) by strand according to linkage groups.

changes, suggesting that the main mode of lifespan alterations from these compounds happen trough other mechanisms than DNA methylation. However, further studies are needed to investigate the significance of these changes. The function of gene body methylation in insects is still a controversial topic[33,81].

Intergenic DNA methylation seems to be involved in the generation of alternative splice isoforms[82,83]. Our data fits in with that notion, as all genes underlying the DMRs have predicted splice isoforms.

However, the significance of previously reported DMRs have come into question as sequence polymorphism and epialleles seems to play a bigger role than previously anticipated in social insects[84–86]. Our results demonstrate fundamental negative effects of low dose ethanol consumption on lifespan and associated DNA methylation changes, and present a proof-of-principle on how longevity and DNA methylation changes can be restored by the bioactive food component curcumin.

Conclusions

In summary, our results show that bioactive food components can modulate lifespan in the honey bee. Our data opens for the possibility that negative effects of ethanol on lifespan might be facilitated trough changes in DNA methylation, and that these changes are prevented when curcumin is consumed together with ethanol. We present a proof-of-principle on how longevity and DNA methylation changes can be restored by the bioactive food component curcumin. Furthermore, we extend the honey bee as model system for the study of DNA

methylation changes in relation to ethanol consumption by introducing an intervention with curcumin. Our findings provide a fundament for further studies of curcumin in invertebrates.

Data availability

The whole genome bisulfite sequencing data have been deposited into the NCBI Gene Expression Omnibus (GEO) database with accession number GSE169362.

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Author contributions
D.M., E.M.K.R., J.A.D. and G.V.A. designed the study. K.L.S., I.K.P., C.K. and E.M.K.R. carried out the animal experiments and K.L.S. and I.K.P. performed the ELISA. E.M.K.R. constructed the sequencing libraries. E.M.K.R. analyzed the data and generated the figures with support from J.A.D., D.M. and G.V.A. E.M.K.R. wrote the manuscript with assistance from G.V.A., D.M. and J.A.D. All authors read and approved the manuscript before publication.

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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to E.M.K.R., D.M. or J.A.D.

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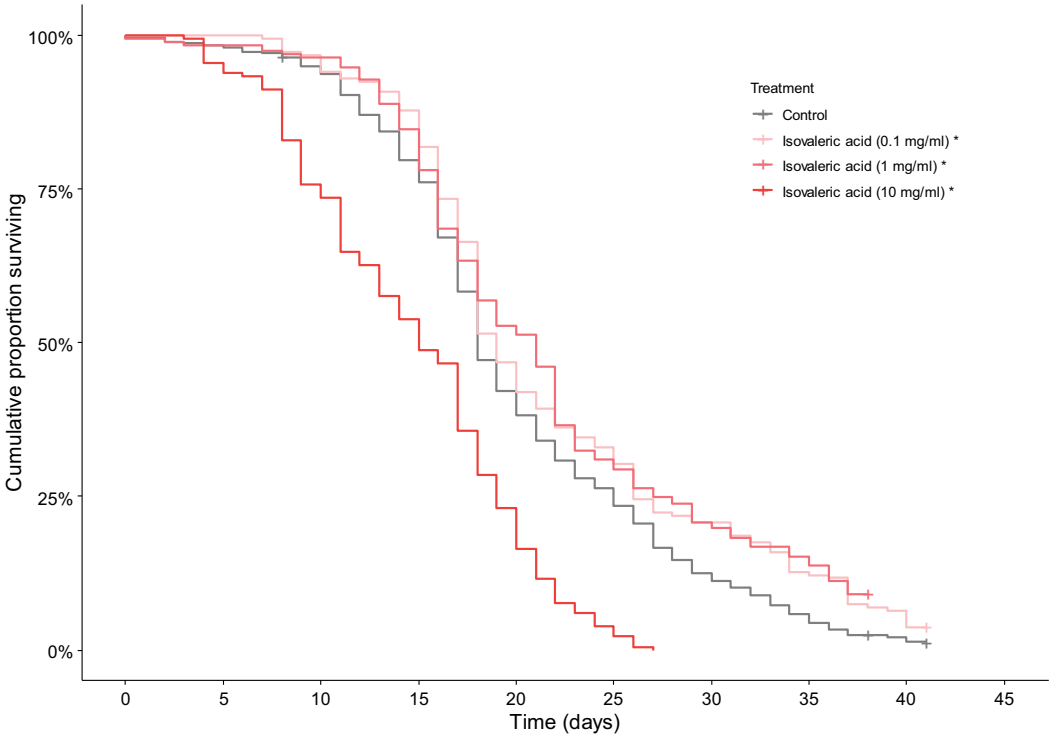
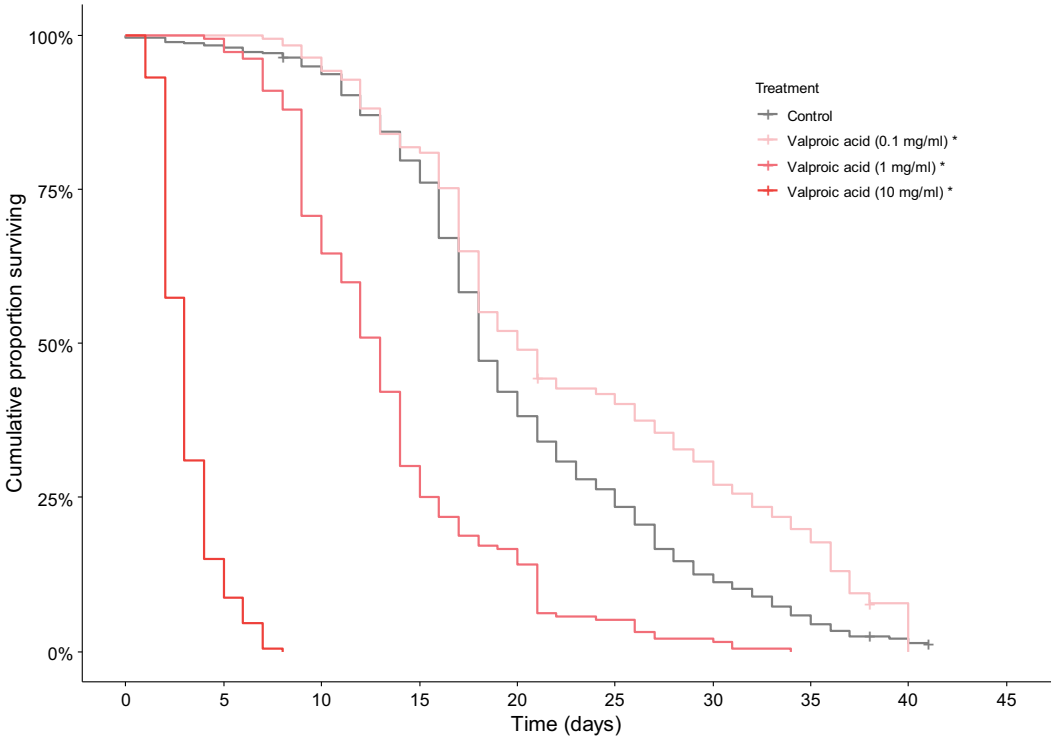
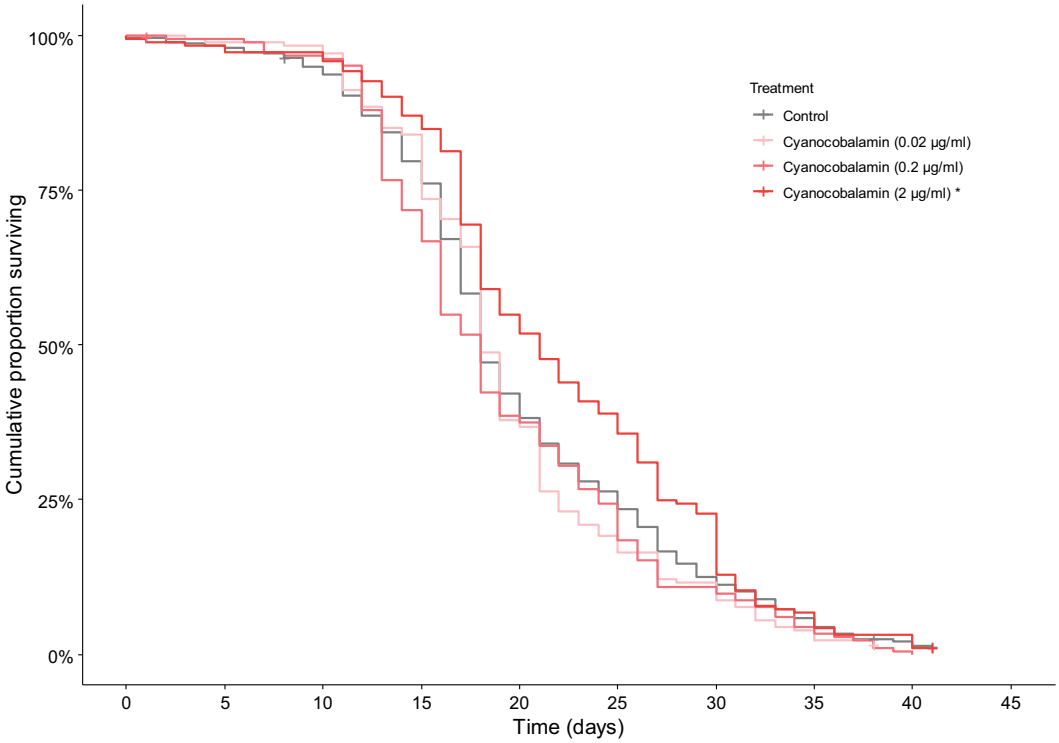
Supplementary information



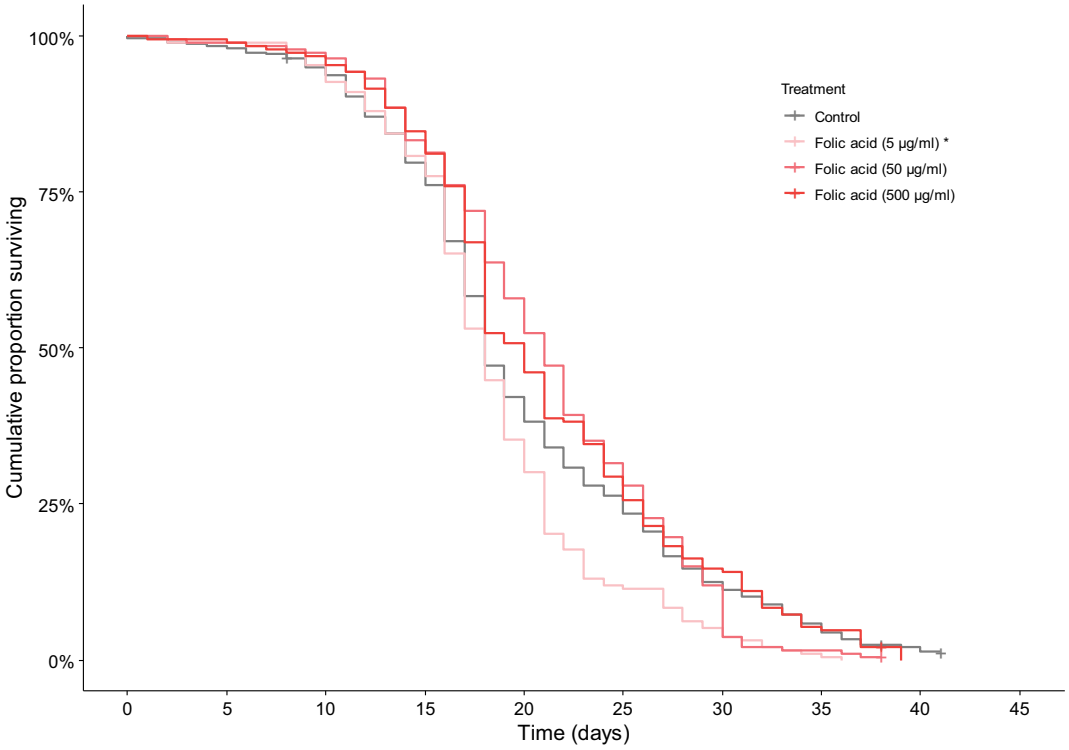
Supplemental figure 1: Cumulative proportion of surviving honey bees exposed to isovaleric acid in feed at 10 mg/ml, 1 mg/ml and 0.1 mg/ml. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival compared to control ($P < 0.05$, Pair-wise Log-Rank test corrected using FDR).



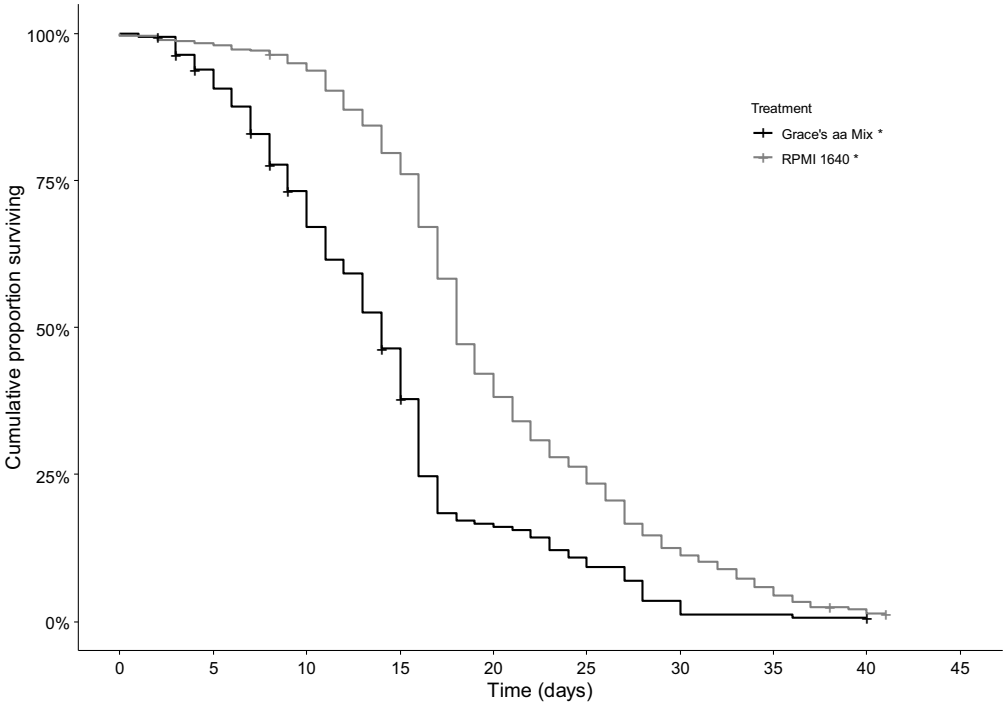
Supplemental figure 2: Cumulative proportion of surviving honey bees exposed to Valproic acid in feed at 10 mg/ml, 1 mg/ml and 0.1 mg/ml. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival compared to control ($P < 0.05$, Pair-wise Log-Rank test corrected using FDR).



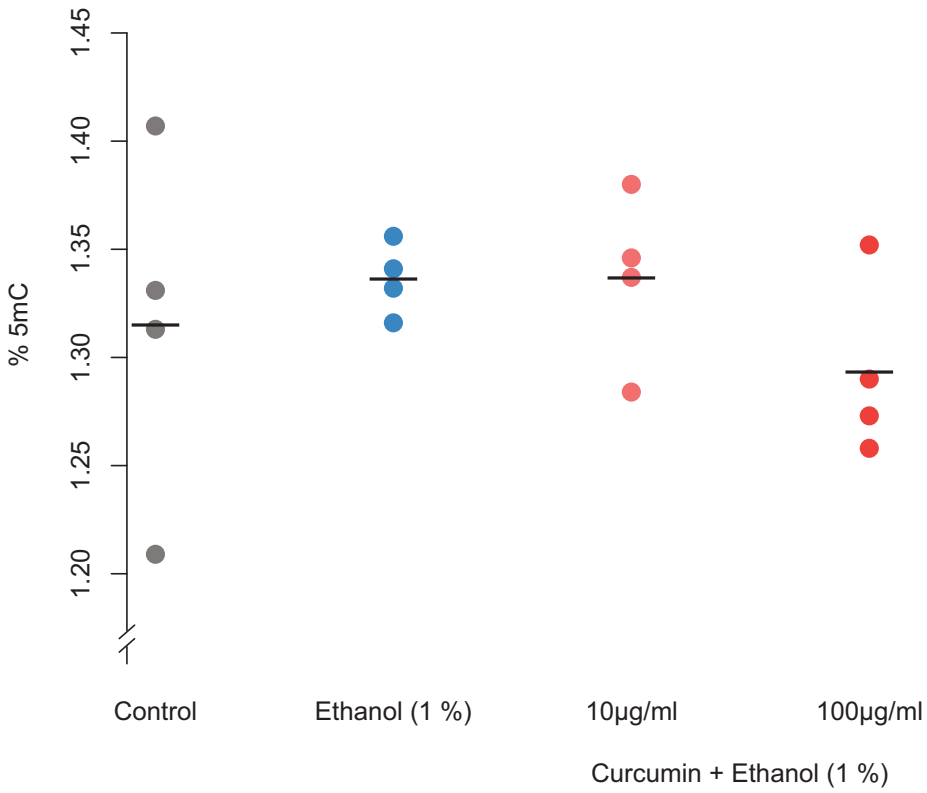
Supplemental figure 3: Cumulative proportion of surviving honey bees exposed to cyanocobalamin in feed at 2 µg/ml, 0.2 µg/ml and 0.02 µg/ml. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival compared to control ($P < 0.05$, Pair-wise Log-Rank test corrected using FDR).



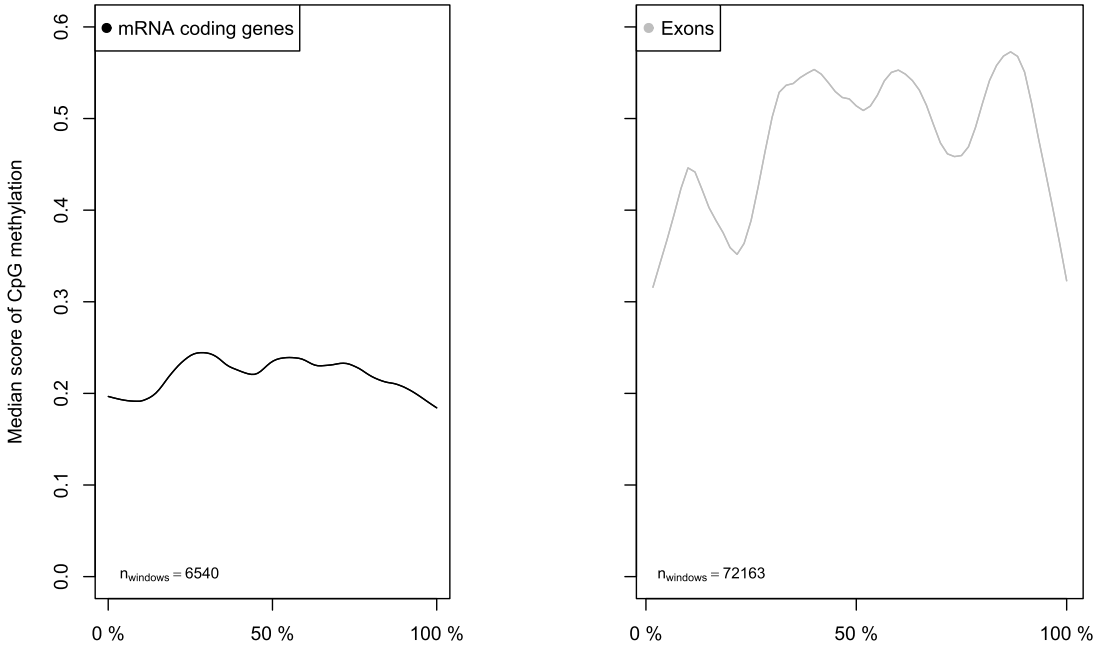
Supplemental figure 4: Cumulative proportion of surviving honey bees exposed to folic acid in feed at 500 µg/ml, 50 µg/ml and 5 µg/ml. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival compared to control ($P < 0.05$, Pair-wise Log-Rank test corrected using FDR).



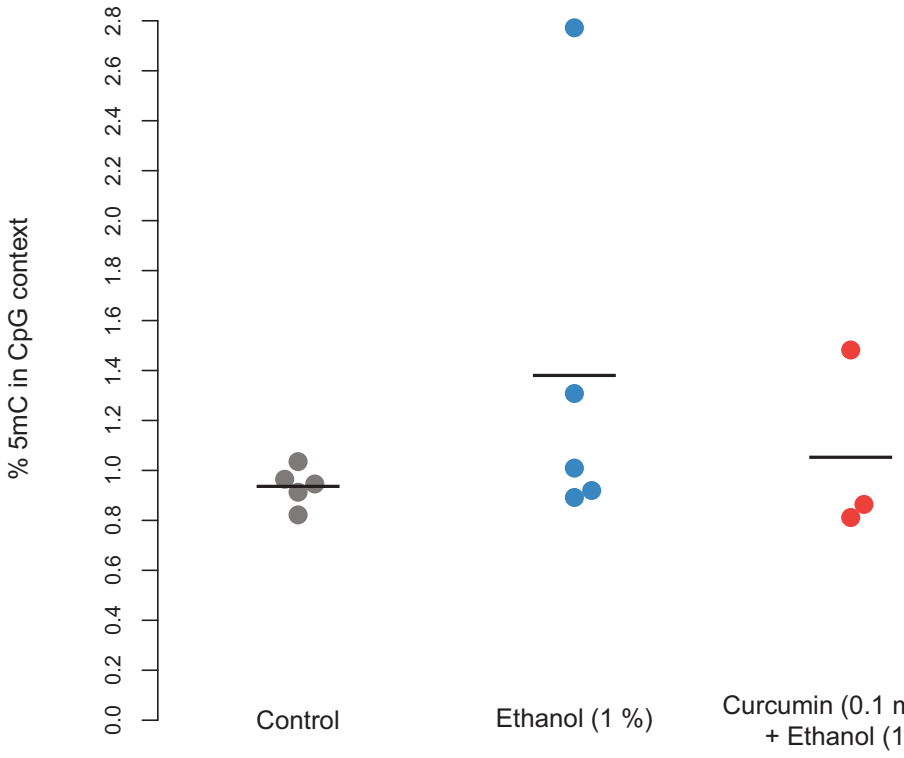
Supplemental figure 5: Cumulative proportion of surviving honey bees exposed to different sources of amino acids. Pluss signs indicate censored honey bees. Asterisks in legends indicates significant effects on survival (Kaplan Meier, $p < 0.0001$).



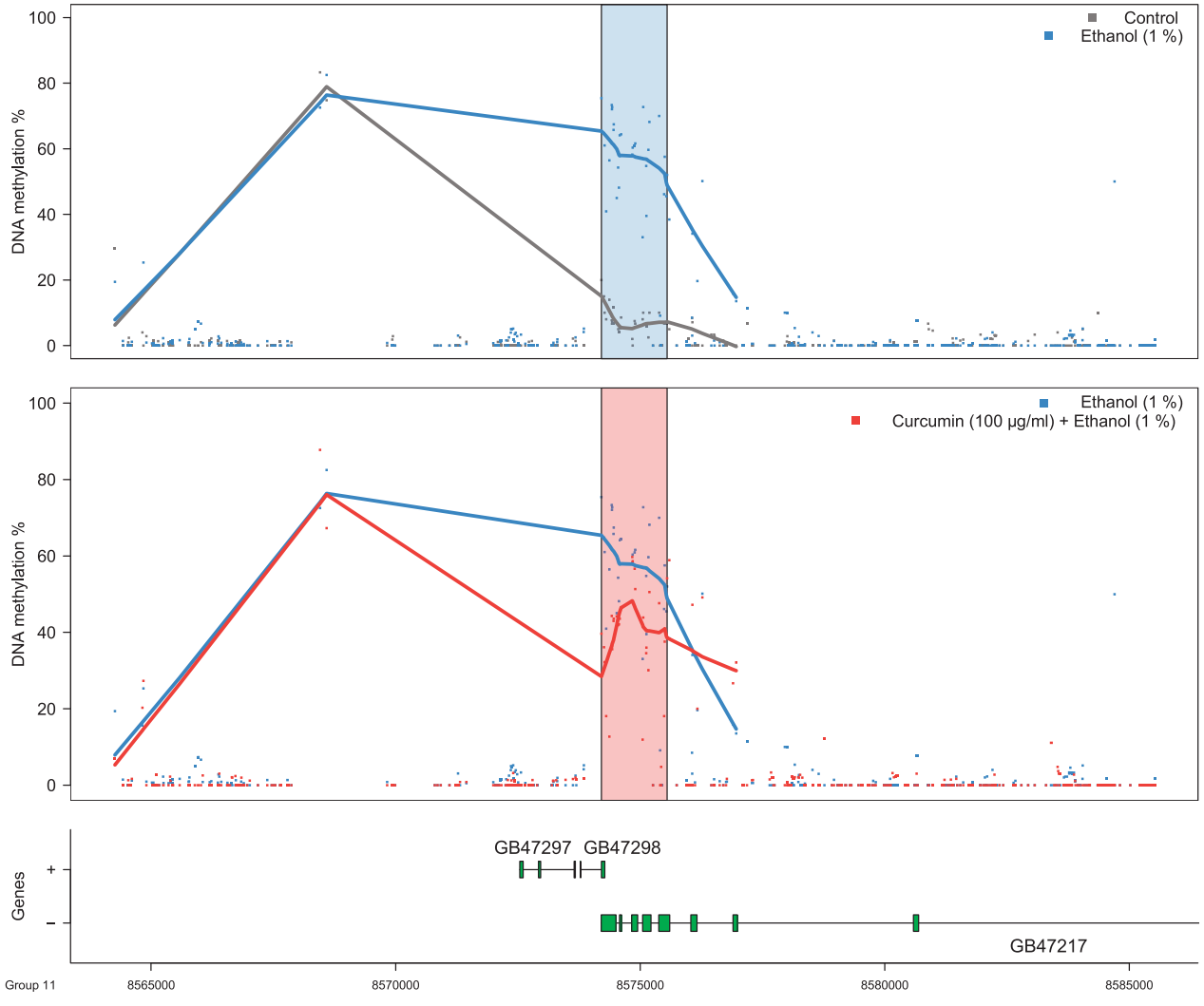
Supplemental figure 6: ELISA methylation percentages. Black lines indicate means. (One−tailed Mann−Whitney test; p>0.05)



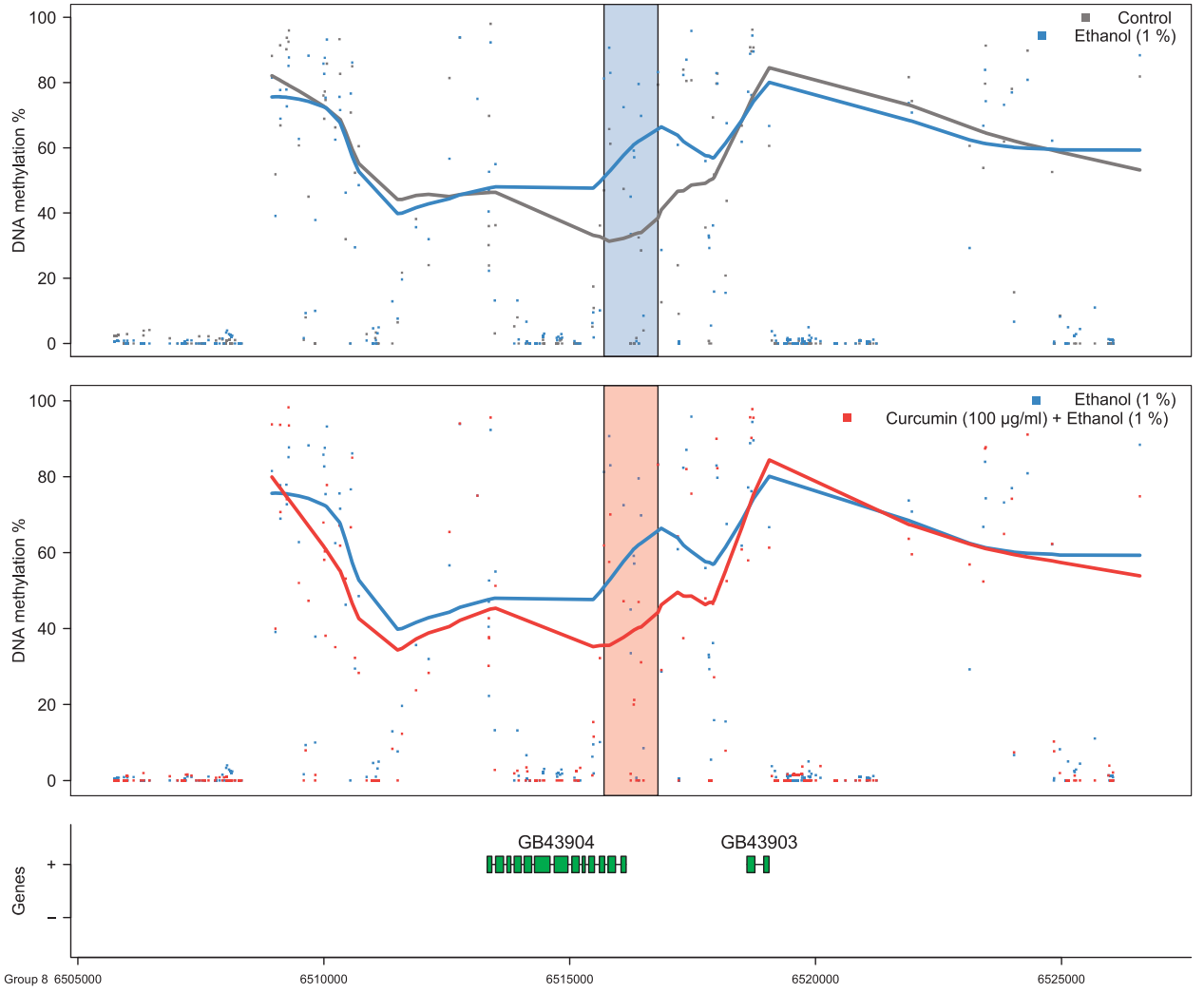
Supplemental figure 7: Median CpG methylation score over mRNA coding genes (left panel) and exons (right panel). mRNA coding genes and exons were divided into a minimum of 3,000 and 60 bins respectively. Scores are re-scaled in the range from 0 to 1 and lines are smoothed using Locally Weighted Scatterplot Smoothing (LOWESS).



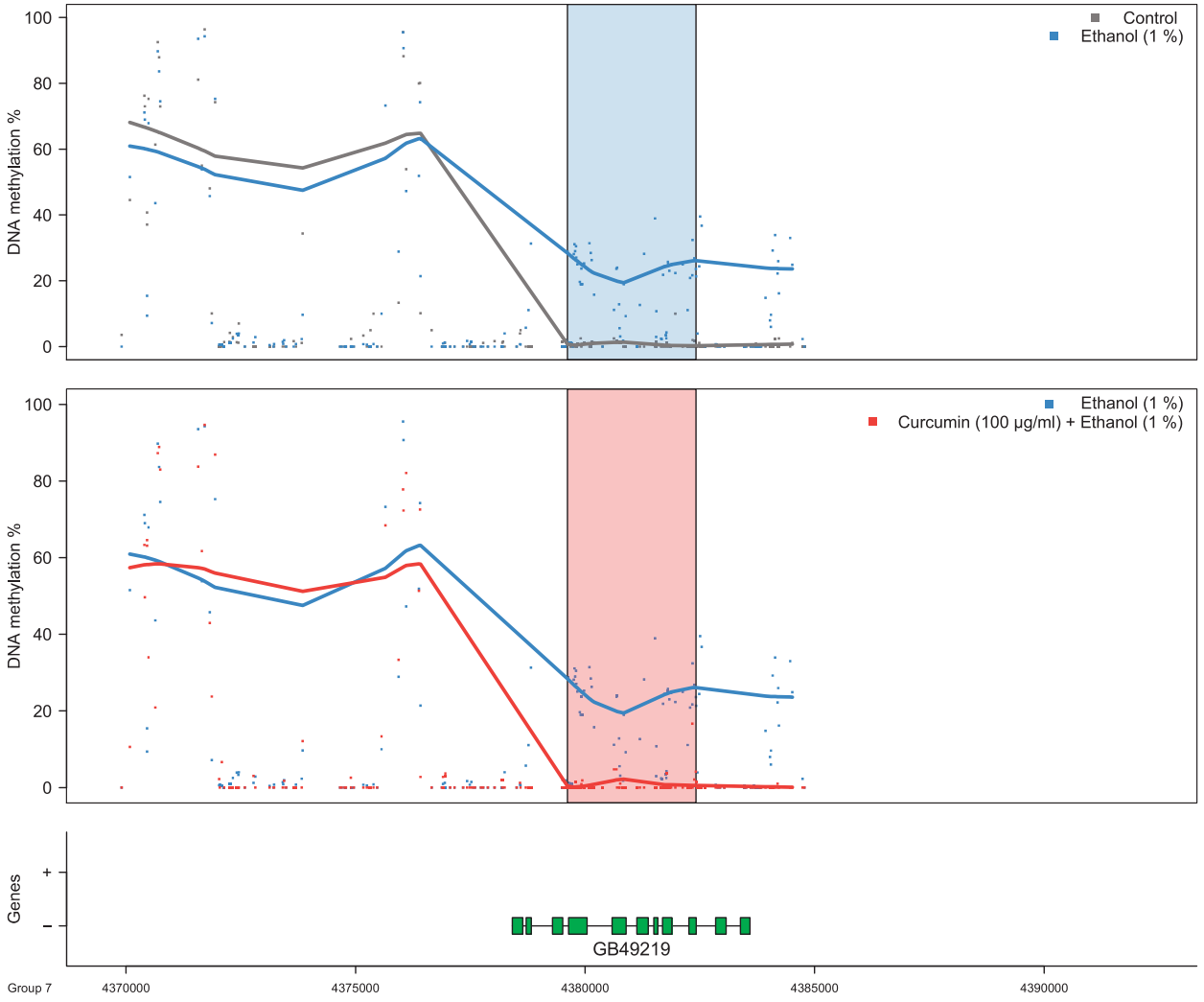
Supplemental figure 8: WGBS CpG methylation percentages. Black lines indicate means. (One−tailed Mann−Whitney tests, p > 0.05)


DNA methylation %
100
80
60
40
20
0
Control
Ethanol (1 %)
DNA methylation %
100
80
60
40
20
0
Ethanol (1 %)
Curcumin (100 µg/ml) + Ethanol (1 %)
Genes
+
–
GB47297
GB47298
GB47217
Group 11
8565000
8570000
8575000
8580000
8585000

Supplemental figure 9: Genomic regions of the DMR at linkage group 11 where the top panel depicts the situation in control (grey) versus ethanol (blue) and the blue shaded area corresponds to the significant DMR in the middle panel. 10 kbp upstream and downstream (if no gaps exist in the Amel 4.5 genome build) are plotted for reference (white background). The middle panel depicts the comparison between ethanol (blue) and the curcumin and ethanol (red) fed honey bees where the significant DMR is in the pink shaded area. The lower panel depicts the gene structures by exons (boxes) by strand according to linkage groups.


Control
Ethanol (1 %)
DNA methylation %
100
80
60
40
20
0
Ethanol (1 %)
Curcumin (100 µg/ml) + Ethanol (1 %)
DNA methylation %
Genes
+
–
GB43904
GB43903
Group 8 6505000
6510000
6515000
6520000
6525000

Supplemental figure 10: Genomic regions of the DMR at linkage group 8 where the top panel depicts the situation in control (grey) versus ethanol (blue) and the blue shaded area corresponds to the significant DMR in the middle panel. 10 kbp upstream and downstream (if no gaps exist in the Amel 4.5 genome build) are plotted for reference (white background). The middle panel depicts the comparison between ethanol (blue) and the curcumin and ethanol (red) fed honey bees where the significant DMR is in the pink shaded area. The lower panel depicts the gene structures by exons (boxes) by strand according to linkage groups.


DNA methylation %
100
80
60
40
20
0
Control
Ethanol (1 %)
DNA methylation %
100
80
60
40
20
0
Ethanol (1 %)
Curcumin (100 µg/ml) + Ethanol (1 %)
Genes
+
–
GB49219
Group 7
4370000
4375000
4380000
4385000
4390000

Supplemental figure 11: Genomic regions of the DMR at linkage group 7 where the top panel depicts the situation in control (grey) versus ethanol (blue) and the blue shaded area corresponds to the significant DMR in the middle panel. 10 kbp upstream and downstream (if no gaps exist in the Amel 4.5 genome build) are plotted for reference (white background). The middle panel depicts the comparison between ethanol (blue) and the curcumin and ethanol (red) fed honey bees where the significant DMR is in the pink shaded area. The lower panel depicts the gene structures by exons (boxes) by strand according to linkage groups.

Supplementary table 1: Detailed makeup of honey bee diets. Compound concentrations in bold were used for ELISA experiments.

Amino acid source	**Compounds**	**Substance solvent**
Grace's amino acid mix	Sodium butyrate 0.01 mg/ml	dH_2O
Grace's amino acid mix	**Sodium butyrate 0.1 mg/ml**	**dH_2O**
Grace's amino acid mix	**Sodium butyrate 1 mg/ml**	**dH_2O**
Grace's amino acid mix	None	dH_2O
Grace's amino acid mix	**Ethanol 1 % (v/v)**	**dH_2O**
Grace's amino acid mix	Curcumin 1 µg/ml	Ethanol 1 % (v/v)
Grace's amino acid mix	**Curcumin 10 µg/ml**	**Ethanol 1 % (v/v)**
Grace's amino acid mix	**Curcumin 100 µg/ml**	**Ethanol 1 % (v/v)**
RPMI 1640	**Cyanocobalamin 0.02 µg/ml**	**dH_2O**
RPMI 1640	**Cyanocobalamin 0.2 µg/ml**	**dH_2O**
RPMI 1640	Cyanocobalamin 2 µg/ml	dH_2O
RPMI 1640	**Folic acid 5 µg/ml**	**dH_2O**
RPMI 1640	Folic acid 50 µg/ml	dH_2O
RPMI 1640	**Folic acid 500 µg/ml**	**dH_2O**
RPMI 1640	**Valproic acid 0.1 mg/ml**	**dH_2O**
RPMI 1640	**Valproic acid 1 mg/ml**	**dH_2O**
RPMI 1640	Valproic acid 10 mg/ml	dH_2O
RPMI 1640	Isovaleric acid 0.1 mg/ml	dH_2O
RPMI 1640	**Isovaleric acid 1 mg/ml**	**dH_2O**
RPMI 1640	**Isovaleric acid 10 mg/ml**	**dH_2O**
RPMI 1640	**None**	**dH_2O**

Supplemental table 2: Sequening statistics. Conversion rate was calcultaed by using a spike-in lambda DNA as unmethylated DNA. For more details please see the GEO submussion form

Sample ID	Species	Tissue	Treatment group	Sequencing library protocol	Sequncer	Library type	Read Length	Total reads	Mapping efficiency	Conversion rate	Selected reads
A6	*Apis mellifera carnica*	Abdominal	Ethanol	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	93,823,416	57.4 %	99.7 %	3,050,348
A7	*Apis mellifera carnica*	Abdominal	Ethanol	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	48,728,070	56.8 %	99.7 %	1,630,273
B1a	*Apis mellifera carnica*	Abdominal	Ethanol	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	23,160,872	58.2 %	98.1 %	721,166
B3	*Apis mellifera carnica*	Abdominal	Ethanol	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	110,567,996	50.4 %	99.8 %	3,152,566
B15	*Apis mellifera carnica*	Abdominal	Ethanol	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	52,288,916	58.2 %	99.4 %	1,838,007
AG-13	*Apis mellifera carnica*	Abdominal	Control	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	36,183,126	53.6 %	99.7 %	912,662
AG-16	*Apis mellifera carnica*	Abdominal	Control	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	55,640,898	54.8 %	99.8 %	1,716,319
BG-7	*Apis mellifera carnica*	Abdominal	Control	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	47,768,046	47.3 %	99.7 %	1,209,955
BG-9	*Apis mellifera carnica*	Abdominal	Control	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	45,910,816	45.4 %	99.8 %	1,187,170
BG-10	*Apis mellifera carnica*	Abdominal	Control	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	54,796,190	46.7 %	99.7 %	1,454,451
C13a	*Apis mellifera carnica*	Abdominal	Curcumin	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	43,776,134	57.4 %	99.3 %	1,503,606
D9	*Apis mellifera carnica*	Abdominal	Curcumin	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	35,154,376	55.7 %	99.8 %	1,170,006
D10	*Apis mellifera carnica*	Abdominal	Curcumin	MethylC	Illumina HiSeq 2500	Paired-end	100 bp	89,321,808	53.7 %	99.6 %	2,740,525

Column1	chr	start	end	value	area	cluster	indexStart	indexEnd	L	clusterL	sig_dif	sig_hyper	sig_hypo	tot_dif	tot_hyper	tot_hypo	slk_p	slk_sidak_p	fdr
6221	11	8574209	8576963	0.689862649692217	24.1451927392276	7760	68834	68868	35	35	-20.6119116171194	0	-20.6119116171194	-25.0228715 38215	0.0115035035885903	-25.0343750418036	4.018e-16	1.643e-14	3.5707966e-12
6421	7	4379616	4382415	0.386992769456874	11.2227903142493	5179	44533	44561	29	29	-1.76041287747123	0	-1.76041287747123	-11.4197996899398	0	-11.4197996899398	1.765e-07	6.425e-06	0.00078427775
6496	8	6515698	6517761	0.402035322194589	6.83460047730802	5863	51116	51132	17	35	-4.75087397719387	0	-4.75087397719387	-6.83975633997661	0.0877105239479423	-6.92746686392455	8.091e-07	3.995e-05	0.0023968239
7107	8	9094851	9097774	-0.601791900249984	9.02687850349475	6056	54043	54057	15	21	4.99660727843152	4.99660727843152	0	7.34706770587181	7.372995434440011	-0.02592772 85282982	2.055e-06	7.163e-05	0.00456569625
6053	6	12440586	12440610	0.854301526770458	1.70860305354092	4698	40477	40478	2	11	-1.70660537512658	0	-1.70660537512658	0	-1.70660537512658	-1.70660537512658	0.0001203	0.4	0.125405444444444
3238	1	12385795	12386839	-0.380870856893337	1.52348342757335	605	5575	5578	4	39	1.53032925013868	1.53032925013868	0	2.2792275757081	2.2792275757081	0	8.168e-05	0.007939	0.125405444444444
5279	11	6081785	6082266	-0.301286526746301	4.51929790119451	7674	68141	68155	15	16	1.02379682429124	1.02379682429124	0	4.62002724641981	4.62002724641981	0	0.000127	0.02654	0.125405444444444
2795	7	3704268	3705547	0.327170104759928	2.29019073331949	5160	44397	44403	7	32	-0.811285673490244	0.640754408731309	-1.45204008222155	-1.6955433816532	0.640754408731309	-2.33629779038451	0.0001175	0.009312	0.125405444444444
1045	15	1282144	1282145	-0.228330404662751	0.228330404662751	9609	85053	85053	1	26	0.257280294830098	0.257280294830098	0	0.257280294830098	0.257280294830098	0	0.0001254	1	0.125405444444444
23	16	3714837	3715669	0.365974937799951	3.29377444019559	10308	91106	91114	9	29	-2.59111500426616	0	-2.59111500426616	-3.23460310638788	0.450892325793077	-3.68549543218096	0.0001588	0.01926	0.14112556
122	8	4035079	4035080	0.560537617371777	0.560537617371777	5763	50482	50482	1	18	-0.561930117789089	0	-0.561930117789089	-0.561930117789089	0	-0.561930117789089	0.0001857	1	0.150028718181818
8086	5	4923088	4923102	0.664356114081784	1.32871222816357	3658	30150	30151	2	74	-1.52387798270541	0	-1.52387798270541	-1.52387798270541	0	-1.52387798270541	0.0002362	0.8207	0.174925783 333333
1289	6	12889152	12889555	0.487065551708054	4.38358996537249	4718	40569	40577	9	9	-1.676914488393	0	-1.676914488393	-4.07396068974 74	0	-4.07396068974 74	0.0003589	0.08675	0.245349561538462
8481	7	4383930	4384515	0.366754380528803	3.30078924759223	5180	44562	44570	9	9	0	0	0	-3.41595658372731	0	-3.41595658372731	0.0004076	0.06854	0.258738657142857
204	12	2741554	2741654	0.53130556331336 13	1.06261112626723	8278	74820	74821	2	17	-1.08105035102734	0	-1.08105035102734	-1.08105035102734	0	-1.08105035102734	0.000599	0.4569	0.3349288125
6015	16	4574919	4575293	-0.263010283901191	1.31505141950596	10382	91949	91953	5	20	0.260789729705 92	0.835507497854024	-0.574717768148104	1.30316880878901	1.87788657693711	-0.574717768148104	0.000603	0.1515	0.3349288125
5448	1	14442977	14443671	0.4836801515839081	3.38576106287357	721	6724	6730	7	16	-2.567815622187 53	0	-2.567815622187 53	-3.465014621 8837	0.218023226122669	-3.683037848 00637	0.0008489	0.1172	0.336680576923077
1632	6	12906291	12907206	-0.251387523792969	2.26248771413672	4723	40620	40628	9	16	1.96028479547453	1.96028479547453	0	2.1046047627699	2.4530076245249	0.348402861755002	0.000985	0.1039	0.336680576923077
5110	4	3605133	3605224	0.555417195077501	1.66625158523225	3050	25569	25571	3	36	-1.678687173676662	0	-1.678687173676662	-1.678687173676662	0	-1.678687173676662	0.0007934	0.5888	0.336680576923077
4636	1	14426687	14426877	0.458958082222205	1.83583232888882	717	6692	6695	4	20	-1.472698011008 08	0	-1.472698011008 08	-1.588135255545082	0	-1.588135255545082	0.0009756	0.4075	0.336680576923077
4248	11	13282119	13282511	-0.452539034618898	1.81015613847559	8061	72219	72222	4	16	1.46047040570441	1.46047040570441	0	1.41939835141463	1.53629504844518	-0.116896697030546	0.0009034	0.2093	0.336680576923077
2484	1	12492548	12493141	-0.435123611920232	2.61074167152139	616	5678	5683	6	17	1.14975653053046	1.14975653053046	0	2.79781171403233	2.79781171403233	0	0.0007085	0.1146	0.336680576923077
3576	14	5943445	5943688	0.174183766438658	0.348367532877315	9313	81859	81860	2	26	-0.405929712977258	0	-0.405929712977258	0	-0.405929712977258	0	0.0008496	0.2998	0.336680576923077
7630	2	15357375	15357376	-0.276568359253747	0.27656835925374 7	2175	18338	18338	1	16	0.308628757487247	0.308628757487247	0	0.308628757487247	0.308628757487247	0	0.0009794	1	0.336680576923077
5851	1	8915977	8915978	0.102199241184284	0.102199241184284	439	3795	3795	1	50	-0.180853234167083	0	-0.180853234167083	0	-0.180853234167083	0	0.0007902	1	0.336680576923077
2876	1	12669572	12670523	-0.356683963111439	3.56683963111439	634	5977	5986	10	10	0	0	0	3.59232223079132	3.59232223079132	0	0.0007391	0.07615	0.336680576923077
1360	11	8729713	8730547	0.278246059379812	1.94772241565868	7774	69073	69079	7	54	-1.530293280764 56	0	-1.530293280764 56	-1.795097983 48257	0.329238767520531	-2.12433675 100311	0.001115	0.1274	0.347205896551724
6455	11	14595885	14596217	-0.165161841997418	0.495485525992 254	8181	74091	74093	3	49	1.00388784997911	1.00388784997911	0	1.59860794387905	1.59860794387905	0	0.001133	0.2937	0.347205896551724
3256	2	13102531	13103267	0.192007329111319	0.768029316445277	2001	16488	16491	4	15	-0.4049353749439	0.295997450740493	-0.700950925684392	-1.1597379050 0203	0.295997450740493	-1.45573535574252	0.001057	0.1361	0.347205896551724
6485	10	11504697	11504963	-0.130286993548686	0.651434967743431	7354	65351	65355	5	98	0.988087355433068	0.988087355433068	0	1.04024238004083	1.55676860200997	-0.516526221969142	0.00119	0.3663	0.352517666666667
5800	13	1122117	1124143	0.377821948815076	5.66732923222615	8742	78317	78331	15	30	-2.746264180804 71	0	-2.746264180804 71	-5.559813692124 18	0.209531456399531	-5.76934514852371	0.001317	0.0641	0.377554161290323
3662	7	2223220	2223771	0.177731730120904	0.533195190362 713	5095	44023	44025	3	44	-0.304629163004644	0	-0.304629163004644	-0.473453907530697	0	-0.473453907530697	0.001396	0.2276	0.387695375
4774	6	18345770	18346222	0.344008985904105	1.37603594361642	4989	42886	42889	4	51	-1.183151010624818	0	-1.183151010624818	-1.777073431 61641	0	-1.777073431 61641	0.001451	0.2791	0.390758696969697
4605	6	16762583	16763875	0.259278724376302	0.777836173128907	4896	42219	42221	3	21	-0.576653322669922	0	-0.576653322669922	-0.791661353 8093838	0	-0.791661353 8093838	0.001622	0.1201	0.423962176470588
806	11	11477525	11478522	-0.358104735434858	3.58104735434858	7897	70063	70072	10	28	1.53096837441022	1.53096837441022	0	3.69219036174818	3.69219036174818	0	0.001803	0.1684	0.425892384615385
7406	8	7790937	7791993	0.235022726142947	3.76036361828716	5962	52453	52468	16	17	-1.398254181164 03	0	-1.398254181164 03	-4.025591042 8303	0	-4.025591042 8303	0.001852	0.1638	0.425892384615385
7754	5	13261894	13262013	-0.189512478326976	0.947562391634881	4153	35737	35741	5	11	0.708307641457991	0.708307641457991	0	1.3983904712303	1.3983904712303	0	0.001801	0.7864	0.425892384615385
2583	15	3130429	3130667	0.192250035692809	0.769000142771234	9715	86281	86284	4	29	-0.616111967776379	0	-0.616111967776379	-0.712562894958776	0.149424351798712	-0.861987246757488	0.001869	0.551	0.425892384615385
4023	3	11989616	11990098	-0.42978237697154	2.14891188485777	2808	23523	23527	5	40	0.317794141223195	0.317794141223195	0	2.57698201793293	0	2.57698201793293	0	0.001713	0.304	0.425892384615385
3029	GroupUn14	38927	39172	-0.309720357519563	0.929161072558689	10610	94146	94148	3	17	0.909058457214002	0.909058457214002	0	0.604556381087792	1.17388723238765	-0.569330851299857	0.001951	0.556	0.427348782608696
666	11	1753683	1755174	0.368161036991957	3.31344933292762	7531	67050	67058	9	20	-0.855429927502211	0	-0.855429927502211	-3.231854997227 22	0	-3.231854997227 22	0.002102	0.1339	0.427348782608696
8794	8	12835587	12836741	0.339605075502419	4.75447105703387	6230	55638	55651	14	29	-0.750337649217363	0	-0.750337649217363	-4.999878275 08904	0	-4.999878275 08904	0.00212	0.1709	0.427348782608696
3809	11	2800220	2801744	0.210920599948838	2.53104719938605	7575	67294	67305	12	17	-0.743720260690662	0	-0.743720260690662	-2.949767073747 29	0.273166637647505	-3.222933711394 79	0.002121	0.1323	0.427348782608696
2985	1	13913191	13913212	0.422551131457595	0.845102262915 191	686	6433	6434	2	58	-0.740248761079136	0	-0.740248761079136	-0.995093491687867	0	-0.995093491687867	0.002167	1	0.427348782608696
2586	GroupUn188	21997	22376	0.231925111814843	0.695775335444529	11305	100321	100323	3	19	-0.618689395721634	0	-0.618689395721634	-0.965032842722 8	0	-0.965032842722 8	0.002002	0.4164	0.427348782608696
7830	1	21802648	21803174	0.191752024704	1.1505121482 24	1006	9284	9289	6	25	-0.611678610066106	0	-0.611678610066106	-0.958866853988544	0.124871441589474	-1.083738295578 02	0.002212	0.3487	0.427348782608696
3854	1	10766980	10767666	0.399800106597994	7.19640191876 39	517	4313	4330	18	30	-1.667233817848 54	0	-1.667233817848 54	-6.755522130 06542	0	-6.755522130 06542	0.002547	0.3153	0.47954252
3050	11	8717634	8718464	-0.35402352956714	2.12414117740284	7771	69006	69011	6	23	0.866439297304077	0.866439297304077	0	2.29716842681438	2.29716842681438	0	0.002659	0.2787	0.47954252
5966	14	2607448	2607524	-0.144009085266377	0.288018170532754	9171	80597	80598	2	29	0.131183366778 65	0.131183366778 65	0	0.498624054442085	0.498624054442085	0	0.002698	0.9732	0.47954252
4419	8	12734152	12734468	-0.229101922156019	0.687305766468056	6211	55458	55460	3	29	-0.043747576110 3823	0.329210681546 14	-0.372958257656522	0.136872129972267	0.509830387628789	-0.372958257656522	0.00262	0.5708	0.47954252
962	14	6921749	6922551	-0.358798366781579	3.58798366781579	9379	82555	82564	10	16	1.919313548396 44	1.919313548396 44	0	3.66204377209865	3.85415634507273	-0.192112572974079	0.002933	0.3114	0.511089627 45098
2322	16	3679983	3680440	0.264360531883266	0.793081595649799	10305	91083	91085	3	15	-0.877383061033767	0	-0.877383061033767	-1.213911113 36693	0	-1.213911113 36693	0.002995	0.4876	0.511857019230769
6801	11	14401301	14401903	0.360134514845 78	3.96147966330358	8161	73803	73813	11	13	-1.341611819068 71	0	-1.341611819068 71	-4.001759198937 94	0	-4.001759198937 94	0.003209	0.4196	0.525766387096774
6048	11	3686325	3687067	-0.430199540538154	2.58119724322892	7617	67624	67629	6	19	1.314587099980 73	1.314587099980 73	0	2.76183871134328	2.80171100474137	-0.039872293980964	0.003381	0.3719	0.525766387096774
5677	2	10972556	10974062	-0.370557154287192	3.33501438858473	1894	15837	15845	9	18	1.165840677999 26	1.165840677999 26	0	3.38224208872859	3.42678117948518	-0.044539097565935	0.003505	0.2114	0.525766387096774
4885	GroupUn94	46785	47105	0.265853674479442	1.06341469791777	10959	97059	97062	4	10	-0.783525830591584	0	-0.783525830591584	-1.132900284748 21	0	-1.132900284748 21	0.003668	0.6896	0.525766387096774
6550	11	13199946	13200623	0.304886792352444	2.13420754646711	8045	71933	71939	7	96	-0.773791461316614	0	-0.773791461316614	-2.311974522 49852	0	-2.311974522 49852	0.00319	0.3817	0.525766387096774
343	10	10423465	10423466	0.575049415723672	0.575049415723672	7270	64536	64536	1	9	-0.575049415723672	0	-0.575049415723672	-0.575049415723672	0	-0.575049415723672	0.003654	1	0.525766387096774
8139	11	9313229	9313598	0.161183593802705	1.28946875042164	7809	69350	69357	8	12	-0.428291582272654	0	-0.428291582272654	-1.765733313 48533	0.351435643741762	-2.117168778 59506	0.003622	0.6328	0.525766387096774
5487	3	9604907	9604908	0.287010137470378	0.287010137470378	2698	22553	22553	1	26	-0.411515787674979	0	-0.411515787674979	-0.411515787674979	0	-0.411515787674979	0.003412	1	0.525766387096774
7771	11	14282447	14282448	0.268745449725952	0.268745449725952	8154	73687	73687	1	21	-0.268721616193673	0	-0.268721616193673	-0.268721616193673	0	-0.268721616193673	0.003362	1	0.525766387096774
82	11	13077623	13077837	0.224112895823047	1.12056447911523	8026	71358	71362	5	57	0	0	0	-1.241035819 73966	0.321476905750726	-1.562512725 49039	0.003486	0.8103	0.525766387096774
270	2	7050108	7050919	0.275258134392099	1.65154880635259	1707	14630	14635	6	8	0.143085040016197	0.331203060653012	-0.188118020636814	-0.561504387273611	0.331203060653012	-0.892707447926623	0.003784	0.3789	0.533784253968254
5118	9	5070990	5072994	-0.319831755990993	11.1941114596848	6425	57265	57299	35	37	0	0	0	11.81262347588 11	11.85661133921 99	-0.043987863 33880051	0.003868	0.1788	0.5371080625
7456	1	6647508	6647862	-0.275244158026949	1.37622079013474	342	3119	3123	5	23	1.572004983738 71	1.572004983787	0	1.30754393142 54	1.656140749068 01	-0.348596817642616	0.003939	0.6789	0.5385522
197	6	16317636	16318033	0.229034521816401	1.60324165271481	4842	41558	41564	7	49	-1.628255607744 88	0	-1.628255607744 88	-2.658164922 72886	0	-2.658164922 72886	0.004078	0.6496	0.549108878 787879
1177	1	14464128	14467281	-0.266831500163186	6.13712450375328	723	6767	6789	23	59	1.569787591949 46	1.569787591949 46	0	6.81379740674 6	7.41997255532948	-0.606175148583479	0.004268	0.1291	0.557789941 176471

Column1	chr	start	end	value	area	cluster	indexStart	indexEnd	L	clusterL	sig_dif	sig_hyper	sig_hypo	tot_dif	tot_hyper	tot_hypo	slk_p	slk_sidak_p	fdr
565	11	8574209	8575551	-0.243855009477988	7.31565028433965	7713	68748	68777	30	35	1.3574252802016	1.3574252802016	0	7.42135396064412	7.53689950352955	-0.115545542885431	8.965e-17	8.438e-15	8.6861885e-13
5271	8	6515698	6516795	-0.431712947722895	4.74884242495185	5824	51044	51054	11	35	1.272643978931901	1.272643978931901	0	4.56929028442776	4.56929028442776	0	2.147e-08	1.994e-06	0.000104011415
2323	7	4379616	4382415	-0.392624416764101	11.3861080861589	5146	44469	44497	29	29	2.6357924518556	2.6357924518556	0	11.5459190411058	11.5459190411058	0	1.765e-07	6.425e-06	0.000570036166666667
8071	6	12889152	12889237	-0.350290020662818	1.05087006198845	4689	40518	40520	3	9	0	0	0	1.03790266726724	1.03790266726724	0	3.805e-05	0.04459	0.0921666125
608	8	9094851	9095993	0.203517224818111	1.22110334890866	6016	53968	53973	6	21	0	0	0	-0.956373038542581	0	-0.956373038542581	4.913e-05	0.004373	0.095204114
6299	16	3714837	3715593	-0.153305897091664	1.22644717673331	10242	90975	90982	8	29	0	0	0	1.24269175368857	1.24269175368857	0	6.291e-05	0.008442	0.101589165
3257	6	12440586	12440610	-0.670351438489205	1.34070287697841	4669	40426	40427	2	11	0	0	0	1.33743064760586	1.33743064760586	0	0.0001203	0.4	0.166512385714286
3078	8	4035079	4035080	-0.375423918542121	0.37542391854212 1	5727	50414	50414	1	18	0	0	0	0.373949237335546	0.373949237335546	0	0.0001857	1	0.2249059125
3239	4	5865466	5865633	0.363177416227743	0.72635483245548 6	3153	26531	26532	2	7	-0.6528572135969 67	0	-0.6528572135969 67	-0.861636404441693	0	-0.861636404441693	0.000306	0.1703	0.238080261111111
7036	11	6081523	6082266	0.226945767214199	3.63113227542719	7627	68054	68069	16	16	-0.530531983107823	0	-0.530531983107823	-3.86997493874343	0	-3.86997493874343	0.0003384	0.04535	0.238080261111111
4959	8	12835587	12835920	-0.364312246341065	3.27881021706959	6190	55563	55571	9	29	0.413160851675104	0.413160851675104	0	3.71311171560808	3.71311171560808	0	0.00041	0.1179	0.238080261111111
5865	11	8729934	8730050	0.104727257832465	0.31418177349739 5	7727	68984	68986	3	54	-0.290812610438657	0	-0.290812610438657	-0.486126963461433	0	-0.486126963461433	0.000293	0.2269	0.238080261111111
375	6	12905980	12906473	0.123274638228623	0.98619710582898 4	4694	40566	40573	8	16	0	0	0	-1.08639901647915	0.197858653931689	-1.28425767041084	0.0004423	0.08736	0.238080261111111
1118	1	14442977	14443369	-0.148016746648676	0.74008373324338 1	720	6720	6724	5	16	0	0	0	1.140034616392 69	1.140034616392 69	0	0.0002547	0.06405	0.238080261111111
1696	5	4923088	4923102	-0.165080116500226	0.33016023300045 2	3638	30111	30112	2	74	0	0	0	1.372621911007 95	1.372621911007 95	0	0.0002362	0.8207	0.238080261111111
1733	5	7672330	7672331	-0.135772375091514	0.13577237509151 4	3799	31839	31839	1	22	0	0	0	0.331777471649793	0.331777471649793	0	0.0004308	1	0.238080261111111
5149	7	4383930	4384515	-0.379783760395419	3.41805384355877	5147	44498	44506	9	9	0	0	0	3.546335418732 41	3.546335418732 41	0	0.0004076	0.06854	0.238080261111111
5721	1	12385877	12386839	0.187258790092976	0.56177637027892 7	604	5573	5575	3	39	0	0	0	-0.784749656703927	0	-0.784749656703927	0.000358	0.03721	0.238080261111111
464	12	2741554	2741654	-0.478487614468354	0.95697522893670 9	8229	74724	74725	2	17	0.990867096701152	0.990867096701152	0	0.990867096701152	0.990867096701152	0	0.000599	0.4569	0.29212335
8760	16	4574919	4575293	0.219444425550601	1.09722212775301	10316	91818	91822	5	20	-0.450972157259316	0	-0.450972157259316	-1.668137186415 09	0.004996 2536586714	-1.673133440073 76	0.000603	0.1515	0.29212335
1729	1	12669572	12670523	0.190262825626941	1.90262825626941	633	5974	5983	10	10	0	0	0	-2.00075775094334	0	-2.00075775094334	0.0007391	0.07615	0.325506359090909
7028	1	12492548	12493141	0.193518089643597	1.16110853786158	615	5675	5680	6	17	0	0	0	-1.31095001920086	0	-1.31095001920086	0.0007085	0.1146	0.325506359090909
3138	4	3605133	3605224	-0.536018052611315	1.60805415783394	3035	25537	25539	3	36	1.190541263157227	1.190541263157227	0	1.642540392949478	1.642540392949478	0	0.0007934	0.5888	0.334228373913043
6230	2	13102531	13103267	-0.262441895876672	1.04976758350669	1991	16466	16469	4	15	0.678401278397774	0.678401278397774	0	1.14059141574 94	1.14059141574 94	0	0.001057	0.1361	0.40965092
651	1	13909499	13909751	0.156069871236776	0.46820961371032 9	685	6405	6407	3	58	0	0	0	-0.395838103419429	0.011159 7401628086	-0.406997843582237	0.001047	0.3453	0.40965092
2416	7	2195419	2195628	-0.295338671516925	0.88601601455077 4	5057	43846	43848	3	7	1.45284740138011	1.45284740138011	0	1.45284740138011	1.45284740138011	0	0.001246	0.4555	0.447129407407407
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6384	13	3184383	3184594	-0.145335967321937	0.87201580393162 1	8799	78798	78803	6	24	0	0	0	0.850159250430809	0.850159250430809	0	0.001426	0.498	0.476431517241379
6972	1	14426687	14426731	-0.377514684625387	0.75502936920754	716	6688	6689	2	20	0	0	0	0.726507697525 06	0.726507697525 06	0	0.001393	0.9604	0.476431517241379
1450	5	11701302	11701383	0.115076130257107	0.46030452102842 7	4018	34104	34107	4	58	-0.166565721520966	0	-0.166565721520966	-0.567464812254099	0	-0.567464812254099	0.001477	0.8441	0.477021766666667
2597	3	11989616	11990098	0.248554618308176	1.24277309154088	2796	23494	23498	5	40	-0.542787291571272	0	-0.542787291571272	-1.52627341009285	0	-1.52627341009285	0.001713	0.304	0.535395387096774
7636	11	11477525	11478522	0.364868037237472	3.64868037237472	7850	69970	69979	10	28	0	0	0	-3.781292569949 56	0	-3.781292569949 56	0.001803	0.1684	0.54591459375
9534	11	1753683	1755174	-0.367354839228075	3.30619355305267	7484	66965	66973	9	20	1.304282893349 24	1.304282893349 24	0	3.194989544110 96	3.194989544110 96	0	0.002102	0.1339	0.614524825
1812	8	11696857	11698054	0.148228962807479	1.18583170245984	6137	54984	54991	8	40	-0.816276933905702	-0.816276933905702	0	-0.547192405368497	0.912968852535381	-1.460161257903 88	0.002422	0.1865	0.614524825
2180	1	10766980	10767275	-0.266421714450131	3.46348228785177	516	4310	4322	13	30	0.341354313833754	0.341354313833754	0	3.550290844645 32	3.587967142218 78	-0.037676297573466 6	0.002388	0.5621	0.614524825
1679	8	6580082	6580083	0.195619785885614	0.19561978588561 4	5838	51216	51216	1	20	0	0	0	-0.303791600540557	0	-0.303791600540557	0.002537	1	0.614524825
3524	1	13913191	13913212	-0.116517603023863	0.23303520604772 5	685	6429	6430	2	58	0	0	0	0.286065072334 17	0.286065072334 17	0	0.002167	1	0.614524825
6859	GroupUn188	21908	22376	-0.207521648428351	0.83008659371340 5	11239	100183	100186	4	19	0	0	0	1.123632408423 04	1.123632408423 04	0	0.002284	0.3921	0.614524825
7211	11	11154990	11154991	-0.139818419515543	0.13981841951554 3	7833	69808	69808	1	8	0	0	0	0.157988586163625	0.157988586163625	0	0.00239	1	0.614524825
8272	11	11552860	11553247	-0.180958010073818	0.90452900536909	7855	69990	69994	5	16	0	0	0	0.994916244283747	1.022892915279 37	-0.027976670995619 3	0.002516	0.4848	0.614524825
2679	8	7790937	7792764	-0.261329026195774	4.44259344532816	5923	52380	52396	17	17	1.463193112438 21	1.463193112438 21	0	4.3109642060329	4.320939403851 37	-0.009975197818474 11	0.002667	0.1384	0.6152515
4047	3	10003775	10005082	0.222760285592866	2.67312342711439	2698	22683	22694	12	16	0	0	0	-2.862209599566 76	0	-2.862209599566 76	0.002633	0.1857	0.6152515
4792	11	14621779	14622636	0.279157224482803	2.51241502034523	8139	74104	74112	9	23	-0.497808725384198	0	-0.497808725384198	-2.672496186556 52	0.195369366515419	-2.867865553071 94	0.002906	0.2925	0.639914409090909
1683	1	12372377	12372378	0.113736207028306	0.11373620702830 6	602	5509	5509	1	16	0	0	0	-0.072267991816219 64	0	-0.072267991816219 64	0.002863	1	0.639914409090909
9243	11	13400307	13400860	-0.382058484152048	0.76411696830409 5	8027	72479	72480	2	43	0.501555344481869	0.501555344481869	0	0.817404202021327	0.817404202021327	0	0.003417	0.4677	0.643701927272727
959	2	10972556	10974062	0.261643559941831	2.35479203947648	1886	15818	15826	9	18	-0.42715245944312	0	-0.42715245944312	-2.827365634441 83	0	-2.827365634441 83	0.003505	0.2114	0.643701927272727
4206	11	13199946	13200623	-0.263135047828796	1.84194533480157	7997	71839	71845	7	96	0.371729069459529	0.371729069459529	0	2.144706202567 56	2.171447968971 42	-0.026741766403862 4	0.00319	0.3817	0.643701927272727
1107	3	9604907	9604908	-0.112919255218529	0.11291925521852 9	2687	22526	22526	1	26	0	0	0	0.107006490578924	0.107006490578924	0	0.003412	1	0.643701927272727
3217	1	12503096	12503438	0.268050130063375	1.07220052025 33	618	5714	5717	4	43	0	0	0	-0.608598804307 61	0.346442788161172	-0.955041592468782	0.003098	0.6031	0.643701927272727
4398	16	3679983	3680440	-0.1626055149597	0.48781654487909 9	10240	90953	90955	3	15	0	0	0	0.959972584234093	0.959972584234093	0	0.002995	0.4876	0.643701927272727
4462	11	14282447	14282448	-0.246955856957924	0.24695585695792 4	8106	73593	73593	1	21	0	0	0	0.246863958066011	0.246863958066011	0	0.003362	1	0.643701927272727
7732	11	3686325	3687067	0.260655772787546	1.56393463672528	7570	67539	67544	6	19	0	0	0	-1.574614140432 58	0	-1.574614140432 58	0.003381	0.3719	0.643701927272727
7741	11	9313229	9313598	-0.184463674197872	1.47570933582 98	7762	69258	69265	8	12	0	0	0	1.646630065902 29	1.984469359398 02	-0.337839293495725	0.003622	0.6328	0.643701927272727
8488	1	14465135	14465319	0.442603743706927	0.88520748474138 55	722	6771	6772	2	59	0	0	0	-1.087740709764 07	0	-1.087740709764 07	0.003574	0.8622	0.643701927272727
8715	10	10423465	10423466	-0.400516490524193	0.40051649052419 3	7223	64451	64451	1	9	0	0	0	0.400516490524239	0.400516490524239	0	0.003654	1	0.643701927272727
2708	5	7095585	7095612	0.137518117917262	0.41255435375178 7	3754	31034	31036	3	36	0	0	0	-1.624115733264 24	0	-1.624115733264 24	0.00385	1	0.66611875
3939	GroupUn6	20907	20974	0.241496025521595	0.96598421008637 9	10461	93119	93122	4	26	0	0	0	-0.916460643093869	0	-0.916460643093869	0.003956	0.9976	0.672450596491228
91	1	7039500	7039550	0.132493928431478	0.52997571372591 1	365	3320	3323	4	29	0	0	0	-0.605902659904621	0	-0.605902659904621	0.004197	0.9998	0.677594655737705
2836	6	16317636	16318033	-0.11715828526756	0.82010799682792 3	4811	41504	41510	7	49	0	0	0	1.164916656494 39	1.164891987041 267	-0.484003213919 18	0.004078	0.6496	0.677594655737705
4826	3	2357291	2357595	0.503569090789139	2.51784545394 57	2289	19389	19393	5	19	0	0	0	-2.628626954902 31	0	-2.628626954902 31	0.004266	0.7613	0.677594655737705
4831	1	14466430	14467281	0.218364198867593	1.96527778980834	722	6777	6785	9	59	0	0	0	-1.861955502598 76	0.007692836436817 73	-1.869648339035 57	0.004137	0.3912	0.677594655737705
3893	14	6921549	6922551	0.179374715240603	2.15249658288723	9317	82437	82448	12	16	-0.246540067265418	0	-0.246540067265418	-1.827570263182 15	0.384165558358679	-2.211735819740 83	0.004454	0.3648	0.696045258064516
7264	11	13282119	13282591	0.341442074118292	1.70721037059146	8013	72125	72129	5	16	-1.04363981241357	0	-1.04363981241357	-1.565414621947 18	0	-1.565414621947 18	0.005063	0.6656	0.698134576086957
7921	11	13373181	13373528	0.143924767959967	0.86354860775908 04	8024	72352	72357	6	41	-0.970817803817994	0	-0.970817803817994	-0.536428517751455	0.726318873533476	-1.262747391284 93	0.004599	0.7416	0.698134576086957
3385	14	5260526	5260944	-0.426154557019567	5.11385468423 48	9224	81427	81438	12	12	0.942380140303933	0.942380140303933	0	5.131949461546 24	5.131949461546 24	0	0.00516	0.7166	0.698134576086957
3474	11	14297513	14297763	-0.144385228590706	1.01069660013494	8108	73669	73675	7	29	0.920926172033377	0.920926172033377	0	1.085739570530 94	1.148005616997 52	-0.062266046466573 2	0.005841	0.9081	0.698134576086957
6253	11	13336172	13336657	-0.355338351648991	2.13203010989395	8018	72245	72250	6	16	0.545849339312974	0.545849339312974	0	2.188082976885 18	2.188082976885 18	0	0.005629	0.6944	0.698134576086957

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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