Sexual diversification of splicing regulation during embryonic development in honeybees (Apis mellifera), A haplodiploid system

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Abstract
The honeybee is a haplodiploid organism in which sexual development is determined by the complementary sex determiner (csd) gene and realized by sex-specific splicing processes involving the feminizer (fem) gene. We used high throughput transcriptome sequencing (RNA-Seq) to characterize the transcriptional differences between the sexes caused by the fertilization and sex determination processes in honeybee (Apis mellifera) embryos. We identified 758, 372 and 43 differentially expressed genes (DEGs) and 58, 176 and 233 differentially spliced genes (DSGs) in 10–15-h-old, 25–40-h-old and 55–70-h-old female and male embryos, respectively. The early difference in male and female embryos in response to the fertilization and non-fertilization processes resulted mainly in differential expression of genes (758 DEGs vs. 58 DSGs). In the latest sampled embryonic stage, the transcriptional differences between the sexes were dominated by alternative splicing of transcripts (43 DEGs vs. 233 DSGs). Interestingly, differentially spliced transcripts that encode RNA-binding properties were overrepresented in 55–70-h-old embryos, indicating a more diverse regulation via alternative splicing than previous work on the sex determination pathway suggested. These stage- and sex-specific transcriptome data from honeybee embryos provide a comprehensive resource for examining the roles of fertilization and sex determination in developmental programming in a haplodiploid system.

KEYWORDS
alternative transcript splicing, differential gene expression, embryo development, embryonic transcriptome, fertilization

INTRODUCTION
How sexual development is regulated is one of the major questions in biology. Despite the existence of general principles of female and male development, different species show diverse primary sex determination signals (Beye et al., 2003; Cline, 1979; Cline & Meyer, 1996; Hall et al., 2015; Krzywinska et al., 2016; Meccariello et al., 2019; Sanchez, 2010; Sharma et al., 2017). A common theme is that diverse primary signals directly or indirectly regulate the sex-specific expression of the dsx gene repeatedly via transformer (tra) homologous genes (Concha & Scott, 2009; Gempe et al., 2009; Geuverink, Kraaijeveld, et al., 2018a; Geuverink, Verhulst, et al., 2018b; Gotoh et al., 2016; Hasselmann et al., 2008; Hediger et al., 2010; Jia et al., 2016; Lagos et al., 2007; Li et al., 2013; Liu et al., 2015; Luo et al., 2017; Morita et al., 2019; Morrow et al., 2014; Pane et al., 2002; Petrella et al., 2019; Ruiz et al., 2007; Schetelig et al., 2012; Shukla & Palli, 2012; Suzuki et al., 2015; Tian & Maniatis, 1993; Verhulst et al., 2010).

Honeybees are haplodiploid organisms in which females are derived from fertilized eggs and males from unfertilized eggs, a
mechanism that is found in approximately 20% of animal species (Bull, 1983; Cook, 1993; Dzierzon, 1845). The sex determination process in honeybees relies on the complementary sex determiner gene (csd), which controls sexual fate via its heterozygous and homozygous/hemizygous genotypes. In heterozygous females, the Csd proteins direct the sex-specific splicing of feminizer (fem), the tra homolog in A. mellifera (Beye et al., 2003; Gempe et al., 2009; Hasselmann et al., 2008). The fem gene regulates female-specific splicing of dsx transcripts. In the absence of Fem protein, dsx is spliced by default (Gempe et al., 2009). Dimorphic spliced transcripts of dsx encode the transcription factors Dsx and DsxM, which differ in their C-terminal ends (Cho et al., 2007). In female honeybees, the dsx gene does not control external sexually dimorphic traits. Disruption of the zinc finger domain via CRISPR/Cas9-mediated mutation had no effect on the head differentiation of female workers, while the development of reproductive organs was masculinized (Roth et al., 2019), suggesting that at least one other gene beside dsx is required to control the development of sexual morphology. This study aims to examine the patterns of gene regulation associated with haplodiploidy and sex determination. To do so, we deeply sequenced the transcriptomes of male and female embryos using RNA-Seq at different developmental stages. We studied expression differences in haploid and diploid embryos at 10–15 h after egg laying (AEL) to identify genes that are regulated by the fertilization process. This time window includes the blastoderm stage (at 10 h AEL; Fleig & Sander, 1985; Nelson, 1915; Schnetter, 1935) and the initial phase of csd expression (12 h AEL; Beye et al., 2003). We also examined sexually regulated transcripts of embryos at 25–40 h AEL to identify genes that are controlled by the csd gene. At this developmental stage (with the end of blastoderm at 33 h AEL and gastrulation at 33–40 h AEL; Fleig & Sander, 1985, 1986), female-specific splicing of fem is induced by the csd gene (Gempe et al., 2009). Moreover, we compared male and female transcriptomes of embryos at 55–70 h AEL to identify expression differences that are controlled by the fem gene. Female-specific splicing of dsx transcripts, which is controlled by the Fem protein, is present at 60 h AEL (Tanja Gempe, personal communication; Gempe et al., 2009). This time window represents the stage during which larval body development is completed (from 55 h AEL on; Fleig & Sander, 1986).

**RESULTS**

**The number of sex-biased spliced genes increases with the progression of embryonic development**

For each of the 10–15, 25–40 and 55–70 h AEL time windows, we sequenced two replicates of pooled embryos with an average of 72 million 100 bp reads per sample (Table 1). On average, 69% of the reads could be mapped to the honeybee genome assembly v.4.5. We obtained FPKM values (fragments per kilobase of exon per million reads mapped) using Cuffdiff software (Trapnell et al., 2012). We found that FPKM values between replicates were highly correlated (Spearman’s $\rho$ 0.92–0.95; Table S1). These correlation coefficients were all well above 0.9 and in the range of correlations observed in previous studies on the honeybee transcriptome (He et al., 2019; Vleurinck et al., 2016), indicating the reliable quality of our sequencing approach.

FPKM comparisons revealed that in embryos at 10–15 h, 758 AEL genes were differentially expressed in the two sexes ($p < 0.001$. Table 2). We found 372 genes in embryos at 25–40 h AEL and 43 genes in embryos at 55–70 h AEL that were sexually differentially expressed. For a subset of DEGs, we analysed the differential expression by RT-PCR and confirmed it in 22 out of 24 (91.6%) cases (Table S2). These results demonstrate that the number of sexually dimorphically expressed genes decreases with the progression of embryonic development.

When we analysed splice junctions using Spanki software (Sturgill et al., 2013), we found 58 differentially spliced genes in males and females at 10–15 h, 176 genes at 25–40 h and 233 genes in 55–70 h AEL embryos (Table 2). This result shows that the number of sex-specific spliced genes increases with the age of the embryo and progression of development.

Next, we studied whether the same genes are expressed in a sex-biased manner in more than one of the developmental time windows. For the alternatively spliced genes in males and females, we found the largest overlap of 19 genes that were consistently sex-biased spliced in embryos at both 25–40 h AEL and 55–70 h AEL. Among those 19 genes was the fem gene that has been previously studied for sex-specific splicing (Gempe et al., 2009). The dsx gene was sex-biased spliced only 55–70 h AEL. We observed an overlap of 55 differentially expressed genes in 10–15 h- and 25–40 h-old embryos (Figure 1), which was the highest number of consistently differentially expressed transcripts in the two analysed time windows. Only one gene was differentially expressed in all three sampled stages, and no gene was consistently alternatively spliced in male and female embryos. These results indicate that there is consistent usage of a set of sexually differentially expressed genes in the first half of honeybee embryogenesis (15–40 h AEL) that is lost during later stages. In contrast, differences in gene regulation in later stages (25–75 h AEL) rely on sets of alternatively spliced genes.

Gene ontology terms related to the molecular function of nucleic acid binding are enriched in sexually regulated transcripts

We performed a Gene Ontology (GO) term enrichment analysis on the differentially regulated genes in males and females that encode open reading frames. GO molecular function terms were inferred from Drosophila melanogaster orthologues and were tested for overrepresentation utilizing DAVID online (Tables S3–S8; Huang et al., 2009). We assigned GO terms to 43 out of 58 differentially spliced genes in embryos at 10–15 h AEL and found that in this group, the molecular function of phosphatase activity (GO:0004722) was overrepresented.
For 108 of the 167 alternatively spliced genes in male and female embryos at 25–40 h AEL, we observed that the molecular functions of protein binding and phospholipid binding (GO:0005515, GO:0005543) were enriched. GO terms for 161 out of 220 sexually differentially expressed genes in 55–70 h-old embryos showed an enrichment for mRNA/nucleotide binding and actin/actin filament binding function (GO:0003729, GO:0003779, GO:0000166, GO:0051015).

### Table 1

| Condition and replicate | Number of sequenced reads | Number of mapped reads | Proportion of mapped reads |
|-------------------------|---------------------------|------------------------|---------------------------|
| M1-a                    | 74761874                  | 51516899               | 68.9%                     |
| M1-b                    | 88100392                  | 63812966               | 72.4%                     |
| M2-a                    | 71080138                  | 47779051               | 67.2%                     |
| M2-b                    | 63409444                  | 42994429               | 67.8%                     |
| M3-a                    | 64624747                  | 46872807               | 72.5%                     |
| M3-b                    | 69666200                  | 54207595               | 77.8%                     |
| F1-a                    | 73545785                  | 48017220               | 65.3%                     |
| F1-b                    | 69219183                  | 46402985               | 67.0%                     |
| F2-a                    | 86089063                  | 58358971               | 67.8%                     |
| F2-b                    | 70893558                  | 47295742               | 66.7%                     |
| F3-a                    | 68941625                  | 45300195               | 65.7%                     |
| F3-b                    | 69103047                  | 46149672               | 66.8%                     |
| Mean                    | 72452546                  | 49892378               | 68.8%                     |

Note: 1: 10–15 h-old embryos; 2: 25–40 h-old embryos; 3: 55–70 h-old embryos. a and b replicates of the same condition.
Abbreviations: F, female; M, male.

### Table 2

| Differentially expressed | 10–15 h after egg deposition | 25–40 h after egg deposition | 55–70 h after egg deposition |
|--------------------------|-----------------------------|-----------------------------|-----------------------------|
| Total                    | 758                         | 372                         | 43                          |
| Protein coding           | 739                         | 346                         | 39                          |
| ncRNA                    | 18                          | 36                          | 4                           |
| Pseudogenes              | 1                           | —                           | —                           |
| Differentially spliced   | 58                          | 176                         | 233                         |
| Protein coding           | 56                          | 167                         | 220                         |
| ncRNA                    | 2                           | 9                           | 13                          |

*aCut-off value *p* < 0.001.

### Figure 1

Comparison of differentially expressed (a) and alternatively spliced (b) genes in males and females at three embryonic stages.
For the differentially expressed genes in embryos at 10–15 h AEL, 514 out of 739 protein-encoding genes could be associated with a GO term. The molecular functions of ATP binding and protein binding were overrepresented in this set of genes (GO:0005524, GO:0005515). In embryos at 25–40 h AEL, we observed a higher abundance of differentially expressed genes with the molecular function of DNA binding and transcription regulation (GO:0043565, GO:0003700, GO:0000977). Here, for 234 out of 346 genes, a GO term could be assigned. We assigned GO terms for 21 out of 39 genes that were differentially expressed in embryos at 55–70 h AEL and found that the function of cuticula formation was overrepresented in this group (GO:0042302, GO:0008010). In general, we observed an overrepresentation of nucleotide binding functions in genes with sex-biased splicing and differential expression in the 25–40 h AEL period.

DISCUSSION

In this study, we identified genes that are differentially regulated in the sexes through differential expression and/or alternative splicing during the key embryonic processes of fertilization and sex determination in honeybees.

In the time window of 10–15 h AEL, we observed the effect of fertilization on embryonic transcription, which occurs prior to 6 h AEL during early cleavage (Fleig & Sander, 1985; Pires et al., 2016). During this stage, we mainly observed regulatory differences at the level of differential expression of transcripts (DEGs: 758 vs. DSGs: 58). Our findings imply that the fertilization process already induces substantial differences in gene expression between the sexes. The overrepresentation of the GO terms ATP binding and kinase activities in this gene set suggests that these differences may manifest in sex-biased changes in phosphorylation and dephosphorylation processes. Examples of regulators of phosphorylation with major roles in development include the Erk (extracellular signal-regulated kinase), which controls the fate and posterior identity of embryonic cells of D. melanogaster (Johnson & Toettcher, 2019), and PI 3-kinase (Class Ia, phosphoinositide 3-kinase), which regulates imaginal disc cell size, cell number and organ size in D. melanogaster (Weinkove et al., 1999).

We found that the molecular functions DNA binding and transcriptional regulation were overrepresented in the group of genes differentially expressed at 25–40 h AEL. The overrepresentation of these functions could represent genes that encode further developmental regulatory activities for sexual differentiation. These genes may operate in parallel to the sex determination pathway and may be differentially expressed in response to fertilization and nonfertilization processes.

The increase in differential splicing of genes in embryos of different sexes at 10–15 and 25–40 h AEL is consistent with the initiation of the sex determination cascade at 12 h AEL and the sex-specific splicing activity provided by the csd and fem genes (Beye et al., 2003; Gempe et al., 2009). The number of genes that are alternatively spliced is further increased in the 55–70 h AEL stage, the next embryonic stage that we examined. At this stage, we observed that 233 genes were alternatively spliced between the sexes as compared to 176 genes in 25–40 h-old embryos. Furthermore, we found that the differential expression of the genes declined in this later stage compared to that in 25-40 h-old embryos, since only 43 DEGs were detected. The observed decline in DEGs but increase in DSGs throughout embryonic development suggests that gene regulation differences in males and females are dominated by alternative splicing in late honeybee embryos. Furthermore, we found an overrepresentation of mRNA binding proteins in the group of DSGs in embryos at 55–70 h AEL, which may suggest that in addition to the identified components of the canonical sex determination pathway, fem and the csd gene, there may be other splice regulators involved in transducing and/or executing the sexual signal (Bopp et al., 2014; Gempe et al., 2009; Gempe & Beye, 2011; Hasselmann et al., 2008). From the increase in alternative splicing processes and overrepresentation of genes with RNA-binding functions with the progression of embryonic development, we conclude that the role of alternative splicing in sexual differentiation is more complex and important than previously appreciated. We identified 19 genes with consistent sex-biased splicing in embryos at 25–70 h AEL when we examined these stages in combination. These genes could play a role in the maintenance of the sexual signal or be targets of the fem gene, which is expressed and sex-specifically spliced from 33 h AEL to the pupal stage (Gempe et al., 2009; Gempe & Beye, 2011). We conclude that these 19 genes could contain new regulators that play a role in sexual differentiation.

The sex-specific transcriptome data of honeybee embryos presented here provide comprehensive information about the early regulation of embryonic sexual differentiation. Using the recently developed CRISPR/Cas9 method or transgenic methods in honeybees (Roth et al., 2019; Schulte et al., 2014), we can now systematically test these candidate genes for their functions. This will allow us to determine which of the differentially spliced and expressed genes are required for which aspects of female and male differentiation. Indeed, studies that employed a similar transcriptome comparison between males and females in Bombyx mori and Anopheles gambiae identified new functionally important regulators of sexual development (Kawamoto et al., 2015; Kiuchi et al., 2014; Krzywinska et al., 2016).

EXPERIMENTAL PROCEDURES

Collection of embryos

Haploid and diploid embryos of A. mellifera carnica were collected at the beekeeping facility of Arizona State University (ASU, USA). We used sister queens that were either inseminated by drones from a single colony or treated with CO2 to induce the laying of unfertilized eggs. To gather embryos, the queens were caged in a Jenter egg collection box (Jenter Queen Rearing Kit, Karl Jenter GmbH, Frickenhausen, Germany). After 5 or 15 h, the eggs were collected and reared in an incubator (Schulte et al., 2014). The embryos were
immediately frozen in liquid nitrogen at 10–15, 25–40 h or 55–70 AEL.

RNA isolation and sequencing

We pooled thirty 55–70 h, sixty 25–40 h and one hundred 10–15-h-old embryos derived from three different mother queens for each sex. Total RNA was isolated according to a TRizol-based protocol, followed by a further purification step using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as previously described (Vleurinck et al., 2016). Quality checks for degradation were performed on 18S and 28S ribosomal RNAs using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Library preparation and 100 bp single-end sequencing were conducted by the Biological and Medical Research Centre (BMFZ) at Heinrich-Heine University Düsseldorf, Germany. The TruSeq Stranded mRNA Kit (Illumina, San Diego, CA, USA) was used for library preparation. Sequencing of two biological replicates for each condition was performed on an Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA) was used for library preparation. Sequencing of two biological replicates for each condition was performed on an Illumina HiSeq 2500 system (Illumina). We achieved an average Phred score of 35 for all 12 samples, which was calculated using CLCbio Genomics Workbench software (v8.0.2, Qiagen). The data are accessible via the Gene Expression Omnibus under the accession number GSE159387.

Bioinformatic analysis of RNA-Seq data

We used honeybee genome assembly v.4.5 from the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/ARCHIVE/ANNOTATION_RELEASE.102/Assembled_chromosomes/seq/). The chromosomes 1–16 were concatenated and unplaced sequences as well as the mitochondrial genome were excluded (Vleurinck et al., 2016). The reads were mapped to the genome using TopHat (2.1.1) and Bowtie2 (2.2.6; Langmead, 2010; Trapnell et al., 2012). We created a de novo transcript model from our dataset using the Cufflinks package (v2.2.1; Trapnell et al., 2012), which also integrates information from the NCBI annotations (http://biomirror.aarnet.edu.au/biomirror/ncbigenomes/Apis_mellifera/GFF//ref_Amel_4.5_top_level.gff3.gz). Differentially expressed genes (DEGs) were identified using the cuffdiff command implemented in the Cufflinks package (Trapnell et al., 2012). We normalized for sequencing depth using the upper quartile counts option instead of total read counts. Details on different parameter settings can be found in Vleurinck et al. (2016). DEGs with p < 0.001 were further analysed. Sorting and visualization of data were performed with the R package CummeRbund (Trapnell et al., 2012). To verify our results, we performed RT-PCR on the transcripts of a subset of identified genes under standard conditions using total RNA from pools of 0–15, 25–40 and 55–70 h-old male and female embryos (Gempe et al., 2009). For comparison between samples, the amount of template and the number of cycles were adjusted according to the reference gene elongation factor 1-alpha. To detect differently spliced junctions, we employed Spanki software (v.0.5.0; Sturgill et al., 2013). We studied only those splice junctions that met the criteria of anchor ≥8, hamming 5’ ≥3 and entropy ≥2 and that had an intron–exon boundary of GT-AG dinucleotides (Sturgill et al., 2013). Splice junctions with a q-value < 0.01 were analysed further. Gene IDs were manually assigned to the junctions via the Integrative Genomics Viewer (Thorvaldsdottir et al., 2013) and the NCBI database to obtain a list of alternatively spliced genes (DSGs).

GO analysis

Annotated amino acid sequences of DEGs and DSGs were obtained from NCBI. Orthologues from D. melanogaster were identified with Blast2GO software (Conesa et al., 2005). GO terms were assigned to identify overrepresented molecular functions of the orthologues using the DAVID online tool (v.6.8, https://david.ncifcrf.gov/; Huang et al., 2009). Genes with no assigned GO terms either had no orthologue in D. melanogaster or an orthologue with no annotated molecular function.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data are openly available from the Gene Expression Omnibus (GEO) under the accession number GSE159387.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

Table S1. Spearman correlation of expression values between the replicates and conditions.

Table S2. Verification of DEGs with RT-PCR.

Table S3. Gene Ontology (GO) terms for molecular function of differentially spliced genes in 10-15 hold male and female embryos.

Table S4. Gene Ontology (GO) terms for molecular function of differentially spliced genes between male and female embryos 25-40 h after egg deposition.

Table S5. Gene Ontology (GO) terms for molecular function of differentially spliced genes between male and female embryos 55-70 h after egg deposition.

Table S6. Gene Ontology (GO) terms for molecular function of differentially expressed genes between male and female embryos 10-15 h after egg deposition.

Table S7. Gene Ontology (GO) terms for molecular function of differentially expressed genes between male and female embryos 25-40 h after egg deposition.

Table S8. Gene Ontology (GO) terms for molecular function of differentially expressed genes between male and female embryos 55-70 h after egg deposition.

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