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Active genic machinery for epigenetic RNA modifications in bees

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Abstract

Epitranscriptomics is an emerging field of investigation dedicated to the study of post-transcriptional RNA modifications. RNA methylations regulate RNA metabolism and processing, including changes in response to environmental cues. Although RNA modifications are conserved from bacteria to eukaryotes, there is little evidence of an epitranscriptomic pathway in insects. Here we identified genes related to RNA m^{6}A (N6-methyladenine) and m^{5}C (5-methylcytosine) methylation machinery in seven bee genomes (*Apis mellifera*, *Melipona quadrifasciata*, *Frieseomelitta varia*, *Eufriesea mexicana*, *Bombus terrestris*, *Megachile rotundata* and *Dufourea novaeangliae*). In *A. mellifera*, we validated the expression of methyltransferase genes and found that the global levels of m^{6}A and m^{5}C measured in the fat body and brain of adult workers differ significantly. Also, m^{6}A levels were differed significantly mainly between the fourth larval instar of queens and workers. Moreover, we found a conserved m^{5}C site in the honeybee 28S rRNA. Taken together, we confirm the existence of epitranscriptomic machinery acting in bees and open avenues for future investigations on RNA epigenetics in a wide spectrum of hymenopteran species.

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1. Introduction

Post-transcriptional RNA modifications were first reported in the late 1950s (Davis and Allen, 1957; Amos and Korn, 1958; Biswas *et al*., 1961; Birnstiel *et al*., 1963; Desrosiers *et al*., 1974; Lavi and Shatkin, 1975; Wei and Moss, 1977). Recent advances in high-throughput methods (see Chen *et al*., 2019a, and references therein) have allowed RNA modifications to be analysed on a large-scale in prokaryotes (Deng *et al*., 2015), viruses (Kennedy *et al*., 2017), yeasts (Clancy *et al*., 2002), plants (Fray and Simpson, 2015), vertebrates (Brzezicha *et al*., 2006) and invertebrates (Hongay and Orr-Weaver, 2011), giving rise to the epitranscriptomic field.

RNA epigenetics depend on a cellular machinery containing RNA binding proteins referred to as writers (acting as single-nucleotide 'modifiers'), readers (as 'recognizers') and erasers (as 'removers'), responsible for the existence of more than 170 types of RNA modifications that are currently known (Boccaletto *et al*., 2018; Huang *et al*., 2020). Methylations of the N6 nitrogen of adenosine (N6-methyladenosine, m^{6}A) and of the C5 carbon of cytosine (5-methylcytosine, m^{5}C) are the most studied epigenetic marks in RNAs (Helm and Motorin, 2017; Vandivier and Gregory, 2017).

m^{6}A is the most common modification found in mRNAs, and its occurrence is mediated by a complex formed by two methyltransferase writers, METTL3 (methyltransferase like 3) and METTL14 (methyltransferase like 14), along with a regulatory subunit WTAP (Wilms' tumour 1–associating protein) and KIAA1429 (functional spliceosome-associated protein; virilizer homolog, also called VIRMA—vir like m^{6}A methyltransferase associated) protein. This complex transfers SAM (S-adenosylmethionine cofactor) methyl groups to adenosine residues (Narayan *et al*., 1994; Bokar *et al*., 1997; Liu *et al*., 2014; Ping *et al*., 2014; Schwartz *et al*., 2014). Reader proteins of YTH (YT521-B homology domain-containing proteins), hnRNP (heterogeneous nuclear ribonucleoproteins), IGF2BP (insulin-like growth factor 2 mRNA-binding proteins) and FMR1families recognize

m^6A sites (Alarcón *et al*., 2015a; Wang *et al*., 2015; Xiao *et al*., 2016; Edupuganti *et al*., 2017; Hsu *et al*., 2017; Li *et al*., 2017; Shi *et al*., 2017; Huang *et al*., 2018), and these methylations may also be removed by erasers, FTO (fat mass and obesity associated protein) (Jia *et al*., 2011) and ALKBH5 (alkB homolog 5) (Zheng *et al*., 2013) RNA demethylases. In eukaryotes, m^6A modification is related to cap-independent translation (Meyer *et al*., 2015), alternative splicing (Zhao *et al*., 2014) and miRNA processing (Alarcón *et al*., 2015a, 2015b), among other processes.

The m^5C modification is more frequent in nonprotein coding RNAs (ncRNA), such as in tRNAs and rRNAs (Edelheit *et al*., 2013), but it is also found in mRNAs (Li *et al*., 2017; Courtney *et al*., 2019). The m^5C methylation is catalysed by the m5C-MTase writers. Among them, the DNMT2 (DNA methyltransferase 2) and the proteins of NSUN (NOP2/Sun RNA methyltransferase member) family (NSUN1, also known as NOP2, and NSUN2 to NSUN7) are primarily responsible for generating m^5C modifications in eukaryotic RNAs (Brzezicha *et al*., 2006; Goll *et al*., 2006; Motorin *et al*., 2010). ALYREF (Aly/REF export factor) and YBX1 (Y-box binding protein 1) proteins are readers of m^5C sites (Yang *et al*., 2017; Zou *et al*., 2020), which in turn may also be oxidized by eraser enzymes of the TET (ten-eleven translocation) family, which create an intermediate 5-hydroxymethylcytosine (5-hmrC) modification (Fu *et al*., 2014a). In general, the m^5C RNA modifications are related to increased stability of the secondary structure of tRNAs and rRNAs. Methyl groups prevent hydrolysis of the transcripts by nucleases, and they also block hydrogen bonds between nucleotides, ensuring an adequate architecture of these RNA molecules and, consequently, correct functioning in protein synthesis (Micura *et al*., 2001; Schaefer *et al*., 2010).

Previous reports demonstrated the functions of m^6A and m^5C modifications in various different contexts, such as reproduction (Harris *et al*., 2007; Hongay and Orr-Weaver, 2011; Khosronezhad *et al*., 2015), development (Tuorto *et al*., 2012; Haussmann *et al*., 2016; Kan *et al*., 2017) and longevity (Schosserer *et al*., 2015; Lence *et al*., 2016). These modifications occur in response to environmental cues (Baldridge and Contreras, 2014; Cao *et al*., 2016).

The honeybee (*Apis mellifera*) is a useful model species for studies of RNA epigenetics due to the fact that nutrition-based stimuli influence phenotypic plasticity and life cycle changes, such as female caste development and division of labour in adult workers (Even *et al*., 2012; Flatt *et al*., 2013).

Caste determination and differentiation in *A. mellifera* depends on the quality and quantity of food received during the larval stages. Until larval stage 3 (L3) individuals are fed on royal jelly. From L3 onwards, larvae that continue to be fed exclusively with royal jelly become queens, while those fed a mixture of royal jelly, nectar and pollen become workers (Jay, 1964). Differential feeding is, therefore, the external stimulus for the development of female morphotypes that differ considerably in size, physiology, behaviour, hormone levels, gene expression, number of ovarioles, development of corbiculae, longevity, among others, in individuals with the same genome (reviewed by Winston, 1987; Evans and Wheeler, 2001; Page and Peng, 2001).

Young adult honeybee workers feed mainly on pollen (a source of proteins, lipids and other nutrients) and perform intra-colony tasks, such as cleaning, comb construction and brood feeding for approximately two weeks. From the third week onwards, workers experience a behavioural transition and become foragers, consume nectar (rich in carbohydrates and low in protein) and collect pollen, nectar, resins and water (Winston, 1987; Page and Peng, 2001). The fat body of insects is a metabolic centre and a nutrient sensor (Azeez *et al*., 2014), together with the brain (a cognitive centre), it controls processes related to longevity and adult behavioural transitions in *A. mellifera* (Ament *et al*., 2010; Ament *et al*., 2011).

We investigated the RNA methylation machinery as a potential novel layer of epigenetic regulation in bees. We identified genes related to m^6A- and m^5C-machinery in the genomes of seven bee species and confirmed their expression using public transcriptome data. In *A. mellifera*, we encountered a conserved methylation of cytosine in a rRNA subunit, validated the expression of methyltransferase transcripts and found that global m^6A and m^5C levels differ between phenotypes. Taken together, our findings confirmed the existence of epitranscriptomic machinery in bees.

2. Results

2.1 *Searches for orthologs of epitranscriptomic machinery in* Apis mellifera

Based on the searches in HGNG platform using the initials of the gene families as keywords (METTL, NSUN, DNMT, TET, FTO, ALKBH, WTAP, KIAA1429, YTH, hnRNP, IGF2BP, FMR1, ALYREF and YBX), 87 genes were identified in the human genome, including 72 for m^6A and 15 for m^5C.

Among m^6A genes, we found 34 METTL writers (METTL1 to METTL26, with some paralogs such as METTL21A, METTL21B and METTL21C), two members of the m^6A complex (WTAP and KIAA1429), the erasers FTO (a single gene) and ALKBH (ALKBH1 to ALKBH8), and the readers of the families YTH (YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3), hnRNP (HNRNPAB, HNRNPA0, HNRNPA1, HNRNPA1L2, HNRNPA2B1, HNRNPA3, HNRNPC, HNRNPCL1, HNRNPD, HNRNPDL, HNRNPF, HNRNPH1, HNRNPH2, HNRNPH3, HNRNPL, HNRNPLL, HNRNPM and HNRNPR), IGF2BP (IGF2BP1, IGF2BP2 and IGF2BP3), and a single FMR1 gene. Among m^5C genes, we found eight m5C-MTase writers (DNMT2, NOP2, NSUN2 to NSUN7), three erasers of the TET family (TET1 a TET3),

readers of the YBX family (YBX1, YBX2 and YBX3) and a single ALYREF gene. Based on this set of 87 human genes, we found 50 orthologs in *D. melanogaster* and 49 in bee genomes.

Though all genes cited above are members of gene families related to m^6A and m^5C modifications, there was no information about their functions and/or whether they participate directly in RNA modification events. For example, METTL10, METTL12, METTL20, METTL21A, METTL21B, METTL21C and METTL22 are specific MTases Lys (K) (KMTs) and promote the methylation of lysin residues of proteins (Cloutier *et al*., 2014; Shimazu *et al*., 2014; Jakobsson *et al*., 2015; Małecki *et al*., 2015). Other METTLs such as METTL13, METTL17 and METTL23, are related to processes such as tumour suppression, oestrogen receptor regulation and intellectual disability, respectively (Bernkopf *et al*., 2014; Reiff *et al*., 2014; Du *et al*., 2015; Zhang *et al*., 2016).

Thus, we selected only those genes for which there was evidence of specifically participating in m^6A and m^5C modifications of RNAs for our analyses. Consequently, the list was reduced to 32 human genes, including 17 and 15 related to m^6A and m^5C, respectively. Of these 32 human genes, 21 are found in *D. melanogaster*, and 20 in all species of bees that we analysed. As expected, only the NSUN7 gene did not meet our search criteria. However, we included the NSUN7 gene because it is known as the least conserved member of the NSUN family (Harris *et al*., 2007). Table 1 summarizes the orthologs of the epitranscriptomic machinery for m^6A and m^5C modifications found in the genomes of *D. melanogaster* and bees.

2.2 RT-PCR

We amplified unique fragments with correct expected sizes for: METTL14, METTL3-X1, DNMT2-X1, NOP2, NSUN3, NSUN3, NSUN4, NSUN5 and NSUN7 (Fig. 1). However, the primer pairs for DNMT2-X2 and for the METTL3-X2 amplified more than one fragment (Fig. 1). These extra amplicons were sequenced, revealing third transcript variants of METLL3 and DNMT2 that were not previously predicted by databases (Figs S1–S3).

The annotation of gene structures related to m^6A and m^5C methyltranferases, including all primer positions and the novel variants, is presented in Figs S4–S6.

2.3 Digital expression of epitranscriptomic machinery orthologs in bees

Methyltranferases expression related to m^6A and m^5C modifications in different bees was searched in SRA libraries deposited in a public database (NCBI). We found evidence of expression of all methyltranferases in all bees analysed (Fig. S7), except for *Melipona quadrifasciata*, as reported in the 'Experimental procedures' section.

2.4 Global m^6A levels

Larval comparisons between caste samples showed that 0.12–0.35% of total RNA is methylated in queens and 0.13–0.26% in workers (Fig. 2). The two-way ANOVA analysis revealed that 'caste' and 'developmental stage' factors, as well as the interaction between them, significantly affect m^6A levels (caste, $F(1, 12) = 8.895$, $p = 0.0114$; developmental stage, $F(2, 12) = 31.44$, $p < 0.0001$; interaction $F(2, 12) = 4.438$, $p = 0.0361$). The Bonferroni post hoc test showed significant differences in m^6A levels between L4 stages of queen and workers ($t = 4.109$, $p < 0.01$). In queens, the L4 stage also differed compared to L2 ($t = 7.443$, $p < 0.0001$) and L3 ($t = 5.457$, $p < 0.001$) stages. In workers, L2 and L4 stages also differed in m^6A global levels ($t = 3.455$, $p < 0.05$).

In adult workers, 0.04–0.18% of total RNA of fat body samples and 0.01–0.07% of brain samples were found in m^6A sites (Fig. 3). A two-way ANOVA analysis revealed that 'age' and 'tissue' factors significantly affect the m^6A levels, but without interaction between them (age, $F(1, 8) = 15.93$, $p = 0.004$; tissue, $F(1, 8) = 14.44$, $p < 0.0052$; interaction $F(1, 8) = 4.042$, $p = 0.0792$). We observed significant differences between fat body samples of 8 and 29 days-old workers ($t = 4.244$, $p < 0.01$), as well as between brain and fat body samples of 29 days-old workers ($t = 4.109$, $p < 0.01$).

2.5 Global m^5C levels

In relation to caste samples, 0.009–0.035% of total RNA was found to be methylated in queen larvae and 0.002–0.027% in workers (Fig. 4). No significant differences in m^5C were found between the samples (two-way ANOVA; caste, $F(1, 12) = 1.201$, $p = 0.2946$; stage, $F(2, 12) = 0.7981$, $p = 0.4727$; interaction $F(2, 12) = 0.2850$, $p = 0.7570$). In adult workers, from 0.0013 to 0.0017% of the total RNA of fat body samples and from 0.009 to 0.1% of brain samples contain m^5C sites (Fig. 5). We found significant differences in m^5C levels for 'age', 'tissue' and 'interaction' factors (two-way ANOVA; age, $F(1, 8) = 38.73$, $p = 0.0003$; tissue, $F(1, 8) = 52.07$, $p < 0.0001$; interaction $F(1, 8) = 21.26$, $p = 0.0017$). Significant differences (Bonferroni post hoc test) in methylation were found between brain samples of 8 and 29-days-old workers ($t = 7.661$, $p < 0.001$) and also between brain and fat body samples of 29-days-old workers ($t = 8.363$, $p < 0.0001$).

2.6 Conserved m^5C site in rRNA

We identify a conserved m^5C site in 28S rRNA of *A. mellifera* (Fig. 6). Based on our sequencing results, the methylated cytosine is located at position 2 393 (named C2393) of the 28S rRNA (based on the deposited sequence AJ302936.1 at GenBank-NCBI) (Fig. S8). Based on sequence alignments, we confirmed that this m^5C site found in *A. mellifera* is conserved between

Table 1.; Epitranscriptomic machinery (m^6A and m^5C) orthologs between humans and insects

Modification	Human gene	Function	Main target	*Drosophila melanogaster*	*Apis mellifera*	*Frieseomelitta varia*	*Melipona quadrifasciata*	*Eufriesea mexicana*	*Bombus terrestris*	*Megachile rotundata*	*Dufourea novaeangliae*
m^6A	METTL3	Methyltransferase (writer)	mRNA	42844	551911	KAF3427832.1	KOX70831.1	108555041	105665938	100883865	107189048
	METTL14		mRNA	34138	409900	KAF3422349.1	KOX80044.1	108548758	100643430	100883193	107192389
	WTAP	m^6A complex		36527	552833	KAF3420093.1	KOX67774.1	108546999	100648687	100875261	107191917
	KIAA1429			47869	411612	KAF3420191.1	KOX78185.1	108555974	100645381	100879088	107185687
	YTHDF1 YTHDF2 YTHDF3	Reader		42995	551840	KAF3426044.1	KOX78134.1	108549048	100651571	100883522	107193511
	YTHDC1			38420	411754	KAF3420202.1	KOX78177.1	108555992	100646303	100878542	107194478
	YTHDC2			47873	409028	KAF3429904.1	MQUA21365-RA*	108551855	100646905	100883335	107192091
	HNRNPA2B1			43385	552027	KAF3430466.1	KOX75468.1	108550073	100643287	100883215	107187494
	HNRNPC			2768945	726153	KAF3425044.1	Not predicted but present in the genome**	108556251	100649273	100881697	107194047
	IGF2BP1 IGF2BP2 IGF2BP3			32009	410398	KAF3421954.1	KOX75655.1	108553296	100647990	100877662	107192751
	FMR1			37528	410580	KAF3426775.1	KOX79781.1	108545540	100649034	100879570	107190537
	FTO	Eraser		-	-	-	-	-	-	-	-
	ALKBH5			-	-	-	-	-	-	-	-
m^5C	DNMT2	Methyltransferase (writer)	mRNA, tRNA	34632	410512	KAF3424430.1	KOX71481.1	108548714	100651101	100875695	107191859
	NOP2		mRNA, rRNA	36365	726212	KAF3426642.1	KOX72944.1	108547093	100642423	100882725	107187352
	NSUN2		mRNA, tRNA, mt-RNA	45064	411579	KAF3427563.1	KOX72241.1	108555797	100650195	100879322	107192196
	NSUN3		tRNA, mt-tRNA	-	-	-	-	-	-	-	-
	NSUN4		rRNA	33293	102655259	KAF3430370.1	KOX80057.1	108554153	100644316	100883736	107190087
	NSUN5		rRNA	7354421	410262	KAF3425199.1	KOX74880.1	108551186	100646720	100880125	107192324
	NSUN6		tRNA	40507	-	-	-	-	-	-	-
	NSUN7		rRNA, tRNA	39825	413907	KAF3420598.1	KOX78472.1	108545959	100647731	100880076	107191535
	ALYREF	Reader		44029	725396	KAF3423346.1	KOX73851.1	108546939	100645586	100882325	107185834
	YBX1 YBX2 YBX3			39377	409854	KAF3428894.1	KOX67189.1	108553898	100647589	100878915	107186850
	TET1 TET2 TET3	Eraser		38347	412878	KAF3424387.1	KOX75173.1	108551398	100642293	100883443	107185337

Machinery gene sequences found in humans served as input for BLASTP searches against sequences of a fly (*Drosophila melanosgaster*) and bees (*Apis mellifera*, *Melipona quadrifasciata*, *Frieseomelitta varia*, *Eufriesea mexicana*, *Bombus terrestris*, *Megachile rotundata* and *Dufourea novaeangliae*). Orthologs are identified by Gene ID or protein accession number from NCBI. The functions of the orthologs and the main targets of methyltransferases are reported according to the literature.

*The gene was not predicted by the NCBI database but was predicted by the Hymenoptera Mine data base (http://128.206.116.3:8080/hymenopteramine/begin.do). The IDs in the Table correspond to Hymenoptera Mine.

**The gene was found in the genome but was divided into two scaffolds (LIRP01012754.1 and LIRP01012753.1—NCBI).

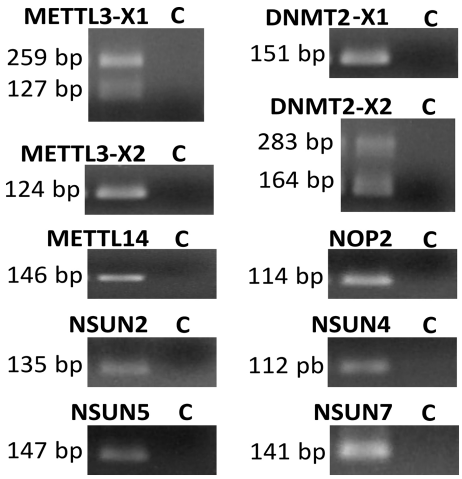

Figure 1. Amplification of specific fragments of different genes of the m^{6}A and m^{5}C epitranscriptomic machinery in *Apis mellifera* by conventional RT-PCR. The amplified fragments are indicated in base pairs (bp) and matched to the expected sizes based on gene structure annotations (see Figs S1–S3). C represents the negative control of the PCR without addition of the cDNA template.

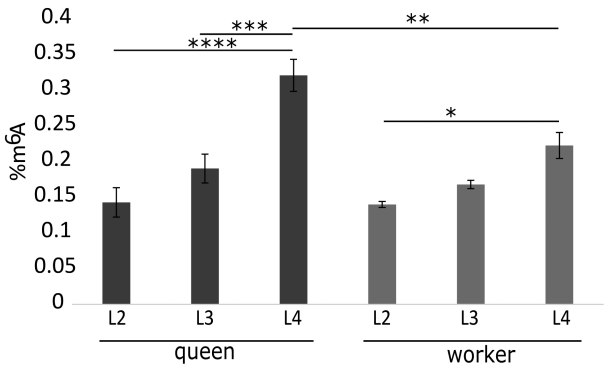

Figure 2. The average percentage (% ± SEM) of global m^{6}A methylation in total RNA samples from L2, L3 and L4 stages of queens and workers ($n = 3$ per condition). Asterisks indicate significant differences (two-way ANOVA, post-hoc Bonferroni test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

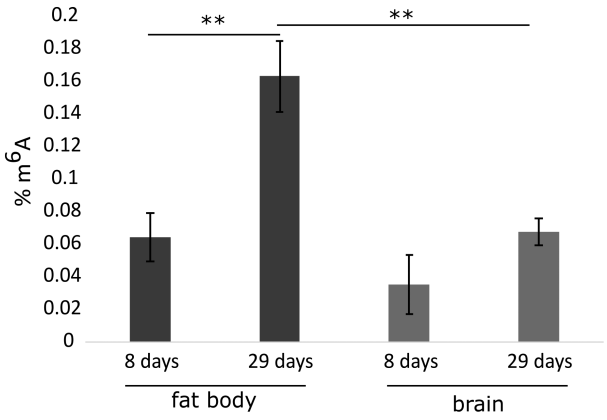

Figure 3. The average percentage (% ± SEM) of global m^{6}A methylation in total RNA samples from fat body ($n = 3$) and brain ($n = 3$) of adult workers analyzed at different ages (8 and 29 days). Asterisks indicate significant differences (two-way ANOVA, post-hoc Bonferroni test, **$p < 0.01$).

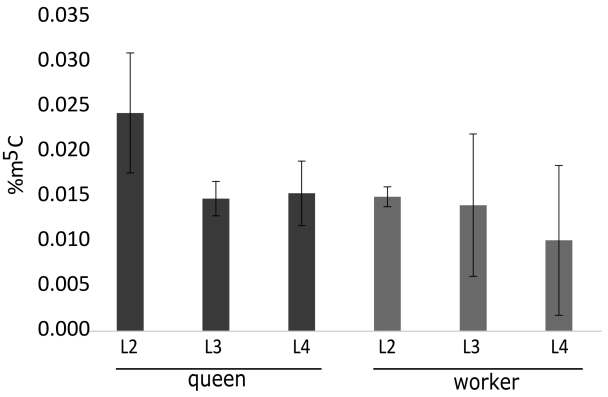

Figure 4. The average percentage (% ± SEM) of global m^{5}C methylation in total RNA samples from L2, L3 and L4 stages of queens and workers ($n = 3$ per condition). No statistically significant differences were found.

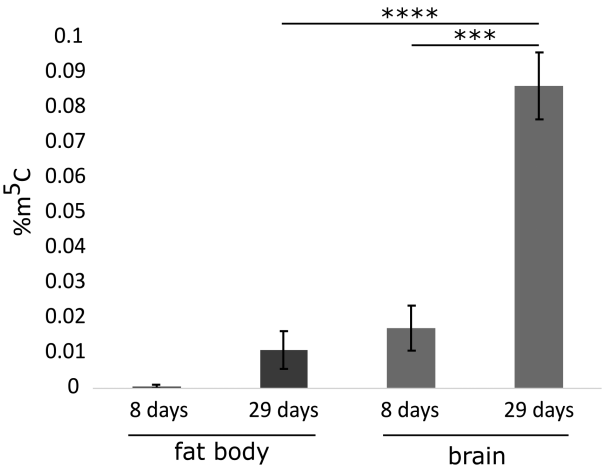

Figure 5. The average percentage (% ± SEM) of global m^{5}C methylation in total RNA samples from fat body ($n = 3$) and brain ($n = 3$) of adult workers analyzed at different ages (8 and 29 days). Asterisks indicate significant differences (two-way ANOVA, post-hoc Bonferroni test, ***$p < 0.001$, ****$p < 0.0001$).

Saccharomyces cerevisiae (AH005487.2—C3501), *Caenorhabditis elegans* (NR_000055.1—C2381) and *Drosophila melanogaster* (NR_133553.1—C2659), as previously reported (Sharma *et al*., 2013; Schosserer *et al*., 2015). In *A. mellifera* we also identified a neighbouring m^{5}C site (C2392), not previously identified in any species (Fig. 6).

3. Discussion

We identified epitranscriptomic gene machinery for m^{6}A and m^{5}C modifications in the genome of all seven bee species, suggesting a genetic conservation that does not depend on the levels of social organization (Kapheim *et al*., 2015; de Paula Freitas *et al*., 2020). In fact, social and nonsocial Hymenoptera tend to be extremely similar

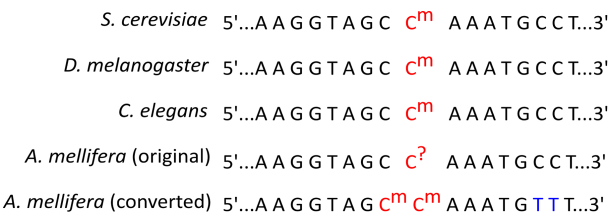

Figure 6. Conserved m^5C methylation site in rRNA. Alignment of the conserved region of 25S rRNA (*Saccharomyces cerevisiae*), 26S rRNA (*Caenorhabditis elegans*) and 28S rRNA (*Drosophila melanogaster* and *Apis mellifera*) with the conserved methylated cytosine indicated in red. The *A. mellifera* original sequence is the sequence from NCBI. The *A. mellifera* converted sequence is the result of sequencing after bisulfite treatment. The cytosines that were sequenced as cytosine after the treatment represent methylation (red) and the cytosines sequenced as thymine (blue) represent the absence of methylation.

in terms of gene repertoires (Sadd *et al*., 2015). All genes found are supported by transcriptional expression evidence, indicating the roles of the epitranscriptomic machinery in the regulation of fundamental cellular processes in these bee species. We also found evidence for epitranscriptomic activity in *Apis mellifera* related to m^6A and m^5C marks. A larger number of m^6A- and m^5C-gene sets are found in humans than in insects, suggesting some gene family expansions during evolution. However, we consider that bees contain a sufficiently complete set of genes at least for regulation of m^6A and m^5C modifications, except for m^6A erasers, FTO and ALKBH5, which are not found in *D. melanogaster* (Lence *et al*., 2016) or bees (this study). We also performed additional searches using TBLASTN alignments against an insect database and no FTO gene was identified. We found ALKBH5 orthologs only in two species of termite (*Cryptotermes secundus* and *Zootermopsis nevadensis*), two beetles (*Anoplophora glabripennis* and *Leptinotarsa decemlineata*) and two bugs (*Diaphorina citri* and *Halyomorpha halys*). It is known that m^6A modifications are reversible, through the action of eraser proteins. No ALKBH enzyme besides ALKBH5 has so far been associated with m^6A RNA demethylation; ALKBH1 is known for demethylating m^6A in DNA (Müller *et al*., 2017) and an ortholog is found in bees (including in *Apis mellifera*, NCBI Gene ID: 408980). Whereas TET demethylase is responsible for m^5C reversion in DNA and RNA (Ito *et al*., 2011), it is possible that ALKBH1 also could act as m^6A eraser in both nucleic acids. More investigation is necessary for the understanding of erasers in insects.

We found transcriptional activity of the epitranscriptomic machinery based on conventional RT-PCR data, showing that all writer genes that we tested are expressed in the fat body of *A. mellifera* adult workers. These results together with the BLAST (orthologs) and SRA data in the various bee species demonstrate the existence and the expression of epitranscriptomic machinery genes related to m^6A and m^5C modifications.

When we compared global m^5C measures between the analysed larval stages of castes, we did not observe any significant difference. However, m^5C was detected in all samples, suggesting potential functions associated with basic regulatory mechanisms of cells, such as regulation of expression and translation (Tuorto *et al*., 2012).

The levels of m^6A were differed significantly between caste samples and larval stages, with the largest differences occurring in the L4 stage between queens and workers and between L4 with other larval stages of the same caste. Other studies also concluded that the L4 stage is critical for caste differentiation in *A. mellifera* (Barchuk *et al*., 2007; Shi *et al*., 2013).

m^6A affects the interaction of RNA with proteins or regulatory factors, resulting in changes in the stability and half-life of mRNA. Methylation can even affect the secondary structure of RNAs, which also alters RNA-protein interactions (Fu *et al*., 2014b; Roignant and Soller, 2017). This modification has various types of effects on gene expression, which can range from degradation to increased translation of methylated mRNAs (Fu *et al*., 2014b; Roignant and Soller, 2017). Therefore, methylation differences may also reflect differences in gene expression found between queens and workers (Barchuk *et al*., 2007).

It is well-documented that m^6A is involved in the processing of microRNA (miRNA). *In vitro* knockdowns of METTL3 and HNRNPA2B1transcripts in human cells results in a reduction of mature miRNAs (Alarcón *et al*., 2015b). The differences in m^6A levels found between castes may explain previously reported differences in queen and worker patterns of miRNA expression (Ashby *et al*., 2016).

The larval stage is characterized by intense feeding and molting processes (five larval instars). Between L2 and L4 stages, honeybee larvae increase considerably in size and there is a marked development in the body structures that will give rise to the legs and wings (Myser, 1954). During this period, the endocrine system of queen larvae develops more rapidly than in worker larvae, including the *corpora allata*, a head gland that produces and secretes juvenile hormone (Dogra *et al*., 1977). Several studies have already shown the importance of RNA modifications for development, mainly involving the correct formation of organs and structures, such as the brain (Cui *et al*., 2016; Chen *et al*., 2019b) and correct body size (Tuorto *et al*., 2012). In particular, m^6A modifications are involved with the control of cell development, and organismal metabolism, survival and growth (Fu *et al*., 2014b). Differences in m^6A levels observed between different larval stages of the same caste suggest relation to morphological changes triggered during the development of immature individuals.

We observed that the global levels of m^6A and m^5C measured in the fat body and brain of adult workers differ significantly. The m^6A levels are higher in fat bodies when compared to the brain, while m^5C levels exhibit the opposite trend. We also found that age and tissue affect the

levels of RNA modifications in *A. mellifera*, as also observed previously in humans (Dominissini *et al*., 2012; Min *et al*., 2018), mice (Meyer *et al*., 2012) and plants (Li *et al*., 2014; Wan *et al*., 2015).

m^6A has a role in the regulation of metabolic pathways, including lipid and carbohydrate metabolism (Wu *et al*., 2020). Nutrition can alter levels of RNA methylation (Wu *et al*., 2020). Thus, we assume that the significant differences in m^6A in the fat body between young and old workers is due to the consumption of different diets at each stage of adult life.

Foragers have an increased cognitive capacity (memory and learning) compared to nurse bees (Zayed and Robinson, 2012), which is consistent with their role of acquiring resources outside the colony. In various organisms, it has been found that the development of the nervous system, including cognitive and behavioural aspects and learning and memory skills, involves reprogramming (spatial and temporal) of the epitranscriptome (Jung and Goldman, 2018; Leighton *et al*., 2018). These changes are also supported by our findings of differences in m^5C levels detected in worker brains apparently related to the maturation of the nervous system throughout adulthood (Withers *et al*., 1993).

We found a conserved m^5C site in the 28S rRNA of *A. mellifera* that ensures correct structure and stability of rRNAs (Gigova *et al*., 2014; Schosserer *et al*., 2015). We also described another neighbouring m^5C site in the rRNA, that is probably unconserved; its structural importance for 28S rRNA remains to be explored. Taken together, this evidence demonstrates the existence and action of RNA methylation machinery in *A. mellifera*.

The epitranscriptomic universe is still poorly investigated in insects (Hongay and Orr-Weaver, 2011; Haussmann *et al*., 2016; Kan *et al*., 2017; Li *et al*., 2019). Our study adds new information to this field of research and broadens our understanding of some aspects of bee biology that have not been fully elucidated.

4. Experimental procedures

4.1 Bioinformatics

Considering that the epitranscriptomic machinery is well documented in humans (Li and Mason, 2014), we first performed searches in the *HGNC* (*Human Genome Organization* [*HUGO*] *Gene Nomenclature Committee*: https://www.genenames.org/) platform using the following gene family names as keywords: METTL, NSUN, DNMT, TET, FTO, ALKBH, WTAP, KIAA1429, YTH, hnRNP, IGF2BPs, FMR1, ALYREF and YBX. The amino acid sequences for the resulting proteins were recovered in FASTA format and used as input to BLASTP alignments against the GenBank-NCBI (https://www.ncbi.nlm.nih.gov/) database in order to detect Reciprocal Best Hits (RBH), as a proxy for orthology relationships (Bataglia *et al*., 2018), for *D. melanogaster*, *A. mellifera* and another six bee species with available genomic sequences (*Melipona quadrifasciata*, *Frieseomelitta varia*, *Eufriesea mexicana*, *Bombus terrestris*, *Megachile rotundata* and *Dufourea novaeangliae*), respectively. BLASTP analyses were performed based on the conditional compositional score matrix adjustment method and the following criteria: score > 200 bits, *e*-value < $e - 50$, query cover > 40%, identity > 35%, matrix: BLOSUM62, and no masking filters used for low complexity regions. For double check validation, BLASTP alignments were also performed against the *FlyBase* (http://flybase.org/) and the HymenopteraMine (https://hymenoptera.elsiklab.missouri.edu/: Elsik *et al*., 2016). The resulting RBH sequences (both as nucleotides and amino acids) found for fly and bees were recovered in FASTA format, and NCBI Gene ID or protein accession number were listed. *Drosophila* sequences were included for comparison because this fly is the insect species that has the most information about epitranscriptomic machinery in the scientific literature and databases, which makes it

Table 2.; Primer sequences designed for PCR amplification of methyltransferase genes (and some variants, X1 or X2) in *Apis mellifera*

Primer name	Sequence 5′ → 3′	Amplicon size (bp)	Annealing temperature (°C)
METTL3-X1-F	GCGTTATGTTACTTCGTTAGC	127	52
METTL3-X1-R	GCTTGTATCTCTTCAAAAGCG		
METTL3-X2-F	CAAATATTATCGCAAAGATATG	124	55
METTL3-X2-R	AAGAGTAAATAATTCATGTCG		
METTL14-F	ATTTTGATCGAGCCCCCGTT	146	60
METTL14-R	CTACTGCCGCACCAGAGAAA		
DNMT2-X1-F1	AATGTGGATGTAAATAAACAGA	151	52
DNMT2-R*	GTATAATTCCAACACTCTCATC		
DNMT2-X2-F2	CCATTACTGAATAATCAGTGG	164	
NOP2-F	CGCGTCGTCGTGATTTAGC	114	60
NOP2-R	CACCCATGGGAACTTGCGAT		
NSUN2-F	CCACCAAAACCTGAGGATGC	135	60
NSUN2-R	TGATGCACGTTCCCAAGGTA		
NSUN4-F	TTCAATCTGAGATTTTAGCGA	112	55
NSUN4-R	GCTACTTGTACAACACCATC		
NSUN5-F	ATGTGTGCAGCTCCTGGAAT	147	60
NSUN5-R	AGTTTCGACACAAGAAGCGTT		
NSUN7-F	CTGTTTCCGAGCTCGTTTGT	141	60
NSUN7-R	GTCGAGGTGCAAGTGAATGC		

*The same R primer was used to amplify fragments of two different DNMT2 variants.

appropriate for validation of results. Next, the architectures of the genes identified in *A. mellifera* were manually annotated, including the exon-intron boundaries, using Artemis platform (http://www.sanger.ac.uk/science/tools/artemis) (Rutherford *et al*., 2000) and nucleotide FASTA sequences of genes and genomic data (assembly Amel_HAv3.1). The gene structure served as a template to design specific intron-spanning pairs of primers to check expression by PCR amplification of cDNA fragments of the m^6A methyltransferases (METTL3 and METTL14) and m^5C methyltransferases (DNMT2, NOP2 and NSUN2 to NSUN7) transcripts (Table 2). In addition, additional primers were designed to discriminate alternative transcripts whenever a gene model had an indication by GenBank-NCBI of the possible expression of more than one variant. The specificity of each primer was checked by NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and additional quality parameters such as formation of hairpins, self-dimers and cross dimers were analysed by Oligo Analyser 3.1 software (https://www.idtdna.com/pages/tools/oligoanalyser).

4.2 Samples

Honeybee samples (*A. mellifera*) were collected from the experimental apiary of the Departamento de Genética of Faculdade de Medicina de Ribeirão Preto of the Universidade de São Paulo (FMRP-USP). Each colony (with unrelated genetic background) contained one naturally mated queen; some queen less colonies were also used when necessary (for details see caste sampling below).

For caste larvae sampling, a controlled queen laying was used in five different colonies, with the Jenter queen rearing system (equipment that can be used to raise a large number of queens) (Wakjira *et al*., 2019). In each colony, the queen was confined in an area for 5 h. After 72 h (period from the end of oviposition to larval hatching), the removable artificial cell plugs from the Jenter kit containing newly eclosed L1 larvae were transferred to standard queen cell cup holders and attached to wooden cell bars. These cell bars were placed in queen less colonies until the sampling time. All other L1 larvae located in nonremovable cells plugs of the Jenter system were returned to queenright colonies until sampling. This strategy allowed us to collect different caste samples in a paired manner. We collected whole larvae at L2 (28 ± 2.5 h after hatching), L3 (45 ± 2.5 h after hatching) and L4 (68 ± 2.5 h after hatching) of queens and workers. For each larval development stage, five larvae from the same colony were pooled for RNA extraction. Thus, we obtained three biological replicates ($n = 3$ pools containing five larvae each) for each stage per caste.

For adult worker sampling, newly emerged workers were marked on the thorax and returned to their original colonies. The marked workers were collected at eight and 29-days old from three different colonies. After sampling, the bees were anesthetized at 4 °C for a period of 15 min for dissections. Batches of five dissected brains or abdominal fat bodies from workers of the same colony were prepared in triplicates ($n = 3$ independent pools containing five tissues each) for RNA extraction.

4.3 Total RNA extraction

The pooled RNA samples were extracted according to the Trizol® (Invitrogen) protocol. The purity (estimated by the ratio between reading values of 260 and 280 nm) and concentration (μg/μl) of the final solution of each sample was obtained by optical absorbance at 260 nm, in a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc).

4.4 cDNA synthesis and PCR

An aliquot of 2 μg of total RNA per sample of fat bodies was treated with DNaseI (Invitrogen), and cDNA strands were synthesized according to the protocol of the enzyme SuperScript™ II Reverse Transcriptase (Invitrogen). Each cDNA was diluted 10X for use as a template in the PCR. To check expression of m^6A and m^5C methyltransferases, we used fat body cDNA samples for PCR. For each amplification reaction, 10 μl of Master Mix PCR (Promega), 0.8 μl of each primer, forward and reverse (10 pmol/μl), 7.4 μl water and 1 μl cDNA (10 ng/μl) were used, in a 20 μl reaction solution. Reactions were run in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) thermal cycler with the following parameters: 94 °C for 1 min, followed by 38 cycles of 94 °C for 35 s, X °C (see Table 2 for X = annealing temperature) for 35 s, and 72 °C for 35 s. Amplification products were visualized in 1.5% agarose gel electrophoresis in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) stained with UniSafe Dye (20 000X-Uniscience) and using a 100 bp molecular marker (100 bp DNA Ladder, 0.5 μg/μl, Invitrogen). The gels were visualized under ultraviolet light and photo documented with Kodak EDAS 290 software and equipment. Alternative spliced variants were sequenced when detected.

4.5 Sequencing novel RNA modification transcript variants

For sequencing PCR amplicons of potential novel variants, the interrogated extra bands observed on agarose gel were carefully cut out from the agarose gels with a fresh sterile scalpel blade, placed in separate tubes and purified by the PureLink™ Quick Gel Extraction Kit (Invitrogen), following manufacturer's specifications. A total of 100 ng of purified material from each sample was used for both direction sequencing, using the forward or reverse primers for METTL3-X1 and DNMT2-X2 variants in separate reactions. Each sequencing reaction consisted of 2 μl of BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 2 μl of 5X Sequencing Buffer (Applied Biosystems), 1 μl of forward or reverse primer (2.5 pmol/μl), 1 μl of purified template (250 ng) and 4 μl of water, in a final volume of 10 μl. The reactions were run in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) thermocycler, with the cycling parameters: 95 °C for 1 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The products were sequenced in an ABI 3500 XL Genetic Analyser. The electropherograms were visualized in the program BioEdit 7.2, and the resulting FASTA sequences were aligned to the original gene sequences (METTL3-X1 and DNMT2-X2) using CLUSTALW software (http://www.genome.jp/tools-bin/clustalw) and to the genomic sequence using the Artemis platform for determining the architectures of the potential novel variants named METTL3-X3 and DNMT2-X3.

4.6 Digital expression

Digital expression was used to elaborate an expression panel to validate the transcriptional activity of methyltranferase genes in bees. The nucleotide sequences of epitranscriptomomic

machinery genes of bees were used as input for BLASTN alignments against the RNA-Seq data stored in the SRA (Sequence Read Archive) database available at NCBI. In particular, the *F. varia* genome sequence is available (from de Paula Freitas *et al*., 2020) and the predicted protein sequences are in the NCBI. The nucleotide sequences of the methyltranferase genes were recovered from an internal database of our laboratory and the FASTA sequences are available in the Supplementary note 1.

For species that have several SRX files available in the SRA database, we randomly chose three of them. For *A. mellifera*, we searched for expression from SRA data generated from samples of four-day-old queen larvae (SRX1546383), heads of adult workers (SRX7278350) and 55-70 h male embryos (SRX9279212). For *F. varia*, we used samples of worker pharate adult integument (SRX2945336), newly emerged worker integument (SRX4680120), and forager worker integument (SRX4680118). For *B. terrestris*, we used samples of the body of adult workers (SRX2945336), heads of adult workers (SRX5710695) and heads of adult queens (SRX5104318). For *M. rotundata*, we used samples of early diapausing prepupae (SRX3136114), late diapausing prepupae (SRX3136118) and pigmented-eye pupae (SRX5557265). Only a single SRX file derived from the whole body of female adults was found for the bee species *E. mexicana* (SRX1013193) and *D. novaeangliae* (SRX1013325), these were used as input for digital expression searches. Although protein predictions are available for *M. quadrifasciata*, we did not perform alignments for this bee because there are no nucleotide gene models available in NCBI or in any other public database that could be used as input in BLASTN searches (although the protein predictions are available for this bee). We considered as significant BLAST alignments with score >80 (other parameters varied depending on the read lengths generated in the different RNA-Seq projects that we used).

4.7 Global m^6A level quantification

Global m^6A levels in total RNA were measured by an ELISA-like assay using the EpiQuik ™ m^6A RNA Methylation Quantification Kit (EpiGentek) and following the manufacturer's protocol. We quantified m^6A levels in different caste and adult worker conditions, using 300 ng of total RNA for each sample. The kit provides a positive and a negative control. The positive control is an oligonucleotide with all adenines methylated and the negative control is an RNA with no methylation. The global m^6A signal per sample was colorimetrically detected by reading the absorbance at 450 nm in a microplate spectrophotometer (Beckman Coulter DTX 880 Multimode Detector spectrophotometer). The overall methylation percentage was calculated based on mathematical formulas according to the kit manual. The formula considers the absorbance of positive and negative controls to determine the global level of methylations of samples. Statistical analysis of the results was made using GraphPad Prism 8 software. The data were analysed by two-way ANOVA and a post hoc Bonferroni *t*-test at a significance level of $p < 0.05$, using 'age and tissue' (for adult workers) or 'caste and developmental stage' (for queens and workers) as factors.

4.8 Global m^5C level quantification

Global m^5C levels in total RNA were measured using the Methyl-Flash 5-mC RNA Methylation ELISA Easy Kit (EpiGentek), following the manufacturer's protocol. We quantified m^5C levels for different caste and adult worker conditions, using 200 ng of total RNA for each sample. The kit provides a positive and a negative control. The positive control is an oligonucleotide with 2% cytosines that are methylated, and the negative control is an RNA with no methylation. Fluorescence reading was performed on a Cary Eclipse Fluorescence spectrophotometer (Agilent) at $530_{ex}/590_{em}$ nm, and the overall methylation percentage was calculated based on mathematical formulas from the kit manual. A standard curve with six points of dilution (0.05, 0.1, 0.2, 0.5, 1 and 2%) of the positive control was generated and the slope value of this curve and the fluorescence value of the negative control were used to determine the global level of methylations of the samples. Statistical analysis of the results was performed using GraphPad Prism 8 software. The data were analysed by two-way ANOVA and a post hoc Bonferroni *t*-test at a significance level of $p < 0.05$, using 'age and tissue' (for adult workers) 'caste and developmental stage' (for queens and workers) as factors.

4.9 Specific m^5C quantification

Considering the previously described methylation of a specific cytosine conserved in rRNA molecules of various eukaryotic species (Schosserer *et al*., 2015), we investigated whether this modification is also present in *A. mellifera* 28S rRNA (NCBI accession number AJ302936.1). To this end, 1.2 μg of fat body-derived RNA were treated with DNase, followed by bisulphite conversion using the EZ RNA Methylation ™ Kit (Zymo Research), following the manufacturer's instructions (including samples and primer pairs used as controls). Bisulphite RNA was reverse transcribed into cDNA using random hexamer primers and all steps of the SuperScript™ II Reverse Transcriptase (Invitrogen) protocol. For PCR amplification, a pair of primers was designed using the Bisulphite Primer Seeker Program (www.zymoresearch.com/tools/bissulfite-primer-seeker), as follows: 28S rRNA *Apis* Forward—5′AGGGTGTTGAYGTAATGTGATTTTTGTTTAGTG and 28S rRNA *Apis* Reverse—5′TTCCAAACCCRTTCCCTTAACAATAA TTTC, as well as the Platinum® Taq DNA polymerase High Fidelity (Invitrogen). The PCR cycling was 94 °C for 2 min, followed by 45 cycles of 94 °C for 40 s, 48 °C for 40 s and 72 °C for 40 s. After electrophoresis, expected amplicon bands were cut out from the agarose gel and purified, as previously described in the 'Sequencing novel RNA modification transcript variants' section. Purified products were cloned into a pGEM®-T Easy Vector vector (50 ng/μl), using the pGEM®-T Easy Vector System I (Promega) followed by thermal shock transformation into competent *E. coli* (DH5α) cells, plated on solid LB medium containing 25 μl ampicillin (50 ng/μl, USB), 25 μl X-Gal (50 mg ml, Promega), 25 μl IPTG (0.024 g/ml, Eppendorf), incubated overnight at 37 °C. White bacterial colonies were selected for plasmid purification using the QIAprep Spin Miniprep Kit (Qiagen), and the final product was eluted in 30 μl of the EB buffer (Buffer EB—10 mM Tris-Cl, pH 8.5) and the concentration was measured with a NanoDrop® ND-1000 (Thermo Fisher Scientific Inc). A total of 250 ng of each sample was sequenced using the conventional protocol of BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems) and the ABI 3500 XL device system. The electropherograms were visualized in the BioEdit 7.2 program, and the resulting FASTA sequences were aligned by the CLUSTALW software (http://www.genome.jp/

tools-bin/clustalw) with the genome sequences to check for potential specific m^5C sites.

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Author contributions

FMFN conceived and supervised the study. LB performed sampling, experiments, data collection and analysis. ZLPS provided materials and reagents. FMFN and ZLPS acquired funding. FMFN and LB wrote the manuscript. All authors performed data interpretation, reviewed, and edited the manuscript, and approved its final version.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request and in the supplementary material of this article.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1: Supplementary Information.