1   *For Genome Research*

2

3   # Extent and complexity of RNA processing in the development

4   of honey bee queen and worker castes revealed by Nanopore

5   direct RNA sequencing

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8   Xu Jiang He[a,b], Andrew B. Barron[c], Liu Yang[d], Hu Chen[d], Yu Zhu He[a], Li Zhen

9   Zhang[a], Qiang Huang[a], Zi Long Wang[a], Xiao Bo Wu[a], Wei Yu Yan[a], Zhi Jiang Zeng[a,b1]

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11   [a]Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi

12   330045, P. R. of China

13   [b]Jiangxi province Honeybee biology and beekeeping Nanchang, Jiangxi 330045, P. R.

14   of China

15   [c]Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109,

16   Australia

17   [d]Wuhan Benagen Tech Solutions Company Limited, Wuhan, Hubei 430021, P. R. of

18   China

19

20   [1]Corresponding author: bees1965@sina.com

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22   Running title: RNA processing in honey bee caste differentiation

23

24 **Abstract**

25 The distinct honey bee (*Apis mellifera*) worker and queen castes have become a model for the

26 study of genomic mechanisms of phenotypic plasticity. Prior studies have explored

27 differences in gene expression and methylation during development of the two castes, but thus

28 far no study has performed a genome-wide analysis of differences in RNA processing. To

29 address this here we performed a Nanopore-based direct RNA sequencing with exceptionally

30 long reads to compare the mRNA transcripts between honey bee queen and workers at three

31 points during their larval development. We found thousands of significantly differentially

32 expressed isoforms (DEIs) between queen and worker larvae. Most DEIs contained

33 alternative splicing, and many of them contained at least two types of alternative splicing

34 patterns, indicating complex RNA processing in honey bee caste differentiation. We found a

35 negative correlation between poly(A) length and DEI expression, suggesting that poly(A) tails

36 participate in the regulation of isoform expression. Hundreds of isoforms uniquely expressed

37 in either queens or workers during their larval development, and isoforms were expressed at

38 different points in queen and worker larval development demonstrating a dynamic

39 relationship between isoform expression and developmental mechanisms. These findings

40 show the full complexity of RNA processing and transcript expression in honey bee

41 phenotypic plasticity.

42

43 **Keywords:** Honey bees; phenotypic plasticity; direct RNA sequencing; transcriptome

44 complexity; isoform expression

45 **Introduction**

46 Phenotypic plasticity - the capacity of divergent developmental pathways to yield more than

47 one distinct phenotypes - is one of the most interesting aspects of development, but it remains

48 poorly understood (Agrawal 2001; Borges 2005). The challenge is to understand how more

49 than one developmental path can be organized and adaptively regulated by a single genome.

50 Phenotypic plasticity is particularly dramatic in eusocial insects, and the honey bee (*Apis*

51 *mellifera*) has become a valuable system for exploring this phenomenon. Honey bee queens

52 and workers both develop from fertilized (diploid) eggs, but differences in nutrition in their

53 larval diets determines their diverging developmental trajectories that result in different

54 phenotypes (Asencot and Lensky 1984; Brouwers 1984; Maleszka 2008; Mao et al. 2015).

55 The queen is the colony's only fecund female with well-developed ovaries producing

56 1500-2000 eggs per day, whereas workers are infertile females that perform all the essential

57 tasks in the colony such as brood care, foraging, defence, construction and cleaning (Winston

58 1991).

59

60 Prior foundational studies have shown how the process of phenotypic plasticity is

61 epigenetically determined (Chen et al. 2012; Foret et al. 2012; Cameron et al. 2013; Guo et al.

62 2016; He et al. 2017; Wojciechowski et al. 2018). Studies have compared the transcriptomes

63 between honey bee queens and workers using short-length RNA sequencing (RNA-Seq)

64 (Chen et al. 2012; Cameron et al. 2013; He et al. 2017). Thousands of genes were

65 differentially expressed between queens and workers during their development (Chen et al.

66 2012; Cameron et al. 2013; He et al. 2017). Consistently a few key signalling pathways have

67 been implicated as important in queen/worker differentiation. These include target of

68 rapamycin (TOR), the fork head box O (FoxO), Notch, Wnt, insulin/insulin-like signaling (IIS)

69 pathways, mitogen-activated protein kinase (MAKP), Hippo, transforming growth factor beta

70 (TGF-beta) (Patel et al. 2007; Mutti et al. 2011a; Duncan et al. 2016; Xiao et al. 2017; Yin et

71 al. 2018; Wang et al. 2021); Key genes such as *Juvenile hormone esterase precursor (Jhe),*

72 *ecdysone receptor* (*Ecr*), *Vitellogenin (Vg)*, *Hexamerin 70b* (*Hex70b*) *major royal jelly*

73 *proteins* (*Mrjps*) also participate in the determination of queen-worker fate (Barchuk et al.

74 2007; Buttstedt et al. 2013; Mello et al. 2014). Moreover, epigeneic modifications such as

75 DNA methylation, microRNA and alternative chromatin states have been shown to be

76 involved in the regulation of honey bee queen-worker dimorphism (Foret et al. 2012; Guo et

77 al. 2016; Wojciechowski et al. 2018). The latest evidence showed that m6A methylation on

78 RNA also participates in the genomic regulation leading to queen-worker dimorphism (Wang

79 et al. 2021).

80

81 These studies have gone a long way to establish the honey bee as a model for genomic

82 analyses of phenotypic plasticity, but thus far, we know rather little about how RNA

83 processing might be involved in the process. RNA processing determines the mRNA coding

84 sequence, and can lead to different isoforms of a gene. Distinct isoforms can perform different

85 biological functions, and these can be important for animal phenotypic plasticity (Marden

86 2008). Alternative transcripts produced from the same gene can differ in the position of the

87 start site, the site of cleavage and polyadenylation, and the combination of exons spliced into

88 the mature mRNA (Parker et al. 2020). Poly(A) tails are also very important for RNA

89   processing. Length of the Poly(A) tail is correlated with translational efficiency and regulates

90   transcript stability (Workman et al. 2019; Roach et al. 2020).

91

92   Alternative splicing (AS) of genes diversifies the transcriptome and increases protein coding

93   capacity through the production of multiple distinct isoforms (Modrek and Lee 2001; Reddy

94   et al. 2013). AS is involved into many biological processes in plants and animals, and

95   particularly responses and adaptation to changing environments (Modrek and Lee 2001;

96   Reddy et al. 2013). AS of pre-mRNA has been associated with phenotypic plasticity in insects

97   such as the bumble bee *Bombus terrestris* and the pea aphid *Acyrthosiphon pisum* (Grantham

98   and Brisson 2018; Price et al. 2018). Different alternative transcripts are also involved in

99   honey bee caste differentiation, and alternative splicing plays an important role in

100   queen-worker differentiation (Aamodt 2008; Mutti et al. 2011b). These studies have focused

101   on the effects of AS on the regulation of gene expression. Thus far none have investigated

102   how AS determines isoform expression genome wide in honey bees.

103

104   So far honey bee RNA-Seq studies have been based on short-length RNA sequencing, which

105   is not an appropriate method to detect different isoforms or alternative transcripts of a gene.

106   The new direct RNA sequencing technology (DRS) based on the Oxford Nanopore

107   technology (ONT) directly sequences the native full-length mRNA molecules, which offers

108   detailed information on the mRNA and RNA modifications (Garalde et al. 2018; Harel et al.

109   2019; Workman et al. 2019; Parker et al. 2020; Roach et al. 2020; Zhang et al. 2020). It

110   avoids biases from reverse transcription or amplification and yields full-length, strand-special

111   mRNA (Garalde et al. 2018; Harel et al. 2019; Zhang et al. 2020). This method allows a

112   genome wide investigation of RNA processing [e.g. alternative splicing and poly(A) tails] and

113   modifications (e.g. m6A).

114

115   Our objective in this study was to deploy this DRS technology to examine differences in RNA

116   processing between the worker and queen honey bee castes. This allows us to investigate

117   fundamental questions on caste-specific transcriptome patterns in honey bees: how isoforms

118   are formed and expressed differentially in queens and workers, and the extent and complexity

119   of the RNA processing in honey bee development.

120

121   We sampled honey bee queens and workers at different points in their larval development to

122   assess the process of developmental canalization into the different phenotypes. Our results

123   reveal extremely prevalent and highly complex differences in RNA processing between

124   workers and queens. These differences changed across larval development showing a

125   complex dynamical interaction between the epigenomic programs and the developmental

126   pathways

127

128   **Results**

129   *Quality of direct RNA sequencing data*

130   Twelve libraries were generated and sequenced by ONT direct RNA sequencing. The average

131   number of clean reads of each sample was 5,416,798 (Table 1). The average N50 read length

132   was 1296 bp, and the average read quality was 10.63 (Table 1) indicating a high integrity of

133 the sequenced RNAs. On average, 98.13% clean reads were successfully mapped to the *Apis mellifera* reference genome (*Amel HAv 3.1*) (Table 1). The

134 Pearson correlation coefficients between two biological replicates of each group were all over 0.85 (Fig.S1) and the sample cluster tree also showed that two

135 biological replicates of each group were clustered together (Fig.S2), reflecting a good repeatability of biological replicates.

136 Table 1 Overview of direct RNA sequencing

Samples	2Q-1	2Q-2	2W-1	2W-2	4Q-1	4Q-2	4W-1	4W-2	6Q-1	6Q-2	6W-1	6W-2	Average
Clean read numbers	7,268,687	5,793,325	6,113,875	6,093,102	6,067,308	6,945,178	6,207,113	3,807,886	5,039,594	2,057,391	5,910,366	3,697,756	5,416,798
Average length	1061.94	997.4	1001.55	1022.06	1007.76	998.23	967.9	1027.53	1036.56	1069.02	1027.87	976.65	1,016
N50 length	1356	1275	1285	1301	1295	1277	1242	1315	1338	1283	1306	1286	1,296
Max read length	23283	16804	16550	17946	29881	19966	16391	19519	17266	17829	13661	15496	18,716
Average read quality	10.71	10.61	10.75	10.71	10.26	10.53	10.66	10.62	10.77	10.68	10.66	10.64	10.63
Alignment % identity	98.80	98.35	98.42	98.46	98.23	98.63	98.19	98.38	96.85	98.40	98.71	96.19	98.13
Uni mapped genes	8,781	8,690	8,658	8,658	8,431	8,656	8,411	8,234	8,666	7,741	8,539	8,644	8,509
Isoforms	24,058	22,978	22,853	22,939	21,746	23,018	20,498	19,790	22,365	16,487	20,885	20,714	21,527

137

138   *Significantly differentially expressed isoforms (DEIs) and genes (DEGs) identified in*

139   *queen-worker comparisons*

140   Our results showed genome wide differentially expressed isoforms in honey bee caste

141   differentiation. We identified 662, 1855 and 1042 DEIs in 2d, 4d and 6d queen-worker larval

142   comparisons respectively, which were notably more than the DEGs (281, 1369 and 645

143   respectively, see Fig.1A, detailed information see Table S1-6). To compare the differences

144   between DEIs and DEGs, we firstly mapped DEIs to reference genes (DEIGs) and showed

145   that only 271 (34.72%), 977 (53.45%) and 410 (38.32%) genes overlapped between DEIGs

146   and DEGs in 2d, 4d and 6d queen-worker comparisons respectively (Fig.1B,C and D). This

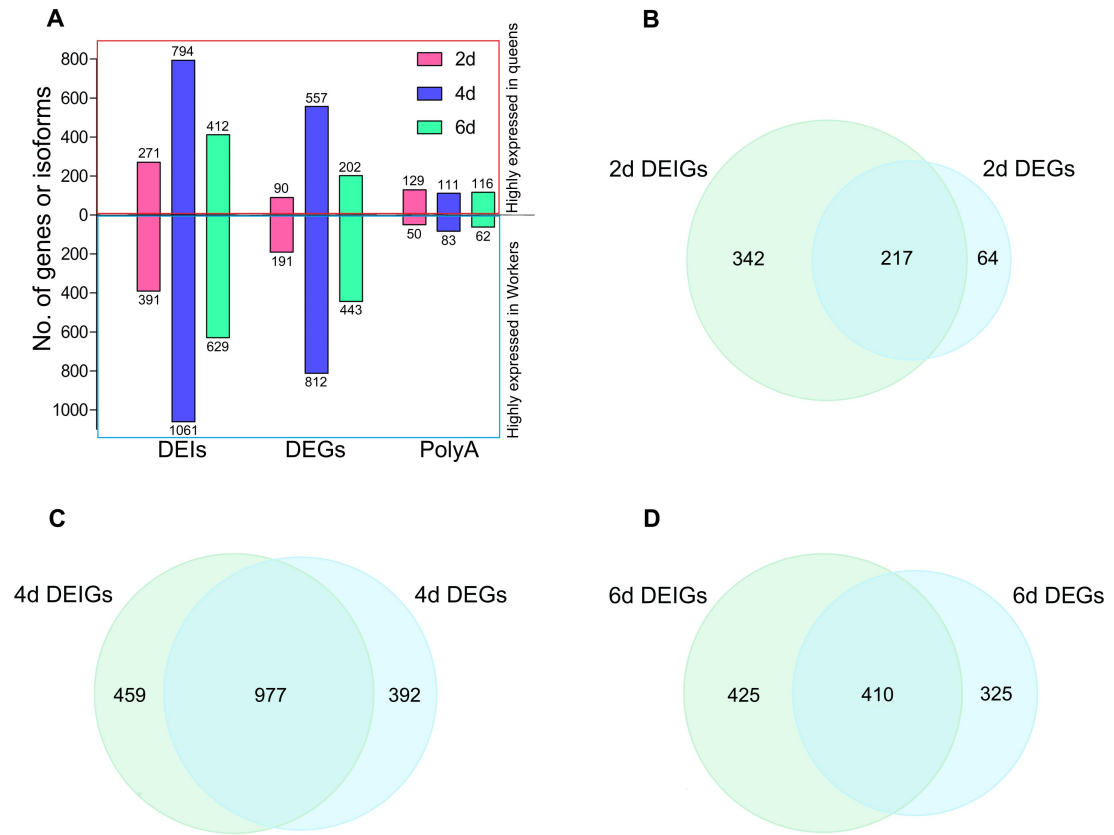147   shows a considerable difference between DEIs and DEGs in queen-worker differentiation.

148

149   Fig. 1

150   We identified DEIs (24, 54, 48 in 2d, 4d and 6d comparisons respectively) that are involved in

151   several key KEGG signaling pathways for honey bee caste differentiation, such as mTOR,

152   Notch, FoxO, Wnt, IIS, Hippo, TGF-beta and MAPK (see Table S1-3). The number of DEGs

153   enriched in these signal pathways were 7, 34, 33 in 2d, 4d and 6d respectively (Table S4-6).

154   Other DEIs mapped to key genes such as *Jhe*, *Mrjps*, *Ecr*, *Vg* and *Hex70b* (see Table S1-3).

155   The number of isoforms with significantly different poly(A) length in 2d, 4d and 6d

156   comparisons showed a negative trend as DEIs and DEGs, with more in queens (Fig.1A,

157   detailed information see Table S7-9) and less in workers.

158

159   DEIs and DEGs were enriched in five key KEGG signaling pathways (mTOR, Notch, FoxO,

160   Wnt, IIS) which have been previous shown to be participating in of queen-worker

161   differentiation. DEIs mapped to 35 enzymes in these five pathways, whereas DEG mapped

162   onto just 17 enzymes (Fig.2A). In detail, 23 enzymes were uniquely mapped by DEIs whereas

163   only 5 were uniquely mapped by DEGs. 12 enzymes mapped to both DEI and DEG (Fig.2A).

164   We also provided detailed information about the expression of DEIs and DEGs enriched in

165   these five key pathways (Fig.2B). Clearly, more isoforms were significantly differentially

166   expressed in these five key pathways compared to DEGs, and the differentially expressed

167   isoforms of each DEIG are presented in Fig.2B.

168

169   These findings suggest that caste-specific isoform expression provided more accurate and

170   detailed information of the real transcriptome differences between honey bee queen and

171   worker castes than measurements of differences in gene expression.

172

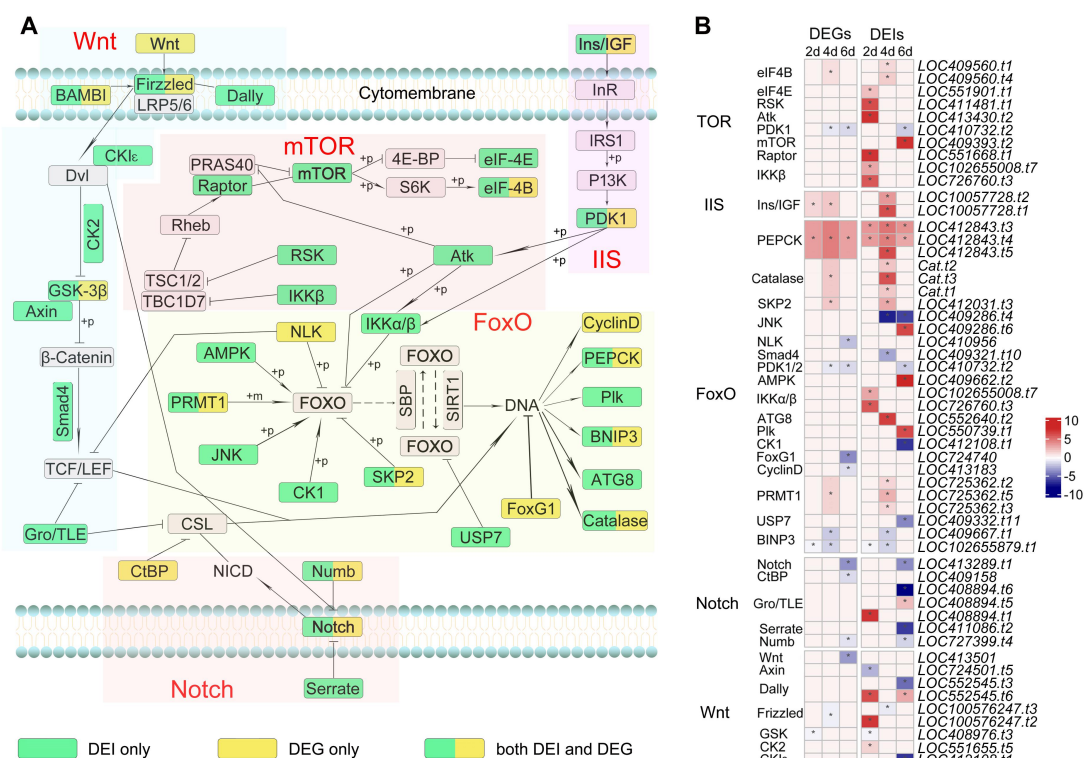173   Fig.2

Uniquely expressed isoforms between queens and workers

We identified 187, 357 and 364 uniquely expressed isoforms in queen or worker larvae at 2d, 4d and 6d age, with more occurring in queens than workers (Fig. 3A). The number of uniquely expressed isoforms in queens reached a maximum at the 4d stage whereas in workers it reached a maximum in the 6d sample. This could suggest that the queen developmental pathway diverges more quickly and at an early larval stage while the developmental pathway of workers diverges slightly later.

Uniquely expressed isoforms were enriched into eight key KEGG signaling pathways such as mTOR and IIS for queen-worker differentiation as well as some key genes such as *DNA methyltransferase 3 (Dnmt3)* and *Ecr* (Fig.3, details see Table S10-12). This indicated that

185   there are isoforms which are likely to be involved in queen-worker differentiation that are

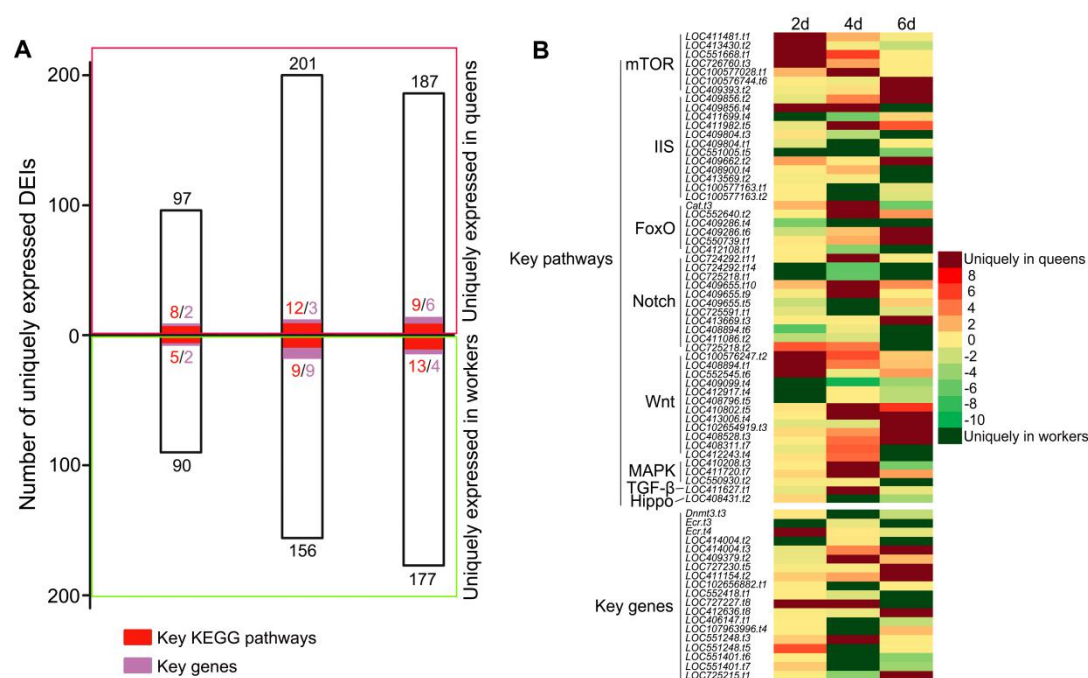186   uniquely expressed in queens or workers during their development.

187

188   Fig. 3

189   *DEIs and alternative splicing*

190   We identified 21,574 isoforms in each sample on average, which was three times more than

191   the number of protein-coding genes (on average 8509) (Table 1). This suggested that the

192   honey bee genome produces various different isoforms from one gene. We therefore detected

193   AS events in the DEIs. The majority of DEIs (73.26%, 67.98% and 63.53% of 2d, 4d and 6d

194   DEIs) contained AS events, and most of them (71.13%, 66.16% and 65.56% in 2d, 4d and 6d)

195   had at least two types of AS in a single isoform. A combination of RI, A5 and A3 in a single

196   DEI was the most common type (Fig.4). This indicates that the formation of transcripts in

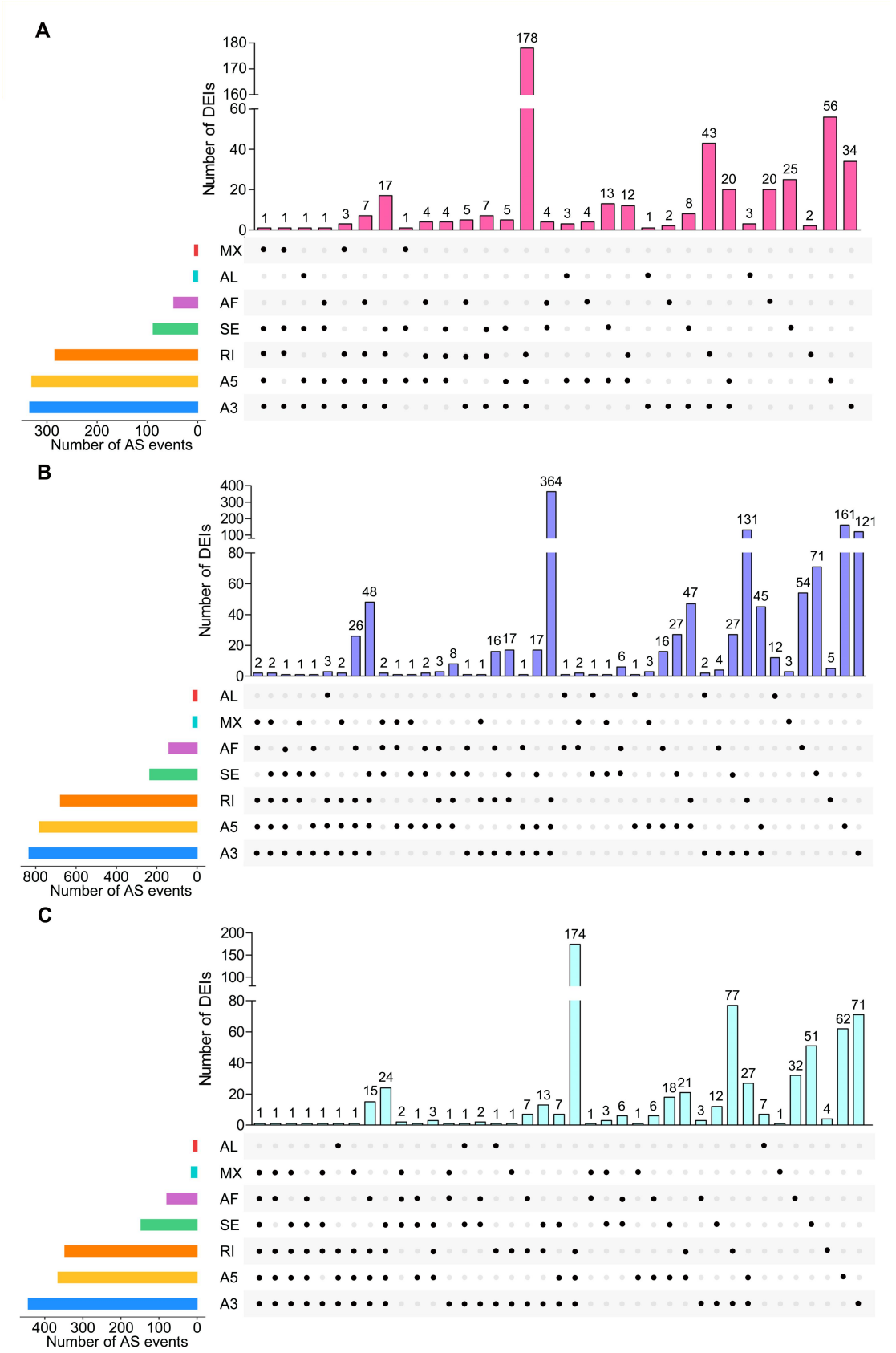197   honey bee development is more complex than previous known.

Fig. 4

201   Of these DEIs, 9.82% (65), 5.71% (106), 9.50% (99) of DEIs from the 2d, 4d and 6d

202   comparisons contained significantly different AS events when comparing workers and queens

203   (Fig. S3, Table S13-15). Similarly, many of them (46.15%, 42.45% and 45.45% in 2d, 4d and

204   6d comparisons respectively) contained at least two types of significantly different AS events

205   (Fig. S4, Table S13-15). Consequently, a part of DEIs were significantly differentially spliced.

206

207   *Correlation between poly(A) length and expression of DEIs and DEGs*

208   We showed a negative correlation between isoform expression and their poly(A) tail length

209   (Fig. S5). Expression of DEIs (Fig.5) and DEGs (Fig.S6) was also negatively correlated with

210   their poly(A) lengths. It is believed that poly(A) tails are involved in the regulation of isoform

211   expression.

212

213   *Correlation of isoform expression and poly(A) lengths for two key genes*

214   Two key genes which participate in queen-worker differentiation are *Jhe* and *Ecr*. These were

215   selected as examples to show the correlation between isoform expression and poly(A) tails.

216   *Jhe* had only one isoform, and was negatively correlated with its poly(A) lengths (Fig. 6A).

217   The *Ecr* gene had 4 isoforms. We selected the most differentially expressed isoform (*Ecr.t4*)

218   to measure the correlation between its expression and poly(A) length. Similarly, the

219   expression of *Ecr.t4* isoform was also negatively correlated with their poly(A) lengths (Fig.

220   6B).

221

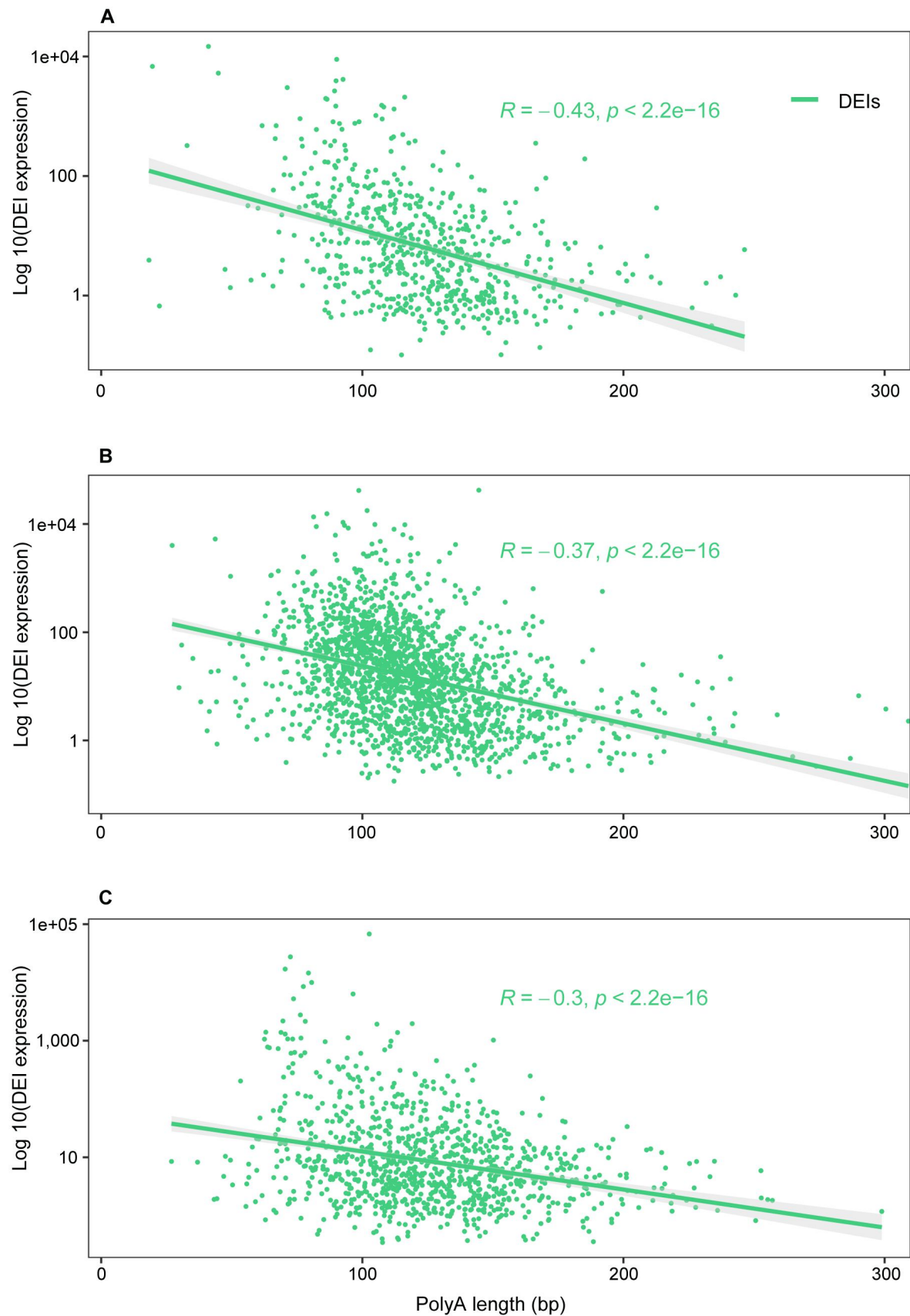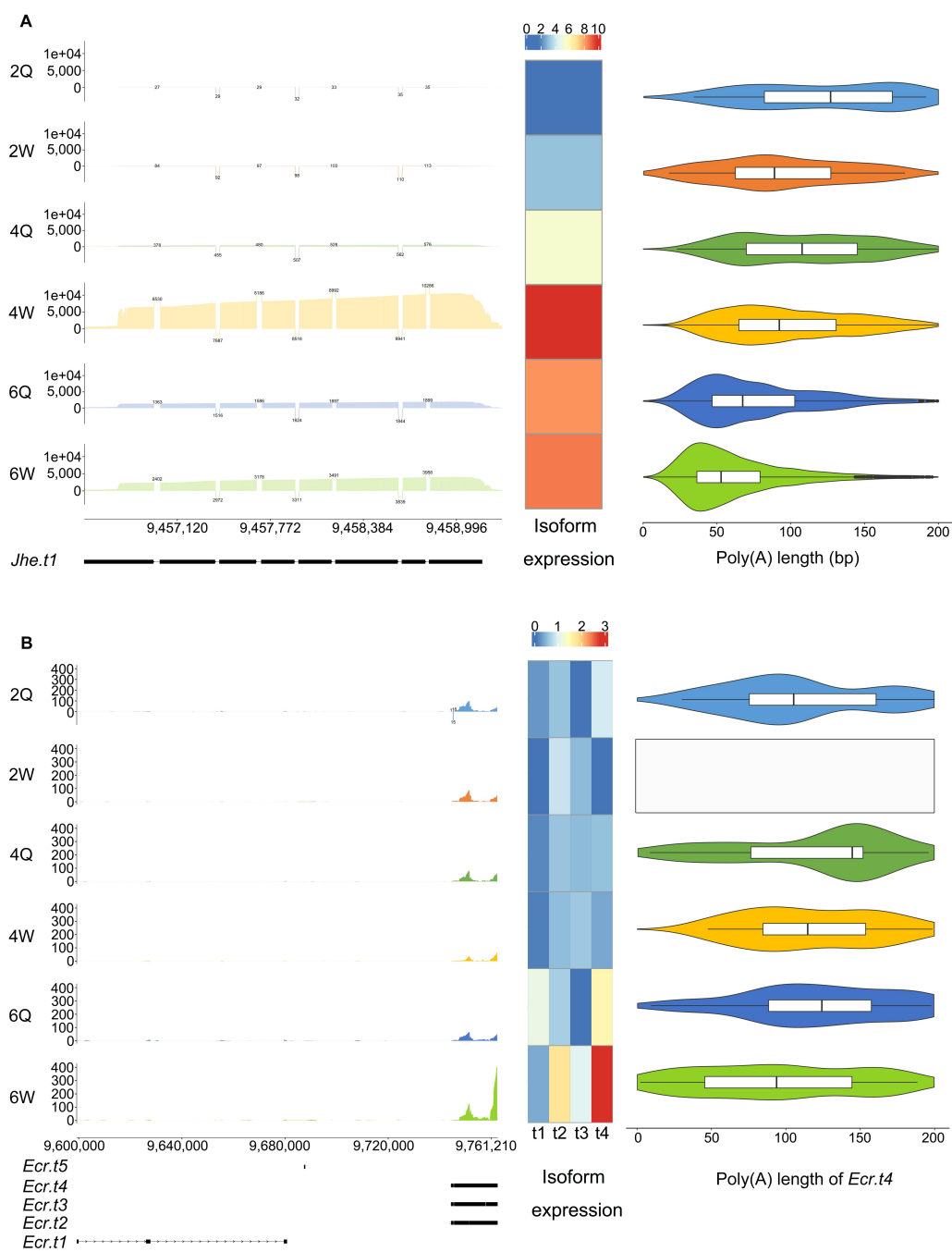222   Fig. 5

Fig. 6

Discussion

RNA processing act as a major driver of animal phenotypic plasticity (Gingeras 2007), but the complexity and characteristics of RNA processing underlying this phenotypic variability is not well understood. The present study has shown the extent of RNA processing in honey bee

229   queen-worker differentiation. Differences in isoform expression revealed the true extent of the

230   differences in the transcriptome between queens and workers during their development,

231   providing more detailed and accurate information on mRNA expression compared to

232   measures of gene expression. We also showed the complexity of alternative splicing patterns

233   in queen-worker differentiation. Most DEIs were formed by more than one type of alternative

234   splicing. Poly(A) length negatively correlated with DEIs expression demonstrating another

235   part of the genomic mechanisms of honey bee caste differentiation.

236

237   Thousands of DEIs were identified between queens and workers, which were notably more

238   than DEGs (Fig.1A). Many genes were not differentially expressed, but different isoforms

239   from these genes were significantly differentially expressed (Fig. 1B-D, Table S1-3),

240   demonstrating how DRS gives a very different perception of the transcriptome differences

241   between developing worker and queen honey bees. DEIs enriched into more enzymes of five

242   key KEGG signaling pathways such as mTOR and IIS than DEGs (Fig 2). The mTOR

243   signaling pathway is the central component of a conserved eukaryotic signaling pathway,

244   regulating cell and organismal growth in response to nutrient status and has been shown to be

245   important for determining the queen-worker developmental paths (Colombani et al. 2003;

246   Oldham and Hafen 2003; Patel et al. 2007). The IIS and Notch signaling pathway have been

247   shown to be involved in the development of honey bee ovaries (Mutti et al. 2011a; Duncan et

248   al. 2016). More DEIs were identified in these key pathways than DEGs, indicating that DEIs

249   could provide more nuanced differences of mRNA expression between honey bee queens and

250   workers during their development. Therefore, compared to DEGs that conceals a more

251 nuanced picture of transcription differences, DEIs show the true nature of transcriptome

252 differences between queens and workers. Consequently, DEIs identified in the present study

253 revealed a more informative knowledge and precise difference in the transcriptome

254 expression of honey bee caste differentiation compared to previous RNA-Seq results (Chen et

255 al. 2012; Cameron et al. 2013; He et al. 2017; Yin et al. 2018).

256

257 Hundreds of uniquely expressed isoforms were identified in queens or workers. Many

258 uniquely expressed isoforms were enriched in mTOR, IIS, Notch, FoxO etc eight key KEGG

259 signaling pathways (Fig.3). Some uniquely expressed isoforms mapped to key genes such as

260 DNA methyltransferase 3 (*Dnmt3*) and *Ecr* genes (Fig.3). *Dnmt3* is a key driver of epigenetic

261 global reprogramming controlling honey bee queen-worker fate (Kucharski et al. 2008). Here

262 we showed one isoform of *Dnmt3* (*Dnmt3.t3*) was uniquely expressed in 4d worker larvae. It

263 is possible that this isoform of *Dnmt3* in worker larvae modifies the DNA methylation status

264 and helps to canalize the worker developmental pathway. For the *Ecr* gene, 2d worker

265 uniquely expressed *Ecr.t3* whereas 2d queen larvae uniquely expressed *Ecr. t4*. Different

266 isoforms from the same gene were expressed in queens and workers, revealing the complexity

267 of of RNA processing in the honey bee queen-worker differentiation. We have documented

268 many genes where, isoform expression was not consistently biased toward either queens or

269 workers throughout all stages of larval development. In some cases, an isoform was uniquely

270 expressed in either queens or workers in one larval stage, but at other developmental stages

271 the isoform occurred in both workers and queens (Fig. 3B). Isoforms like LOC409856.t4 and

272 LOC727227.t8 uniquely expressed in queens at 2d and 4d stages but oppositely expressed in

273 workers at 6d stage (Fig. 3B). This illustrates the dynamic relationship between RNA

274 processing and the developmental process. A simple model might imagine different isoforms

275 of a gene specific to either the queen or worker developmental pathway. Instead we see

276 isoforms featuring in both pathways, but at different stages.

277

278 The number of uniquely expressed isoforms also showed a different trend in queens and

279 workers. It reached a maximum in queens at the 4d larval stage but it peaked later in workers

280 (Fig. 3A). These different trends correlate with the known faster development of queens

281 (Winston 1991) and suggest the queens developmental pathway differentiates at an earlier

282 larvae stage than the worker developmental pathway. Queen larvae at early stage receive

283 significantly more food than worker larvae, and the nutrient contents in royal jelly also differ

284 from worker jelly in terms of sugar, vitamins, proteins, acids, microRNA (Asencot and

285 Lensky 1984; Brouwers 1984; Maleszka 2008; Mao et al. 2015). In Fig. 3B, most isoforms

286 enriched in mTOR signaling pathway which is a signaling pathway in response to nutrient

287 status (Colombani et al. 2003; Oldham and Hafen 2003) were uniquely expressed in queens at

288 2d stage and its number decreased at 4d and 6d stages, which supports our speculation.

289

290 We detected on average 21,574 isoforms from 8509 genes in each sample (Table 1). This is

291 consistent with a previous study that 60.3% of honey bee multi-exon genes are alternatively

292 spliced (Li et al. 2013). The majority of DEIs, contained AS events and over 65% of them had

293 at least two types of AS patterns in a single isoform (Fig. 4). Some DEIs contained more than

294 five different AS types (Fig. 4). A few DEIs were significantly differently spliced between

295   queens and workers by multiple AS patterns (Fig. S3, table S13-15). It means several distinct

296   AS types together contributed to the construction of a single isoform (Fig.4), revealing an

297   extremely complex splicing system in honeybee caste differentiation.

298

299   Poly(A) tails are a regulator of translation and transcript stability (Lim et al. 2016; Tudek et al.

300   2018; Woo et al. 2018). Poly(A) tail length is not stable but dynamic and condition-dependent

301   (Lim et al. 2016; Woo et al. 2018). This study showed that poly(A) tail lengths were strongly

302   and negatively correlated with expression of isoforms in honey bees (Fig. 5). The negative

303   correlation between isoform expression and poly(A) length has been shown in the regulation

304   of *Caenorhabditis elegans* development (Roach et al. 2020). Here we showed that poly(A)

305   tails also participate in the regulatiion of isoform expression, and therefore contributes to

306   complexity of RNA processing in honeybee queen-worker differentiation.

307

308   In summary, RNA processing plays an important role in shaping honey bee phenotypic

309   plasticity, but investigations of gene expression by short-length RNA-Seq fail to reveal the

310   full complexities of   RNA processing (Chen et al. 2012; Cameron et al. 2013; He et al. 2017;

311   Yin et al. 2018). By using DRS, this study has shown the extent of differential isoform

312   expression between workers and queens, complexities of transcript splicing, and

313   polyadenylation. It provides a more detailed understanding of the molecular mechanisms

314   underlying the divergence of the queen and worker developmental paths. It also contributes to

315   our understanding of the extent and complexity of RNA processing in animal phenotypic

316   plasticity.

317

Material and methods

Insects

320   Three healthy honey bee colonies (*Apis mellifera*) each with a mated queen were used for this

321   study. Each colony had ten frames with approximately 35,000 bees. These colonies were

322   maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang,

323   China, according to the standard beekeeping techniques.

324

325   The mother queen was caged onto a plastic frame (Pan et al. 2013) to lay eggs into worker

326   cells for 6 hours. Afterwards, half of these eggs were removed into commercial plastic queen

327   cells to rear queens before hatching. Queen and worker larvae at 48 hrs (2 day), 96hrs (4 day)

328   and 144 hrs (6 day) after hatching were harvested and were flash-frozen in liquid nitrogen. In

329   the DRS experiment, for the 2dQL or 2dWL, larvae were very small, therefore we collected

330   20 2d queen larvae and mixed them into one sample. The 4d and 6d larvae were large enough,

331   and one sample needed only one larva. Each group had two replicates from two colonies,

332   therefore in total 12 samples were collected. Larval samples for short-length RNA-Seq were

333   sampled the same as DRS, but with twice the number of larvae in each sample. We had three

334   replicates for each of these 6 groups: 18 samples in total. Within a colony queens and workers

335   developed from a same mother queen and eggs were laid at the same time. All sampled eggs

336   were reared in a same colony. We compared queen and worker larvae sampled at the same

337   time point: 2dQL vs 2d WL, 4dQLvs 4dWL, and 6dQL vs 6dWL.

338

339 *RNA preparation*

340 Total RNA of 2nd, 4th and 6th instars of queens and workers as extracted in accordance with

341 the standard protocol of the of the Total RNA Kit I (R6834, Omega Bio-tek), respectively.

342 Each sample contained 30 μg total RNA for DRS. RNA was cleaned and concentrated by

343 NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490S) according to the

344 manufacturer's instructions. From each sample we extracted 1 μg of total RNA for the RNA

345 integrity and concentration measurement using Nanodrop (Thermo Fisher Scientific).

346

347 *Direct RNA sequencing*

348 Prepared RNA of each sample was used for a DRS library preparation using the Oxford

349 Nanopore DRS protocol (SQK-RNA002, Oxford Nanopore Technologies). For reversed

350 connector connection, 9 μL prepared RNA, 3μL NEBNext Quick Ligation Reaction

351 Buffer(NEB), 1 μL RT Adapter (RTA)(SQK-RNA002) and 2 μL T4 DNA Ligase (NEB) were

352 mixed together and incubated under 25 ℃ for 10 min. Afterwards, 8 μL 5x first-strand buffer

353 (NEB), 2 μL 10 mM dNTPs (NEB), 9 μL Nuclease-free water, 4 μL 0.1 M DTT (Thermo

354 Fisher) and 2 μL SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044)

355 were added into the above 15 μL reaction system and incubated under 50 ℃ for 50 min then

356 70 ℃ for 10min. Reverse-transcribed mRNA was purified with 1.8*Agencourt RNAClean XP

357 beads and washed with 23ul Nuclease-free water, and subsequently sequencing adapters were

358 added using 8 μL NEBNext Quick Ligation Reaction Buffer, 6 μL RNA Adapter (RMX) and

359 3 μL T4 DNA Ligase. The mix was purified and washed again as above, and 75 μL RRB

360 （SQK-RNA002）were added with 35 μL Nuclease-free water. This final reaction system was

361   loaded into Nanopore R9.4 sequencing micro-array and sequenced for 48-72 hrs using

362   PromethION sequencer (Oxford Nanopore Technologies). This was performed by Wuhan

363   Benagen Tech Solutions Company Limited.

364

365   *Preprocessing and alignments*

366   To assess the read quality, raw reads were base called using Guppy software (version 3.2.6)

367   (Fesenko and Knyazev 2020) and adapter sequences were trimmed by Guppy as well. Low

368   quality reads (Q-value < 7) and short-length reads (<50 bp) were filtered by Nanofilt (version

369   2.7.1) (Coster et al. 2018). The remaining clean reads were corrected using the Fclmr2

370   (version 0.1.2) with our short-length RNA-Seq data (Wang et al. 2018). Afterwards, clean

371   reads from each library were aligned to the genome of honey bees *Apis mellifera* (*Amel

372   HAv3.1*) using Minimap2 (version 2.17-r941) (Li 2018). The alignment ratio of clean reads to

373   the honey bee reference genes were calculated using Samtools (version 1.10) (Li et al. 2009)

374   and showed in Table 1. After clearing redundant sequences using Flair collapse (version 1.5.0)

375   (Tang et al. 2020) and Stringtie (version 2.1.4) (Pertea et al. 2015), the clean reads from each

376   library were merged by Gffcompare (version 0.12.1) (Pertea and Pertea 2020) to obtain the

377   final isoforms.

378

379   *DEG and DEI calculation*

380   Isoform expression was annotated to the honey bee reference genes and quantified using the

381   selective-alignment-base model in Salmon software (version 1.4.0) (Patro et al. 2017). Gene

382   expression was measured according to standard methods as in He et al (He et al. 2017) using

383   DRS raw data rather than RNA-Seq data. Significantly differentially expressed genes (DEGs)

384   and isoforms (DEIs) between queen and worker larvae were identified as those with p-values

385   <0.05 and log2 Fold change values > 1.5 using DESeq2 (version 1.26.0) (Love et al. 2014).To

386   compare the expression level of genes/isoforms, we computed TPM values (transcripts per

387   kilobase of exon model per million mapped reads) based on reads counts as expression. To

388   compare the DEGs and DEIs, all DEIs were referenced to the genes to obtain Differentially

389   Expressed Isoform of Genes (DEIGs). Sequences of the DEGs and DEIGs were blasted to the

390   Swiss-Prot database, non-redundant protein (Nr) and non-redundant nucleotide sequence (Nt)

391   database with a cut-off E-value of 10^{-5}.

392

393   *KEGG pathway enrichment analysis*

394   Five KEGG pathways [TOR, FoxO, Notch, Wnt and IIS] which have been repeatedly

395   associated with honey bee queen-worker differentiation (Patel et al. 2007; Mutti et al. 2011a;

396   Duncan et al. 2016; Xiao et al. 2017; Yin et al. 2018; Wang et al. 2021) were selected and

397   combined into a pathway net according to KEGG pathway database and a previous study

398   (Wang et al. 2021). The statistical enrichment of DEIGs and DEGs in these KEGG pathways

399   were analyzed using a hypergeometric test (Q-value < 0.05) using the KOBAS 2.0 software

400   (Xie et al. 2011). Results were shown in Fig. 3A. Expression of DEIs and genes in these five

401   pathways were also presented in Fig.3B.

402

403   *Alternative splicing identification*

404   The AS events of each comparison were analyzed using SUPPA2 (version 2.3) (Trincado et al.

405   2018). AS events were divided into seven patterns including 3'splice site (A3), 5'splice site

406   (A5), First exon (AF), Last exon (AL), Retained intron (RI), Skipping exon (SE) and

407   Mutually exclusive exon (MX). The PSI values of each AS events in DEIs were calculated

408   after filtering percent spliced in index (PSI) values with 'not available' (NA) or '0' (Trincado

409   et al. 2018). Significant differences in AS events were identified as its dPSI-value >0 and

410   p-value <0.05.

411

412   *Poly(A) length estimation and correlation with expression of genes and isoforms*

413   Poly(A) lengths of each read were calculated by the Nanopolish software (version 0.13.2)

414   (Coster et al. 2018). The 3' poly(A) tail lengths of each read were directly identified using the

415   ionic current signals (Workman et al. 2019). A previous study showed that poly(A) lengths

416   were negatively correlated with isoform expression, therefore, the median of poly(A) length

417   of each isoform was used for correlation and regression analysis with their TPM values

418   (results were shown in Fig.S5) in R package (version 4.0.3). We also evaluated the correlation

419   between poly(A) tail lengths and DEIs or DEGs.

420

421   For this we used median of poly(A) length and TPM or log2Fold change values of DEIs or

422   DEGs for correlation and regression analysis as above. Two key genes (*Jhe* and *Ecr)* for the

423   determination of queen-worker differentiation were selected as an example to show the

424   correlation between expression of isoforms and poly(A) lengths. Results were shown in Fig.

425   5.

426

427 *Short-length RNA sequencing*

428 Total RNA of each sample was extracted using TRIzol reagent (Tiangen, Beijing). The

429 concentration and integrity of RNA were assessed using Qubit 2.0 Fluorometer and Agilent

430 2100 bioanalyzer. Each group of queen and worker larvae had 3 biological replicates.

431

432 Total mRNA of each sample was enriched using oligo (dT) magnetic beads. First-strand

433 cDNA was synthesized by random hexamers, and the second-strand cDNA was synthesized in

434 DNA polymerase I system using dNTPs and RNaseH. Afterwards, cDNA was subjected to

435 end repair and performed with poly(A) tail and ligation sequencing adapter. 200-350 bp

436 cDNA were selected by AMPure XP beads, and then were PCR amplified and purified by

437 AMPure XP beads. Library quality was evaluated using Agilent 2100 bioanalyzer and

438 qRT-PCR. Totally 18 libraries were sequenced by an Illumina NovaSeq 6000 platform. This

439 was performed by Wuhan Benagen Tech Solutions Company Limited also.

440

441 **Data access**

442 All data are available from the corresponding author upon request. The DRS sequence data

443 for all 12 libraries and the fastq sequences of RNA-Seq of 18 libraries have been deposited in

444 the NCBI database under the accession: NCBI Bioproject: PRJNA748829.

445

446 **Competing interest statement**

447 The authors declare no competing interest. All authors were involved in the preparation of the

448 final manuscript.

449

461

462   **References**

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600

601   **Figure legends**

602   **Fig.1A:** Numbers of differentially expressed isoforms (DEIs) and genes (DEGs), different

603   poly(A)-length related isforms in three queen-worker larval comparisons (2d, 4d and 6d). The

604   up bars are DEIs, DEGs and different poly(A) isoforms highly expressed in queens and the

605   down bars are that highly expressed in workers. **B:** The venn diagram of DEIGs (DEI mapped

606   genes) and DEGs from 2d queen-worker comparison. **C:** The venn diagram of DEIGs and

607   DEGs from 4d queen-worker comparison. **D:** The venn diagram of DEIGs and DEGs from 6d

608   queen-worker comparison.

609

610   **Fig. 2A:** DEIs and DEGs enriched in key steps of five KEGG pathways. Five KEGG

611   pathways were shown with transparent boxes and the names of these pathways were red

612   marked. Green boxes inside of transparent boxes are DEI enriched enzymes; yellow boxes are

613   DEG enriched enzymes; green-yellow mixed boxes are both DEI and DEG enriched enzymes;

614   blank boxes are non DEI and DEG enriched enzymes. **B:** Expression of DEIs and DEGs in

615   the five key pathways. The left are pathway and enzyme names, and the right are isoform

616   expression and names. Expression of isoforms and genes are presented with their log2TPM

617   values and shown with color scales. DEIs or DEGs are marked with "*" in the middle of the

618   boxes.

619

620   **Fig. 3A:** Numbers of uniquely expressed isoforms in queen larvae (up bars) or worker larvae

621   (down bars) at 2d, 4d and 6d stages. The red bars are numbers of uniquely expressed isoforms

622   enriched in eight key KEGG signaling pathways (mTOR, IIS, FoxO, Notch, Wnt, MAPK,

623   Hippo and TGF-beta). The purple bars are numbers of uniquely expressed isoforms that are

624   key genes reported in previous studies (details see Table S10-12). **B:** The heat map of

625   expression of uniquely expressed isoforms which are shown in red bars and green bars in A.

626   The red color blocks in heat map are uniquely expressed isoforms in queen larvae at 2d, 4d

627   and 6d age, whereas the dark green blocks are uniquely expressed isoforms in worker larvae.

628   Other color scales are the log2fold change values of isoform expression between queen and

629   worker larvae at 2d, 4d and 6d stages.

630

631   **Fig. 4** The alternative splicing patterns (AS) in DEIs of three comparisons. **A:** DEIs of 2d

632   comparison containing AS events. The colorful bars in the left bottom diagram are the

633   different AS patterns and the number of AS events in DEIs. Seven different AS patterns are

634   3'splice site (A3), 5'splice site (A5), First exon (AF), Last exon (AL), Retained intron (RI),

635   Skipping exon (SE) and Mutually exclusive exon (MX). The pink bars in the top of right

636   diagram are the number of DEIs. The black spots in a column mean one single isoform

637   containing these different AS patterns. Same in B and C. **B:** DEIs of 4d comparison

638   containing AS events. **C:** DEIs of 6d comparison containing AS events.

639

640   **Fig. 5:** Correlation between poly(A) lengths and DEI expression of 2d queen-worker

641   comparison. Expression of each DEI was the log10 TPM value. Same in B and C. **B:**

642   Correlation between poly(A) lengths and DEIs of 4d comparison. **C:** Correlation between

643   poly(A) lengths and DEIs of 6d comparison.

644

645   **Fig. 6A:** Expression and related poly(A) lengths of *Jhe* isoform. The left is the exon coverage

646   of clean reads of *Jhe* gene of 6 larval groups. The *Jhe* has only one isoform (*Jhe.t1*) and its

647   structure is present (see its exons below marked with black color). The middle heat map is the

648   isoform expression of *Jhe.t1* (log2TPM values) in 6 groups and presented with color scales.

649   The right is the poly(A) length distribution of *Jhe.t1* in 6 groups. The mean poly(A) length is

650   presented in the middle of each violinplot. **B:** Expression and related poly(A) lengths of *Ecr*

651   isoforms. The *Ecr* gene has 4 isoforms and their expression is presented using heat map as

652   above. The poly(A) length distribution of *Ecr.t4* in 6 groups is presented on the right side, it

653   was the most significantly differentially expressed isoform of gene *Ecr*. The poly(A) of *Ecr.t4*

654   in 2dW was presented with a blank box which means few clean data of poly(A) was detected,

655   since 2dW did not express *Ecr.t4.*

656

657   **Table 1** Overview of direct RNA sequencing

658

659   **Supplementary**

660   Fig. S1 The pearson correlation coefficient of 2d, 4d and 6d queen (QL) and worker larvae

661   (WL). Each larval group had two biological replicates (A and B).

662

663   Fig. S2 The sample cluster tree of 12 samples. Isoform expression of each sample was

664   compared and its correlation was presented using color scales.

665

666   Fig. S3 DEIs containing significantly different AS events in 2d, 4d and 6d queen-worker

667   comparisons. Blue color presents the proportion of DEIs containing AS events, and red color

668   presents DEIs containing significantly different AS events.

669

670   Fig. S4 The significantly differential alternative splicing patterns in DEIs of three

671   comparisons (similar to Fig.4). **A:** DEIs of 2d comparison containing significantly differential

672   AS events. The colorful bars in the left bottom diagram are the different AS patterns and the

673   number of AS events in DEIs. Seven different AS patterns are 3'splice site (A3), 5'splice site

674   (A5), First exon (AF), Last exon (AL), Retained intron (RI), Skipping exon (SE) and

675   Mutually exclusive exon (MX). The pink bars in the top of right diagram are the number of

676   DEIs. The black spots in a column mean one single isoform containing these different AS

677   patterns. Same in B and C. **B:** DEIs of 4d comparison containing significantly differential AS

678   events. **C:** DEIs of 6d comparison containing significantly differential AS events.

679

680   Fig. S5 Correlation between poly(A) lengths and isoform expression of 12 samples.

681   Expression of each isoform was the log10 TPM value.

682

683   Fig. S6 Correlation between poly(A) lengths and DEG expression of three queen-worker

684   comparisons. Up: 2d comparison, middle: 4d comparison, down: 6d comparison. Expression

685   of each DEG was the log10 TPM value.

686

687   Table S1 DEIs between 2d queen and worker larvae

688   Table S2 DEIs between 4d queen and worker larvae

689   Table S3 DEIs between 6d queen and worker larvae

690   Table S4 DEGs between 2d queen and worker larvae

691   Table S5 DEGs between 4d queen and worker larvae

692   Table S6 DEGs between 6d queen and worker larvae

693   Table S7 Different poly(A) related genes between 2d queen and worker larvae

694   Table S8 Different poly(A) related genes between 4d queen and worker larvae

695   Table S9 Different poly(A) related genes between 6d queen and worker larvae

696   Table S10 Uniquely expressed isoforms in 2d queen or worker larvae

697   Table S11 Uniquely expressed isoforms in 4d queen or worker larvae

698   Table S12 Uniquely expressed isoforms in 6d queen or worker larvae

699   Table S13 DEIs of 2d comparison containing significantly different AS events

700   Table S14 DEIs of 4d comparison containing significantly different AS events

701   Table S15 DEIs of 6d comparison containing significantly different AS events