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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

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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

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
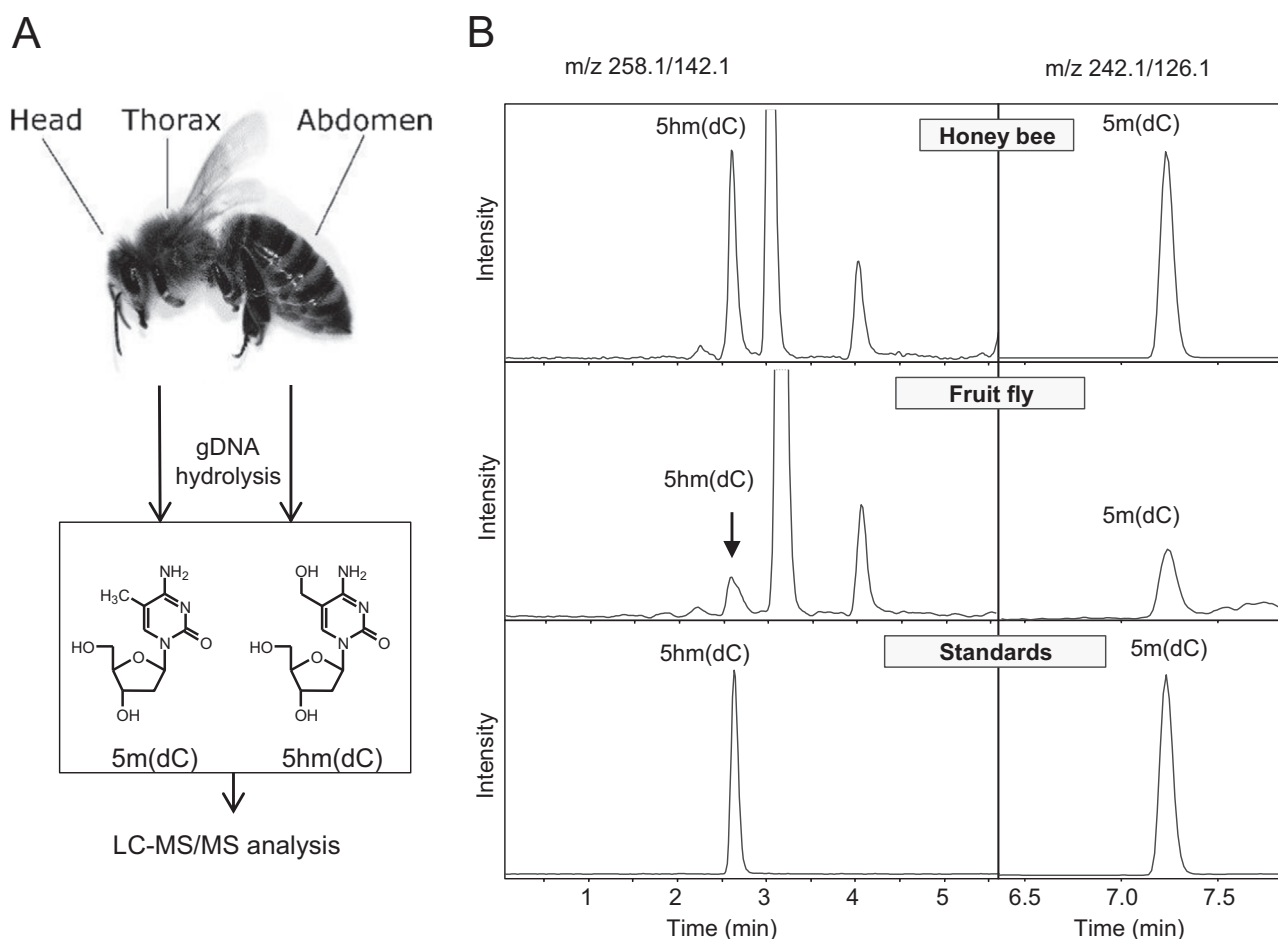

Fig. 1. Detection and quantification of cytosine modifications in honey bee and fruit fly. (A) Overview of the experimental setup. (B) Representative LC/MS/MS chromatograms showing 5hmC (left column) and 5mC (right column) in honey bees (top) and fruit fly (middle). Pure nucleoside standards are shown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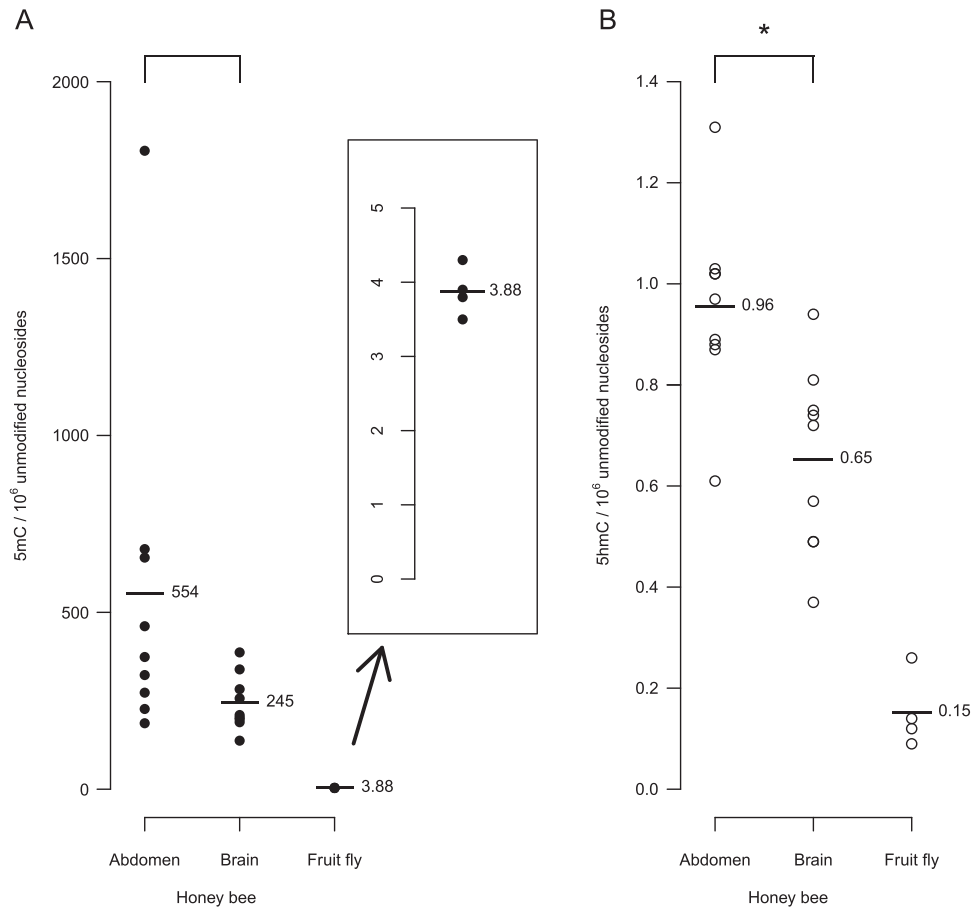
A

B

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.
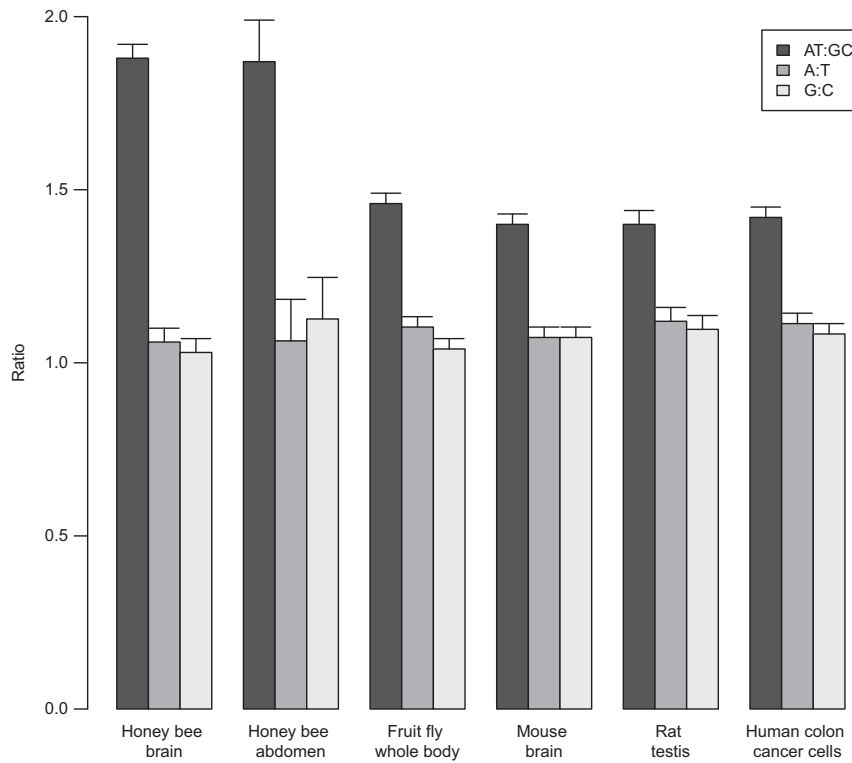

Fig. 3. Unmodified DNA base levels in different taxa. Relative levels of unmodified adenosine, thymine, guanine and cytosine in honey bee brain, honey bee abdomen, fruit fly, mouse brain, rat testis and human cancer colon cells. Error bars=SD.

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.

Acknowledgements

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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