Monoallelic methylation and allele specific expression in a social insect

Kate D. Lee[1†], Zoë N. Lonsdale[2†*], Maria Kyriakidou[2], Despina Nathanael[2], Harindra E. Amarasinghe[2], Eamonn B. Mallon[2]

1 Bioinformatics and Biostatistics Support Hub (B/BASH), University of Leicester, Leicester, U.K.

2 Department of Genetics, University of Leicester, Leicester, U.K.

† **Joint first authors**

∗ **E-mail: Corresponding zl107@leicester.ac.uk**

Abstract

Social insects are emerging models for epigenetics. Here we examine the link between monoallelic methylation and monoallelic expression in the bumblebee *Bombus terrestris* using whole methylome and transcriptome analysis. We found nineteen genes displaying monoallelic methylation and expression. They were enriched for functions to do with social organisation in the social insects. These are the biological processes predicted to involve imprinting by evolutionary theory.

Introduction

Several recent reviews have heralded social hymenopteran insects (ants, bees and wasps) as important emerging models for epigenetics [Glastad et al., 2011, Weiner and Toth, 2012, Welch and Lister, 2014, Yan et al., 2014]. This is based on theoretical predictions for a role for genomic imprinting in their social organisation (e.g. worker reproduction) [Queller, 2003] and on data showing a fundamental role for methylation in their biology [Chittka et al., 2012]. Genomic imprinting is allele specific expression in diploid individuals, with the expression being dependent upon the sex of the parent from which an allele was inherited [Haig, 2000]. In mammals and flowering plants, genomic imprinting is often associated with methylation marks passed from parents to offspring [Reik and Walter, 2001]. There is contraditory evidence for the role of methylation on allele specific expression in social insects. Methylation is associated with allele specific expression in a number of loci in the ants *Camponotus floridanus* and *Harpegnathos saltator*

[Bonasio et al., 2012]. Other work on the honeybee *Apis mellifera* found no link between potentially imprinted loci and known methylation sites in that species [Kocher et al., 2015].

Given the diversity within the hymenoptera, especially in social structure, this field would benefit from an increase in the breadth of the species studied. The recently sequenced genome of the bumblebee, *Bombus terrestris* displays a full complement of genes involved in the methylation system [Sadd et al., 2015]. Our lab has demonstrated that methylation is important in worker reproduction in this bumblebee [Amarasinghe et al., 2014]. We recently also found allele specific expression in *Bombus terrestris* worker reproduction genes using a candidate gene qPCR based approach [Amarasinghe et al., 2015].

In this paper, we examine the link between monoallelic methylation and monoallelic expression by examining two whole methylome libraries and an RNA-seq library from the same bee. MeDIP-seq is an immunoprecipitation technique that creates libraries enriched for methylated cytosines [Harris et al., 2010]. Methyl-sensitive restriction enzymes can create libraries that are enriched for non-methylated cytosines (MRE-seq) [Harris et al., 2010]. Genes found in both libraries are monoallelically methylated, with the methylated allele being in the MeDIP-seq data and the unmethylated allele in the MRE-seq data [Harris et al., 2010]. Monoallelic expression was identified in these loci from the RNA-seq library. If only one allele was expressed then we knew that these loci were both monoallelically methylated and monoallelically expressed in this bee. We also searched more generally for allele specific expression by analysing thirty published RNA-seq libraries from worker bumblebees [Harrison et al., 2015, Riddell et al., 2014].

Results and discussion

In total, we found nineteen genes that were both monoallelically methylated (present in both MeDIP and MRE-seq libraries) and monoallelically expressed (only one allele present in the RNA-seq library), see supplementary table 1. Monoallelic expression was confirmed in *slit homolog 2 protein-like* (AELG01000623.1) by allele specific qPCR [Amarasinghe et al., 2015]. The allele with a guanine at the snp position had a mean expression of 6.04 $\pm$8.28 (S.D.) in four bees from three different colonies. The thymine allele was not expressed at all. This was not due to the efficiency of the primers as the DNA controls of both alleles showed amplification (G mean = 422.70 $\pm$507.36, T mean = 1575.17 $\pm$503.02). Of the nineteen genes, fourteen had the methylated (MeDIP) allele expressed, while five had the unmethylated (MRE-seq) allele expressed. These were blasted against the nr/nt database (blastn). Four returned no

hits. Another four returned noninformative hits ("hypothetical proteins").

A number of these genes had homologs known to be methylated in humans. We found the MeDIP allele of *yippee-like 1* (AELG01001021.1) was expressed. Yippee is an intracellular protein with a zinc-finger like domain. DNA methylation of a CpG island near the *yippie-like 3* promoter in humans represents a possible epigenetic mechanism leading to decreased gene expression in tumours [Kelley et al., 2010]. The MeDIP allele of *slit homolog 2 protein-like* (AELG01000623.1) was expressed. Slit (AELG01000623.1) is produced by midline glia in insects and is involved in cell projection during development [Rothberg et al., 1990]. All three human Slits were found to be hypermethylated in hepatocellular carcinoma cell lines [Zheng et al., 2009]. Methionine aminopeptidases catalyse N-terminal methionine removal [Leszczyniecka et al., 2006]. We found the MeDIP allele of *methionine aminopeptidase 1-like* (AELG01000544.1) was expressed. MAP1D in humans was found to be potentially oncogenic [Leszczyniecka et al., 2006]. Calmodulin-lysine N-methyltransferase catalyses the trimethylation of a lysine residue of calmodulin. Calmodulin is a ubiquitous, calcium-dependent, eukaryotic signalling protein with a large number of interactors. The methylation state of calmodulin causes phenotypic changes in growth and developmental processes [Magnani et al., 2010]. The MRE-seq allele of *calmodulin-lysine N-methyltransferase-like* (AELG01003672.1) was expressed in our study.

Six of the eleven genes with informative hits have functions to do with social organisation in the social insects. We found the MRE-seq allele of *Ecdysone receptor* (AELG01000543.1) to be expressed. In *Drosophila melanogaster*, ecdysone receptor interacts with ecdysone to activate a series of ecdysteroid genes [Takeuchi et al., 2007]. In honeybees, *Ecdysone receptor* is expressed in the brain mushroom bodies of both workers and queens and ovaries of queens [Takeuchi et al., 2007]. Shaker (MeDIP allele expressed, AELG01001021.1) is involved in the operation of potassium ion channel. *Shaker* expression was upregulated in sterile versus reproductive honeybee workers [Cardoen et al., 2011]. The MRE-seq allele of *excitatory amino acid transporter 4-like* (AELG01000969.1) was expressed. Excitatory amino acid transporters are neurotransmitter transporters. *Excitatory amino acid transporter 3* expression was upregulated in sterile versus reproductive honeybee workers [Cardoen et al., 2011]. *Excitatory amino acid transporter 1* expression differences was also associated with worker - queen differentiation in the paper wasp *Polistes metricus* [Toth et al., 2014]. The MeDIP allele of *elongation of very long chain fatty acids protein 6-like* was expressed. The timing of the upregulation of fatty acid metabolism was found to be different in queen and worker honeybees [Li et al., 2010]. The MeDIP allele of *ras GTPase-activating*

protein nGAP-like (AELG01004618.1) was expressed in our sample . *Ras GTPase-activating protein 1* was found to be upregulated in reproductive compared to sterile honeybee workers [Cardoen et al., 2011]. It is involved in oocyte meiosis.

We found the MeDIP allele of *bicaudal D-related protein homolog* (AELG01005399.1) to be expressed in our sample. Bicaudal is involved in embryonic pattern formation in *Drosophila* [Markesich et al., 2000]. It is thought to be involved in the differentiation between soldiers and workers in the termite *Reticulitermes flavipes* [Scharf et al., 2003]. Intriguingly, *bicaudal protein D* has been shown to be methylated more in eggs than sperm in honeybees [Drewell et al., 2014]. To our knowledge this is the first link between gamete specific methylation and monoallelic methylation in the adult found in insects. This would be a key component of any methylation based imprinting system.

We then looked at these 19 genes in our RNA-seq libraries. If they are monoallelically expressed here, we would find only one allele in a given RNA-seq library. 15 of these 19 genes were confirmed to show monoallelic expression in all 30 RNA-seq libraries, see supplementary table 3. We can not rule out that all these genes just happen to be homozygote in all thirty libraries, but this result at least suggests that the finding in the monoallelic analysis can be generalized. The remaining 4 genes (AELG01000620.1, AELG01001021.1, AELG01002224.1a, AELG01002224.1b) were inconsistent; they showed expression of one allele in some *B. terrestris* workers, and expression of two alleles in other workers.

We then search more generally for allele specific expression in the 30 RNA-seq libraries. 533 SNPs were found to show allele-specific expression in $\geq$3 of the 30 RNA-seq libraries (Supplementary Table 2). Blastn against *Bombus terrestris* returned 275 hits, and blastx against *Drosophila melanogaster* returned 301 hits. 127 of the blastx results had GO annotations.

Of particular interest, allele specific expression was found to be present in the *homeobox protein hox-a3-like* gene (AELG01000285.1). *Hoxa3* is thought to be involved in the regulation of gene expression and embryonic development, and is predicted to be maternally imprinted in humans [Luedi et al., 2007, Wang, 2014]. We also found the *paternally-expressed gene 3* (*PEG3*) (AELGO1005183.1) to show allele specific expression. In mammals *PEG3* is highly expressed in the brain, ovaries, and placenta, where it is involved in the transcriptional control of foetal growth [Hiby et al., 2001, Kim et al., 1997, 2013b, Kohda et al., 2001]. *PEG3* shows allele-specific expression in several organs in humans, including the foetal brain [Murphy et al., 2001]. Furthermore, *PEG3* has previously been demonstrated to be maternally imprinted in humans and mice [Kaneko-Ishino et al., 1995, Kuroiwa et al., 1996].

We found 170 GO terms to be enriched in the regions showing allele specific expression (Fishers exact test). Interestingly, the ecdysone receptor-mediated signalling pathway was enriched, as were processes involved in gene transcription (e.g. negative regulation of DNA recombination) (Figure 2). However we would expect most of these genes not to be involved in epigenetic processes. Allele specific expression is known to be caused by a number of genetic as well as epigenetic processes [Palacios et al., 2009]. Cis-acting inherited variants, the main genetic process, in general, involve transcription factor-binding sites or less often untranslated regions altering RNA stability or microRNA binding [Farh et al., 2005]. Figure 2 shows the large number of biological functions associated with these 533 genes.

Conclusions

Our results found that genes which are both monallelically methylated and monoallelically expressed are enriched for functions to do with social organisation in the social insects. For example several genes showed roles in ecdysone pathways. In various social insect species ecdysteriods have been shown to be involved in ovary activation and dominance hierarchy in workers and queens [Geva et al., 2005]. Theory has predicted that imprinting should be associated with these types of functions [Queller, 2003]. Of particular note is *bicaudal protein D*, evidence of a possible first link between gamete specific methylation and monoallelic methylation in the adult found in insects, a key component of any putative methylation based imprinting system.

Materials and Methods

Samples

Data from thirty RNA-seq libraries were used for the allele specific expression analysis (twenty three from Riddell et al. [2014], six from Harrison et al. [2015] and a RNA-seq library from the bee also used for methylation analysis). The Riddell bees came from two colonies, one commercially reared bumblebee colony from Koppert Biological Systems U.K. and one colony from a wild caught queen from the botanic gardens, Leicester. The Harrison bees were from three commercially reared colonies obtained from Agralan Ltd. The bees used for the MeDIP-seq/MRE-seq/RNA-seq experiment and for the qPCR analysis were from separate Koppert colonies. All samples are outlined in Table 1. Colonies were fed *ad libitum* with

pollen (Percie du sert, France) and 50 % diluted glucose/fructose mix (Meliose Roquette, France). Before and during the experiments colonies were kept at 26°C and 60 % humidity in constant red light.

Next generation sequencing

MeDIP-seq, MRE-seq and RNA-seq

RNA and DNA was extracted from a single five day old whole bee (Colony K1). DNA was extracted using an ethanol precipitation method. Total RNA was extracted using Tri-reagent (Sigma-Aldrich, UK).

Three libraries were prepared from this bee by Eurofins genomics. These were MeDIP-seq and MRE-seq libraries on the DNA sample and one amplified short insert cDNA library with size of 150-400 bp using RNA. Both the MeDIP-seq and MRE-seq library preparations are based on previously published protocols [Harris et al., 2010]. MeDIP-seq uses monoclonal antibodies against 5-methylcytosine to enrich for methylated DNA independent of DNA sequence. MRE-seq enriches for unmethylated cytosines by using methylation-sensitive enzymes that cut only restriction sites with unmethylated CpGs. Each library was individually indexed. Sequencing was performed on an Illumina HiSeq2000 instrument (Illumina, Inc.) by the manufacturers protocol. Multiplexed 100 base paired-read runs were carried out yielding 9390 Mbp for the MeDIP-seq library, 11597 Mbp for the MRE-seq library and 8638 Mbp for the RNA-seq library.

Previously published RNA-seq

Full details of the RNA-seq protocols used have been published previously [Harrison et al., 2015, Riddell et al., 2014]. Briefly, for the Riddell bees, total RNA was extracted from twenty three individual homogenised abdomens using Tri-reagent (Sigma-Aldrich, UK). TruSeq RNA-seq libraries were made from the 23 samples at NBAF Edinburgh. Multiplexed 50 base single-read runs was performed on an Illumina HiSeq2000 instrument (Illumina, Inc.) by the manufacturers protocol. For the Harrison bees, total RNA was extracted from whole bodies using a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) following the manufacturers' protocol. The six libraries were sequenced as multiplexed 50 base single-read runs on an Illumina HiSeq 2500 system in rapid mode at the Edinburgh Genomics facility of the University of Edinburgh.

Monoallelic methylation and expression - Bioinformatic analysis

We searched for genes that were monoallelically methylated (present in both methylation libraries), heterozygous and monoallelically expressed (only one allele present in the RNA-seq library).

Alignment and bam refinement

mRNA reads were aligned to the *Bombus terrestris* genome assembly (AELG00000000) using Tophat [Kim et al., 2013a] and converted to bam files with Samtools [Li et al., 2009]. Reads were labelled with the AddOrReplaceReadGroups.jar utility in Picard (`http://picard.sourceforge.net/`). The MRE-seq and MeDIP-seq reads were aligned to the genome using BWA mapper [Li and Durbin, 2009]. The resultant sam alignments were soft-clipped with the CleanSam.jar utility in Picard and converted to bam format with Samtools. The Picard utility AddOrReplaceReadGroups.jar was used to label the MRE and MeDIP reads which were then locally re-aligned with GATK [DePristo et al., 2011, McKenna et al., 2010]. PCR duplicates for all bams (mRNA, MeDIP and MRE) were marked with the Picard utility Markduplicates.jar.

Identifying regions of interest and integrating data

Coverage of each data type was calculated using GATK DepthofCoverage [McKenna et al., 2010]. Only regions with a read depth of at least six in each of the libraries (RNA-seq, MeDIP-seq and MRE-seq) was used. Heterozygotes were identified using Samtools mpileup and bcftools on each data set separately [Li and Durbin, 2009] and results were merged with vcf tools [Danecek et al., 2011]. CpG islands were identified using CpG island searcher [Takai and Jones, 2002]. Regions of mRNA with overlaps of MeDIP, MRE, CpG islands and monoallelic snps were identified with custom perl scripts.

Allele specific expression - Bioinformatic analysis

We created a pipeline to search for heterozygous loci that show allele-specific expression and identify the associated enriched gene ontology (GO) terms in thirty previously published RNA-seq libraries [Harrison et al., 2015, Riddell et al., 2014]. Each RNA library was mapped to the *Bombus terrestris* reference genome (AELG00000000) [Sadd et al., 2015] using the BWA mapper [Li and Durbin, 2009].

Bcftools (version 0.1.19-44428cd), bedtools (v2.17.0), and samtools (version 0.1.19-44428cd) were used

to prepare the RNA libraries and call the SNPs, before the SNPs were filtered based on mapping quality score [Li and Durbin, 2009, Quinlan and Hall, 2010]. Only SNPs with a mapping quality score of $p < 0.05$ and a read depth of ≥ 6 were included in the analyses. The R package, QuASAR, was then used to identify genotypes (according to the Hardy-Weinberg equilibrium), and locate any allele specific expression at heterozygous sites [Harvey et al., 2014]. The loci (the snp position +/- 2900bp) identified as showing ASE in at least three of the thirty libraries, were blasted (Blastx) against *Drosophila melanogaster* proteins (non-redundant (nr) database) [Altschul et al., 1997]. The blast results were compared to the InterPro database, mapped, and annotated using Blast2Go [Gotz et al., 2008]. Fisher's exact test was implemented to identify enriched GO terms, which were then visualised using REVIGO [Supek et al., 2011]. To identify which bumblebee genes the snps were located in, the snp position +/- 25 bp was blasted (Blastn) against the *Bombus terrestris* genome [Sadd et al., 2015].

Candidate gene allele specific qPCR

DNA was extracted from four bees from three Koppert colonies using the Qiagen DNA Micro kit according to manufacturer's instructions. RNA was extracted from a sample of the head with the QIAGEN RNeasy Mini Kit according to manufacturer's instructions. cDNA was synthesized from a 8µl sample of RNA using the Tetro cDNA synthesis Kit (Bioline) as per manufacturer's instructions.

We amplified numerous fragements of the 19 candidate genes. Sanger sequencing results were analyzed using the heterozygote analysis module in Geneious version 7.3.0 to identify heterozygotic nucleotide positions. It was difficult to identify snps in exonic regions of the 19 loci, which could be ampliflied with primers of suitable efficiency. We managed to identify a suitable region in *slit homolog 2 protein-like* (AELG01000623.1 exonic region 1838-2420).

The locus was run for 3 different reactions; T allele, G allele and reference. Reference primers were designed according to Gineikiene et al. [2009]. A common reverse primer (CTGGTTCCCGTCCAATC-TAA) was used for all three reactions. A reference forward primer (CGTGTCCAGAATCGACAATG) was designed to the same target heterozygote sequence, upstream of the heterozygote nucleotide position, leaving the same common reverse primer. The reference primers measure the total expression of the gene, whereas the allele specific primers (T allele:CCAGAATCGACAATGACTCGT, G allele: CAGAATCGA-CAATGACTCGG) measure the amount of expression due to the allele. Thus the ratio between the allele specific expression and reference locus expression would be the relative expression due to the allele.

Three replicate samples were run for each reaction. All reactions were prepared by the Corbett robotics machine, in 96 well qPCR plates (Thermo Scientific, UK). The qPCR reaction mix (20µl) was composed of 1µl of diluted cDNA (50ng/µl), 1µl of forward and reverse primer (5µM/µl each, Table **??**), 10µl 2X SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich, UK) and 7µl dd$H_2$0. Samples were run in a PTC-200 MJ thermocycler. The qPCR profile was; 4 minutes at 95°C denaturation followed by 40 cycles of 30s at 95°C, 30s at the relevant annealing temperature (Table **??**) and 30s at 72°C and a final extension of 5 minutes at 72°C.

Forward primers are different, both in their terminal base (to match the snp) and in their length. It is entirely possible that they may amplify more or less efficiently even if there was no difference in amount of template [Pfaffl, 2001]. To test for this we repeated all qPCRs with genomic DNA (1µl of diluted DNA (20ng/µl) from the same bees as the template. We would expect equal amounts of each allele in the genomic DNA. We also measured efficiency of each reaction as per Liu and Saint [2002].

Median C_t was calculated for each set of three technical replicates. A measure of relative expression (ratio) was calculated for each allele in each worker bee as follows:

$$ratio_{allele} = \frac{E_{allele}^{-Ct_{allele}}}{E_{reference}^{-Ct_{reference}}} \quad (1)$$

E is the median efficiency of each primer set [Liu and Saint, 2002, Pfaffl, 2001]. All statistical analysis was carried out using R (3.1.0) [Team, 2015].

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Data accessibility

All sequence data for this study are archived at European Nucleotide Archive (ENA); Accession no. PRJEB9366 (http://www.ebi.ac.uk/ena/data/view/PRJEB9366). GO-analysis results and lists of differentially expressed transcripts are available as Supporting Information.

Author contributions

EBM designed the project. HA and DN carried out the experiments. EBM, KL, MK and ZL analysed the data. ZL and EBM wrote the initial draft. All authors were involved in the redrafting of the manuscript.

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Figures

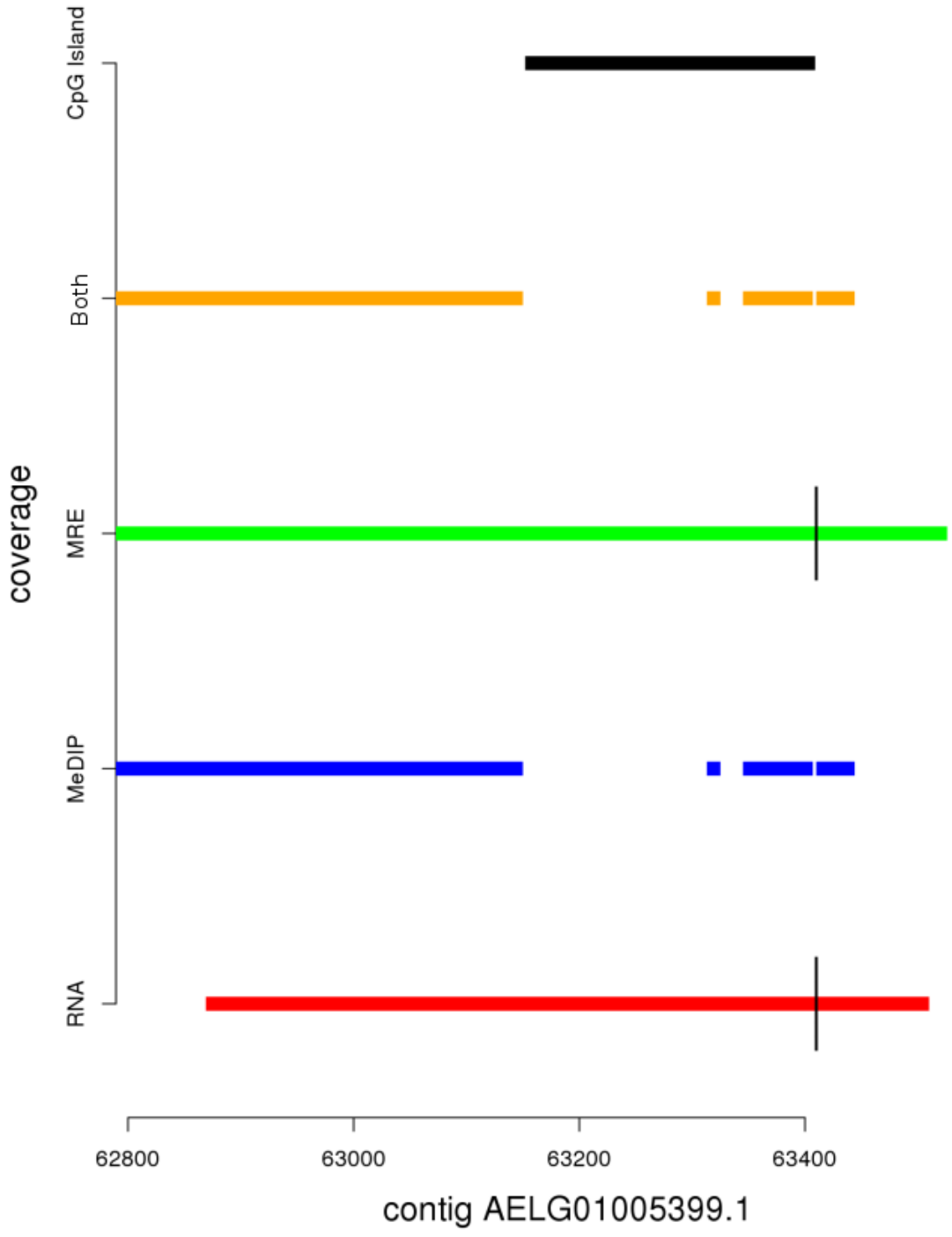

Figure 1. Coverage of three libraries for bicaudal d. Horizontal lines represent available reads for each library over this genomic range (x-axis). The vertical line shows the position of the snp and which genomic library it was found in. Similar graphs for the other 18 genes showing monoallelic methylation and expression are in the supplementary data.

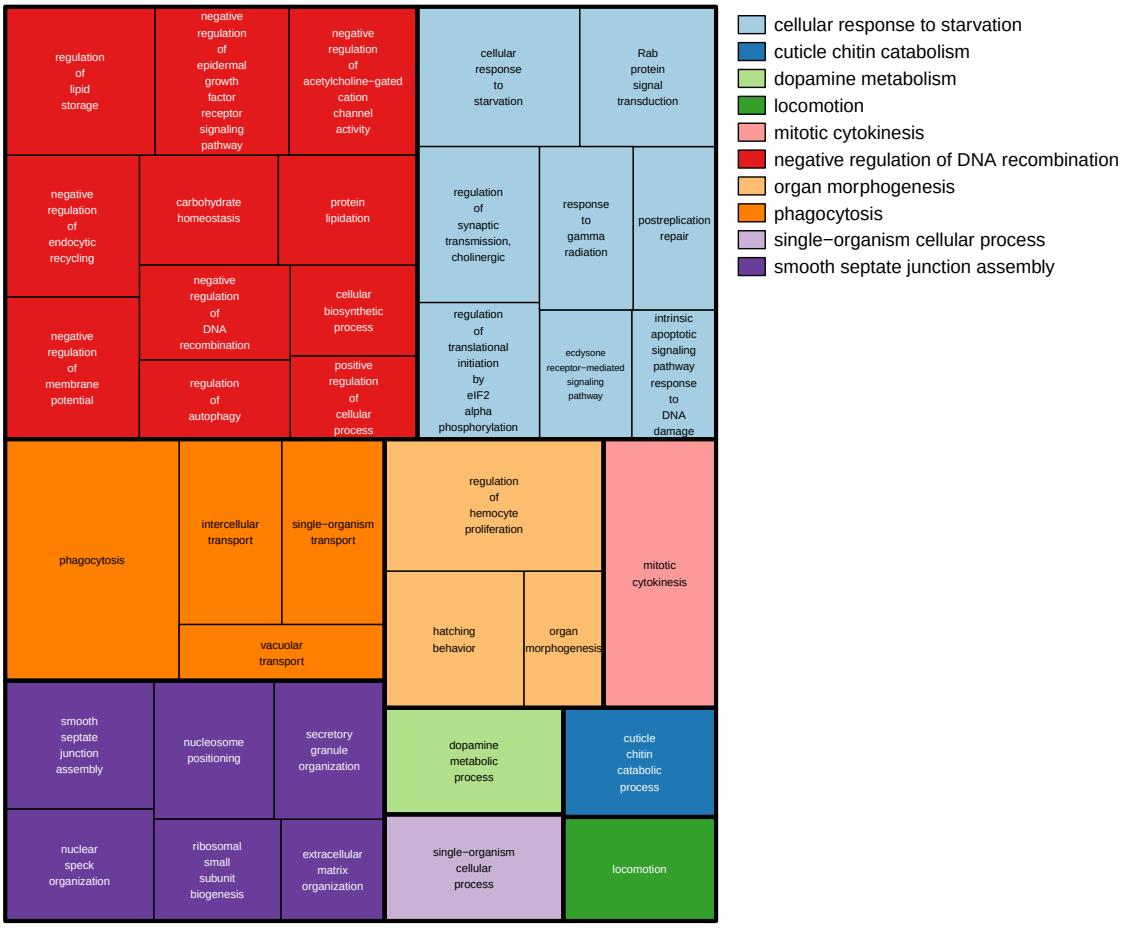

Figure 2. GO terms associated with allele specific expression. A summary of the enriched GO terms ($p < 0.05$, based on Blast2Go annotation) found for genes displaying allele specific expression. This figure was produced using Revigo

Tables

Table 1. Bees used in each experiment. K refers to Koppert, A to Agralan and Q to the wild caught Leicester queen.

Experiment	Number	Colony
Allele specific expression RNA-seq	2	A1
	2	A2
	2	A3
	1	K1
	14	K2
	9	Q1
MeDip/MRE/RNA-seq	1	K1
qPCR	2	K3
	1	K4
	1	K5

Supporting Information Legends