Cellular heterogeneity of the developing worker honey bee (*Apis mellifera*) pupa: a single cell transcriptomics analysis

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Abstract

It is estimated that animals pollinate 87.5% of flowering plants worldwide and that managed honey bees (*Apis mellifera*) account for 30-50% of this ecosystem service to agriculture. In addition to their important role as pollinators, honey bees are well-established insect models for studying learning and memory, behaviour, caste differentiation, epigenetic mechanisms, olfactory biology, sex determination and eusociality. Despite their importance to agriculture,

knowledge of honey bee biology lags behind many other livestock species. In this study we have used scRNA-Seq to map cell types to different developmental stages of the worker honey bee (prepupa at day 11 and pupa at day 15), and sought to determine their gene signatures and thereby provide potential functional annotations for as yet poorly characterized genes. To identify cell type populations we examined the cell-to-cell network based on the similarity of the single-cells' transcriptomic profiles. Grouping similar cells together we identified 63 different cell clusters of which 15 clusters were identifiable at both stages. To determine genes associated with specific cell populations or with a particular biological process involved in honey bee development, we used gene co-expression analysis. We combined this analysis with literature mining, the honey bee protein atlas and Gene Ontology analysis to determine cell cluster identity. Of the cell clusters identified, 9 were related to the nervous system, 7 to the fat body, 14 to the cuticle, 5 to muscle, 4 to compound eye, 2 to midgut, 2 to hemocytes and 1 to malpighian tubule/pericardial nephrocyte. To our knowledge, this is the first whole single cell atlas of honey bees at any stage of development and demonstrates the potential for further work to investigate their biology of at the cellular level.

Introduction

The western honey bee, *Apis mellifera*, is valued for the pollination services it provides to many crops and wild flowers (Kleijn *et al*., 2015; Breeze *et al*., 2011; Ollerton *et al*., 2011; Gallai *et al*., 2009; Klein *et al*., 2007; Corbet *et al*., 1991) as well as *for* its production of honey and wax (Carreck, 2018; Hepburn et al., 1991). Globally there are 11 species of honey bee (Arias and Sheppard, 2005; Engel, 1999) whose distribution is restricted to Asia with the exception of the western honey bee found all over the world and indigenous to Africa, the Middle East and Europe (Seeley, 1985; Ruttner, 1988). Despite the diversity of honey bee species in Asia, the world's beekeeping industry is based almost entirely on one species, *Apis mellifera*. In addition to their importance to agriculture and the economy, honey bees represent a useful model organism for many areas of research (Dearden *et al*., 2009). Although less complex than mammals, honey bees possess a highly evolved social structure, a wide range of behaviours, complex communication and can learn and remember colours, shapes, fragrances and location of sources of forage (Dearden et al., 2009). Similar to the best studied model organism in the phylum Arthropoda, *Drosophila melanogaster,* honey bees are also considered a good model for understanding cognition as they possess a range of complex social and navigational behaviour with a brain that contains ~1 million neurons and are used as a model for olfactory learning (Menzel, 2012). Alternatively, as honey bees and *Drosophila* are over 300 million years diverged (Misof et al., 2014) there are many biological differences between them including eusociality, haplodiploidy, multiple discrete phenotypes from a single

genotype (polyphenisms) and symbolic language (Tautz, 2008). For example, honey bees are excellent models to study polyphenism. Worker honey bees switch between different in-hive tasks eventually progressing to foraging, allowing the mechanisms required for behavioural plasticity of major life history changes to be studied (Elekonich and Roberts, 2005; Simpson et al., 2011) whilst the differential development of queen bees and worker bees is solely dependent on diet (Slater et al., 2020).

The genome for the western honey bee was first published in 2006 by the Honey Bee Genome Sequencing Consortium. This was later improved upon by Elsik et al., 2014 who found c.5,000 more protein-coding genes, 50% more than previously reported. Wallberg et al., 2019 reported a further improvement using Pac-Bio long-reads (Amel_HAv3.1). Parallel to annotating the genome, efforts have also been made to associate phenotypes with genes using omic analyses. Studies have examined changes in gene expression associated with different treatments (pheromones and pesticide) and how they relate to behaviour, phenotype and changes associated with the colony e.g. queen loss (Ma et al., 2019; Chaimanee and Pettis, 2019; Christen et al., 2016). Pheromone and pesticide treatments have also been studied in combination with various conditions [e.g. with seasonal changes (Jeon et al., 2020), infections from Varroa (Navajas et al., 2008; Zhang et al., 2010; Morfin et al., 2019) and Nosema (Badaoui et al., 2017; Li et al., 2016; Azzouz-Olden et al., 2018)]. Mechanisms underlying developmental processes such as embryogenesis, ageing and caste determination have also been analysed (Yin et al., 2018; Evans and Wheeler, 1999; He et al., 2019; Tsuchimoto et al., 2004; Azevedo et al., 2011). Whilst some of the aforementioned experiments have derived transcriptomic data from whole honey bees others have studied tissue-specific differences e.g. analysis of differences in alternate splicing patterns between the brain and fat body (Kannan et al., 2019; Wang et al., 2012; Zayed and Robinson, 2012). However, a comprehensive tissue/cell atlas of the developing honey bee is still lacking.

Bulk tissue transcriptomics atlases have been used effectively to annotate and assign function to poorly annotated genes in pig, sheep, mice, humans and *Drosophila melanogaster* (Freeman et al., 2012; Clark et al., 2017; Su et al., 2002; Chintapalli et al., 2007; Leader et al., 2018). scRNA-Seq enables the classification of cell subtypes and differentiation trajectories that is challenging with solely a bulk RNA-Seq strategy. Single-cell expression atlases have been derived from several tissues like the Tabula Muris which spans 100,000 cells across 20 mouse tissues (Tabula Muris Consortium, 2018). Other efforts like the Fly Cell Atlas have conducted exhaustive scRNA-Seq studies on individual tissues providing a comprehensive atlas, e.g. for the brain (Davie et al., 2018) and midgut (Hung et al., 2020). Studies have also tracked the development of various organisms including Drosophila (Karaiskos et al., 2017), Zebrafish (Raj et al., 2018), cnidarian (Sebe-Pedros et al., 2018) and C. elegans (Packer et

101 al., 2019). Such studies have demonstrated the sensitivity of scRNA-Seq data in tracking cell
102 types and their lineages while also identifying their gene signatures and how conserved they
103 are across species. We have followed a similar approach to construct a single-cell atlas
104 spanning two developmental stages of the worker honey bee (prepupa at day 11 and pupa at
105 day 15). To identify cell types associated with each stage and track them through development
106 we examined the similarity between cells based on their gene expression using a cell-to-cell
107 network, thus revealing different cell types (which were closely connected in the network).
108 Similarly, by using gene co-expression network (GCN) analysis, we identified coexpressed
109 genes i.e. genes sharing a similar expression profile across samples which were likely
110 representative of a common biology as has been shown previously (Patir et al., 2020; Patir et
111 al., 2019). Gene signatures for the various biology associated with cell types from worker
112 honey bee pupae across developmental stages were identified. To our knowledge this is the
113 first analysis of the honey bee transcriptome at single cell level resolution.

114 Material and Methods

115 Whole *Apis mellifera* pupae cell dissociation and sorting

116 Honey bees are holometabolous and worker prepupae at day 11 (S1) and pupae at day 15
117 (S2) were chosen for this study in order to capture the key developmental stages between
118 capping of the larval cell (day 9) and the emergence of the imago on day 21 (Oertal, 1930)
119 (**Figure 1A**). To gather samples, a piece of brood comb containing appropriately staged pupae
120 was collected from a single honey bee colony at the Easter Bush Campus apiary in August
121 2018. Pupae were removed from the comb and placed in microcentrifuge tubes on ice. Each
122 pupa was placed in 0.5 ml HyQTase (GE Healthcare, Chicago, Illinois, USA), finely chopped
123 with small spring scissors for one minute and incubated for 5 min at 25°C. Samples of each
124 stage were centrifuged at 400 RCF for 5 minutes at 4°C. Cell pellets were resuspended in 1
125 ml WH2 medium by drawing liquid into and out of pipette tip fifteen times (Goblirsch *et al*.,
126 2013). Samples (n = 4 per stage) were pooled (total volume 4ml), and the cells passed into a
127 5ml tube through a 70 µm strainer cap (Becton, Dickinson and Company, New Jersey, USA)
128 to remove debris and aggregated cells. Following centrifugation of the filtered cells at 400 RCF
129 for 5 minutes at 4°C, the supernatant was discarded and the cells resuspended in 2ml WH2
130 medium. After further centrifugation at 400 RCF for 5 min at 4°C, cells were resuspended in 1
131 ml WH2 medium and stained with 1:2,000 Sytox Red (Thermo Fisher, Waltham,
132 Massachusetts) for downstream cell viability analysis during cell sorting. Gating strategies
133 sorted cells on the basis of their size (forwards vs side scatter area to exclude debris), single
134 cells (forward scatter area vs height to exclude doublet cells) and viability using a 633nm laser
135 and 660/20 band pass emission filter on an Aria IIIu FACS (Becton, Dickinson and Company,

136 New Jersey, USA (Figure 1B and C). Before sequencing the cells were counted and tested
137 again for viability using a TC20 automated cell counter (Bio-Rad, Hercules, California, USA).

138 Single-cell RNA-Seq data generation, processing and quality control

139 Approximately 7,000 cells at each stage were used for cDNA library preparation using
140 the Chromium platform v2.0 (10X Genomics, Pleasanton, California, USA), as per the
141 manufacturer's instructions. Library quality was confirmed with a LabChip Gx24 bioanalyzer
142 (PerkinElmer, Waltham, Massachusetts, USA). Sequencing (75bp paired-end) was performed
143 using an Illumina NextSeq550 platform using a Mid Output 150 cycle flow cell (Clinical Research
144 Facility, the University of Edinburgh).

145 Binary base call files were pre-processed using the Cell Ranger pipeline (10X
146 Genomics). Reads were assigned to sample index tags to generate FASTQ files. Of the total
147 180 million reads generated, 69 million mapped to sample indices of prepupa (day 11) and 55
148 million to pupa (day 15). For read alignment, the recent *Apis mellifera* reference genome
149 (Amel_HAv3.1) and annotation (GFF file) were downloaded from NCBI. To keep compatibility
150 with Cell Ranger, the GFF file was converted to GTF using the Cufflinks software suite
151 (Tuxedo) (Trapnell et al., 2012) and filtered for non-protein coding regions.

152 The resultant GTF file and reference genome were used to generate an expression
153 matrix for each sample. Raw expression matrices were quality controlled and analysed using
154 the Seurat package v2 in R (Stuart et al., 2019). Data from the two developmental stages were
155 first merged. Cells having a low number of UMI reads ≤700 and ≥10% being mitochondrial
156 were filtered out. Furthermore, genes expressed in ≤ 3 cells were removed. The data was log-
157 normalized and genes having the most variable expression across cells were identified, i.e.
158 possessing a standard deviation >0.5 and an average expression between 0.0125 and 3.
159 Effects from technical factors, including variable library sizes and percent mitochondrial UMIs,
160 were regressed out. The scaled variables were reduced to a lower feature space using
161 principal component (PC) analysis. The most significant PCs (61 in total, P *value* < 0.05) based
162 on JackStraw permutations (Chung and Storey, 2015) were considered and the resultant cell
163 vs PC matrix was loaded into the network analysis tool, Graphia (Freeman et al., 2022). A
164 correlation (Pearson similarity coefficient) matrix was then calculated between cells comparing
165 the PC profile of each cell. Using this cell similarity matrix, a cell-to-cell network was
166 constructed where cells (represented by a node) were connected to the 20 most similar cells
167 by an edge, while only considering similarities beyond a Pearson cut-off threshold r ≥ 0.77.
168 This graph was clustered using Markov clustering algorithm (Van Dongen, 2008) with an
169 inflation value of 1.6. Cells were further filtered to remove those with an edge degree lower

170 than three. For statistical purposes small clusters with less than 10 cells were merged into the
171 closest cluster with the highest sum of weighted edges.

172 Gene co-expression network analysis

173 Gene expression modules associated with biological process and cell types were
174 identified using gene co-expression network (GCN) analysis. For conventional transcriptomics
175 data GCNs are widely used to capture coexpressed clusters of genes associated with a shared
176 biological function (Patir et al., 2019; Nirmal et al., 2018; Patir et al., 2020). However, due to
177 the inherent variability within scRNA-Seq data attributed to the transcriptional heterogeneity
178 of cells and the technical effects of dropouts (false zero expression values) we were unable to
179 capture these coexpressing genes as they are poorly correlated. Hence, we constructed a
180 GCN by averaging reads across cells present in each of the cell clusters described above,
181 thus, focusing on the inter-cell type variations, i.e., the difference amongst the cell clusters,
182 rather than the intra-cell variation. Before averaging reads, various filters were applied to
183 reduce the effects of technical artefacts and low-level signals. First, for a given gene and
184 cluster, cells were assigned a zero expression value if: 1) fewer than three cells within the
185 cluster expressed that gene, 2) the maximum expression across cells was < 0.5 logged TPM
186 and 3) < 5% of cells within the clusters expressed that gene. Moreover, to avoid the influence
187 of outliers or spikes in expression commonly observed in RNA-Seq data we capped the
188 maximum expression of a gene to the 95% percentile from cells of the cluster. The gene
189 expression from the resultant filtered data was then averaged across cells for each cluster.
190 Where a cluster consisted of cells derived from both developmental stages, they were
191 averaged separately for each stage. In this way, the 63 cell clusters identified from the graph
192 analysis of cells, were expanded to 81 stage differentiated cell clusters. Consequently, an
193 expression matrix of genes vs. cell clusters was used to generate a GCN within Graphia. Only
194 genes with a maximum expression above 0.2 average logged TPM were considered. The k-
195 nearest neighbour (kNN) algorithm was applied where each cell was connected to the four
196 most similar cells provided this similarity was $r \geq 0.7$. Subsequently, the graph was clustered
197 using the Louvain cluster algorithm (Blondel et al., 2008) applied with a granularity setting of
198 0.65.

199 Functional gene annotation using *Drosophila melanogaster* homologues

200 Functional annotation of gene clusters from the GCN analysis was provided based on
201 Gene Ontology (GO) enrichment analysis and literature mining. First, each protein of the bee
202 proteome was mapped to the most similar (E score < 10^{-4}) protein in *Drosophila melanogaster*
203 (Release 6 plus ISO1 MT) based on their sequence using BLASTp (Altschul et al., 1990). The
204 resultant nomenclature in combination with studied honey bee genes was used to functionally

205 annotate gene clusters. Furthermore, the *Drosophila* homologues were also used for GO
206 enrichment analysis, this was conducted for each gene cluster using the clusterProfiler
207 package in R (Yu et al., 2012) with the genome wide annotation for *Drosophila* (org.Dm.eg.db)
208 as the reference GO term database (Carlson et al., 2016)

209 Results

210 The expanding cellular diversity of the developing pupa

211 We developed a cell isolation protocol (Methods) from the developmental stages (S1,
212 prepupa at day 11; S2, pupa at day 15) (**Figure 1A**) of the honey bee which provided sufficient
213 cell numbers and viability for processing through the 10x Chromium platform v2.0. Four
214 prepupae or pupae samples were combined for each stage. Briefly, cells from each stage
215 were homogenised and dissociated using HyQTase enzyme, and then resuspended in WH-2
216 medium. The cell solution was passed through a 70 µm strainer to filter out any cell clumps
217 and subsequently stained using Sytox red to estimate cell viability. These cells were then
218 sorted based on their size, granularity and staining to identify viable single-cells (**Figure 1B**
219 **and 1C**). Just before library preparation, the cells went through a second round of counting
220 and variability testing to assure sufficient cells were processed for sequencing.

221 Raw reads from the scRNA-Seq experiment were mapped to the NCBI based *Apis mellifera*
222 (Amel_HAv3.1) genome using Cell Ranger pipeline from 10x. 69 million reads mapped to
223 samples from the day 11 S1 sample and 55 million reads to the day 15 S2 sample. The data
224 was subjected to various quality control measures to remove outlier samples and genes with
225 negligible expression. Only genes expressed in more than 3 cells were considered, leaving
226 9,119 genes for S1 and 9,309 genes for S2. Cells were filtered on their read content, removing
227 cells with a low read count (< 700 per cell) and those with a high mitochondrial gene content
228 (> 10%), leaving 2,148 cells from S1 and 2,178 cells from S2. As the two samples were from
229 a single batch, datasets were merged and followed the standard scRNA-Seq pre-processing
230 steps of normalistion and scaling (for mitochondrial content and library size). To cluster cells
231 based on their gene expression profile, the 1,361 most variable genes were identified and
232 were reduced using principal component (PC) analysis from which the 61 most significant PCs
233 were inspected. These PCs were used to calculate Pearson pairwise similarity between cells
234 across the merged dataset thereby generating a cell-to-cell similarity matrix. The matrix was
235 used to construct a cell-to-cell network (**Figure 2**) where each node represented a cell and
236 those having a Pearson correlation coefficient greater than $r \geq 0.77$ were connected to one
237 another by an edge. Furthermore, for each cell only the 20 nearest neighbours were
238 considered and poorly connected cells, i.e. connected to < 3 other cells, were removed. These
239 steps further helped in removing potential outlier cells that were dissimilar to the majority of

240 cells. The final cell-to-cell graph consisted of 4,149 nodes (cells) (2,045 cells from S1 and
241 2,104 cells from S2) and 31,000 edges.

242 The cell-to-cell graph consisted of one large, interconnected component and 11 smaller
243 components. Cells from the two stages were distributed differently across the network
244 indicative of stage-specific cell types with S2 possessing more heterogenous populations of
245 cell types (**Figure 2A**). On studying the distribution of genes and reads across cells, cells from
246 S2 showed a significant (1.28 times, *P value* < 10^{-3}) increase in the number of genes
247 expressed relative to S1. Clustering of the cell network resulted in 72 clusters potentially
248 representing distinct cell types of states. To improve the statistical power of downstream
249 analyses, smaller cell clusters with less than 10 cells were merged with a neighbouring cluster
250 to which they were highly connected, i.e., had the highest sum total of weighted (based on the
251 Pearson correlation) connections resulting in 63 cell clusters (**Figure 2B**). Interestingly, even
252 though the number of cells from both stages was approximately the same, 51 clusters
253 comprised of cells from S2, while S1 cells were only present in 30 clusters. All together, these
254 results were indicative of the expanding cellular diversity in the developing honey bee pupa.

255

256 Clustering of coexpressing genes and their functional annotation

257 To determine genes associated with specific cell populations or biological processes
258 involved in worker honey bee development, GCN analysis was performed. The approach has
259 been used extensively to study expression data to determine coexpressing genes, i.e., genes
260 sharing a common expression profile across samples and which are likely to represent a
261 common biology (Patir et al., 2019; Nirmal et al., 2020; Patir et al., 2020). Although widely
262 used to study bulk transcriptomics data, genes have shown to be poorly correlated in scRNA-
263 Seq data due to the complexity of single-cell biology and technical artefacts inherent to this
264 technology (Hicks et al., 2018). Hence, we have averaged expression values across cells
265 within a cluster to improve the stability of signals within clusters whilst also highlighting inter-
266 cell type variation rather than the variation within a cell type (Satija and Shalek, 2014). The
267 resultant stage-cluster vs gene expression matrix was used to calculate a gene-to-gene
268 correlation matrix, from which we constructed a GCN. In the network, genes were connected
269 to the four most similar genes by an edge provided they were highly correlated $r \geq 0.7$. The
270 network graph consisted of 3,994 genes which were clustered into 32 gene clusters using the
271 Louvain clustering algorithm with a granularity of 0.65 (**Figure 3 and Table S1**). In addition to
272 GCN analysis, differential gene expression analysis was performed using the default Wilcox
273 test provided in Seurat to gauge the magnitude and specificity of genes towards cell-clusters
274 based on their expression (**Table S2**)

275 Tissues, cell types and biological processes corresponding to the clusters of genes in
276 the GCN were identified from GO enrichment (**Table S1**), public resources and literature
277 mining (**Table S3**), the final annotation of which is summarised in figure 4 & table S3. The
278 enrichment analysis was performed on each gene cluster based on the *Drosophila*
279 *melanogaster* GO reference database. For this analysis, honey bee genes based on their
280 corresponding proteins were first mapped to the *Drosophila melanogaster* proteome using
281 blastp (Altschul et al., 1997) where the most similar mapping was considered for a gene. 26
282 clusters were found to be enriched in various GO terms (*adj. P value* < 0.05) (**Table S1**). For
283 literature mining previous publications and resources were used including the Drosophila
284 FlyAtlas2 (Leader et al 2018) and Honey Bee Protein Atlas (Chan et al. 2013). The analyses
285 revealed gene clusters associated with stage-dependent differences, as well as tissue/cell-
286 specific biology, e.g. neuronal, muscle, cuticle, fat body, alimentary canal and haemolymph:

287 *Stage-specific clusters*: The largest gene cluster, cluster 1 comprised of 708 genes (Figure 3)
288 with a higher expression in cells from S2 relative to S1. GO terms enriched in these genes
289 included those related to development, the top three GO terms being "post-embryonic animal
290 morphogenesis" (*adj. P value* = 2.29×10^{13}), "instar larval or pupal morphogenesis" (*adj. P value*
291 = 3.35×10^{13}) and "regulation of intracellular signal transduction" (*adj. P value* = 5.39×10^{13}).

292

293 ***Neuronal related cell clusters:*** Three gene clusters (gene clusters 2, 3 and 5) contained
294 genes associated with various neuronal biology and were highly expressed in 17 cell clusters
295 identified as being related to neurons and sense organs. All of the cell clusters identified as
296 neuronal or sense organ related expressed both synapsin and the nicotinic acetylcholine
297 receptor alpha 1 subunit, while those annotated as only neuronal expressed the NR1 subunit
298 of the NMDA receptor. Some genes were expressed differentially across developmental
299 stages, including genes from the family of G protein-coupled receptors that bind octopamine
300 and/or tyramine. Octopamine is widely distributed in the nervous system of invertebrates
301 where it acts as a neurotransmitter (Verlinden et al., 2010) and is thought to be the functional
302 homologue of vertebrate adrenergic transmitters. On examining the different classes of these
303 G protein-coupled receptors (Sinakevitch et al., 2017) in invertebrates, OA1 receptor showed
304 a high expression in cell clusters 37 and 60 containing S2 cells, AmTAR1 was highly
305 expressed in cell cluster 33 having cells from both pupal stages, whilst AmTARII showed a
306 high expression in cell clusters 9 and 11 of S1 stage

307 ***Glial related cell clusters:*** Glial cell*s* have an essential role in the development of neurons
308 and are involved in regulation of synaptic plasticity, provide trophic support to neurons and
309 contribute to the blood-brain barrier (Shah et al., 2018). In the honey bee these cells can be

310 labelled using a serum raised against the *Drosophila* glial transcription factor *repo* (Shah et
311 al., 2018), *repo* was highly expressed in several non-neuronal cell clusters (6, 16, 34, 35, 48
312 and 61) identifying them as potentially representing glia or glial-related cells. Gene cluster 16
313 was found to be associated with these cell clusters. Further subclassification of these cells
314 was revealed through genes linked with astrocytes in *Drosophila*, like *Eaat2* and GABA
315 transporters (Gat-a and Gat-1b) highly expressed in cell cluster 35 (Freeman, 2015).

316 ***Sensory organ and compound eye related cell clusters:*** *A* higher average expression of
317 genes from gene cluster 8 was observed in sensory organs relative to neuron related cell
318 clusters. GO terms enriched in genes from this cluster were associated with ciliary biology,
319 the most significant terms being "cilium organization" (*adj. P value* = 6.48×10^{24}), "cilium
320 assembly" (*adj. P value* = 8.53×10^{24}) and "plasma membrane bounded cell projection
321 assembly" (*adj. P value* = 4.36×10^{21}). The modified primary cilium is a structure common to all
322 peripheral sensory neurons in arthropods with the exception of photoreceptors (Keil, 2012),
323 suggesting that cell clusters 27, 29 and 53 were related to sense organs other than the
324 compound eye and ocelli. Four cell clusters (clusters 26, 33, 44 and 49) identified as neural
325 were associated with the compound eye. Genes from gene cluster 16 (80 genes) were
326 specifically expressed in these eye related cell clusters with genes associated with this tissue
327 e.g. *AmPNR-like* (LOC413558) shown by *in situ* hybridization to be expressed in the
328 developing eyes of pupae in either the photoreceptor cells or support cells (Velarde et al.,
329 2006). LOC408804 (1-phoshatidylinositol 4,5 bisphosphate phosphodiesterase epsilon-1)
330 was expressed in these cell clusters and in Drosophila it's homologue (*Plc21c)* has a role in
331 Pigment Dispersing Factor neurons in the circadian photoresponse (Ni et al., 2017).
332 Phosrestin 2 was specifically expressed in cell clusters 17, 26 and 44, and has been
333 associated with the visual system in honey bees where it has a role in circadian rhythms
334 (Rodriguez-Zas et al., 2012).

335 ***Cuticle related cell clusters*****:** Gene clusters 4, 9, 11, 22 and 30 included genes expressed in
336 20 cell clusters associated with the cuticle. Only four of these cell clusters were associated
337 with the S1 prepupal cuticle (cell clusters 36, 38, 39, 51). This could indicate that cell
338 populations from the S1 stage cuticle are less diverse than those from S2 which might consist
339 of heterogenous populations of cells differentiating in different regions of the developing honey
340 bee exoskeleton. The cuticle associated gene clusters included key enzymes in the chitin
341 biosynthetic pathway linked to cuticle development and the moulting process e.g. LOC412215
342 (homologue of Drosophila gene *kkv*, a chitin synthase that catalyses the conversion of UDP-
343 *N*-acetylglucosamine to chitin), LOC552276 (homologue of Drosophila gene *cda5),* a chitin
344 deacetylase that catalyses the conversion of chitin to chitosan (a polymer of *β*-1,4-linked d-
345 glucosamine residues) (Sobala and Adler, 2016) and LOC551964 (homologue of *Drosophila*

346 gene *mmy*, an enzyme required for glycan and chitin synthesis) (Araujo et al., 2005). Chitin
347 (the polymer of N-acetyl glucosamine) is a key component of the honey bee inner procuticle,
348 which together with the outer epicuticle forms the exoskeleton (Locke and Krishnan, 1971)
349 and the difference in cuticle structure in arthropods is due to the different expression of
350 proteins (Magkrioti at al., 2004). In addition to chitin, the cuticle consists of various cuticle
351 structural proteins some of which were present in the cuticle related gene clusters including
352 LOC726451 (homologue of *Drosophila* gene *Cpr57A*) and *Apd-3* (Falcon et al., 2019).

353 ***Fat body related cell clusters*:** In insects this tissue has a similar role to the liver and adipose
354 tissue of mammals as it functions as a store for excess nutrients, synthesizes most of the
355 haemolymph proteins and is responsible for detoxification processes (Arrese and Soulages,
356 2010). Various genes associated with the fat body were found in gene cluster 6 (199 genes)
357 which had a high expression in seven cell clusters. The majority of these clusters comprised
358 cells from the S1 stage (six clusters). The gene *ilp-2* known to be expressed in both oenocytes
359 and trophocytes (cell types found in the fat body) in the adult honey bee and was expressed
360 in all seven fat body related cell clusters (Nilsen et al., 2011). A similar expression was
361 observed for *mmp2* shown to be necessary and sufficient for fat body remodelling during early
362 metamorphosis in *Drosophila* (Bond et al., 2011) and Vitellogenin receptor shown to be
363 expressed in the fat body, ovary and head of adult worker bees (Guidugli-Lazzarini et al.,
364 2008).

365 ***Haemolymph related cell clusters*:** In insects, haemocytes are derived from anterior
366 mesoderm, form part of the immune system and comprise lamellocytes, crystal cells,
367 plasmatocytes and granulocytes (Richardson et al, 2018). Granulocytes are the major
368 phagocytic cells (Richardson et al, 2018). Gene clusters 28 and 32 contained known
369 haemocyte markers (*hml* and *lz*) and the average expression of these genes was higher in
370 cell clusters 20 and 43. The marker *hml* (hemolectin/hemocytin) is specifically expressed in
371 haemocytes in *Drosophila* in embryos and larvae, while *lz* is required for the differentiation of
372 crystal cells (Lebestky et al., 2003) and the absence of its expression results in the
373 differentiation of a plasmatocyte. Whilst both gene were expressed highly in the haemocyte
374 cell clusters, *lz* showed higher levels of expression in cell cluster 43, suggesting that it
375 represented crystal cells.

376 ***Muscle related cell clusters*:** In Drosophila, somatic muscle, visceral muscle and cardiac
377 muscle develop from the mesoderm (Gunage et al., 2017). The largest somatic muscles in the
378 honey bee are two pairs of indirect flight muscles (dorsumventral and anterior-posterior) in the
379 thorax that are responsible for moving the wings up and down (Snodgrass, 1910). Cell clusters
380 5, 12, 24, 28 and 32 were annotated as differentiating muscle cells based on expression of
381 twist (Gunage et al., 2017), mef2 (Crittenden et al., 2018), nautilus (Abmayr and Keller, 1998),

382 TpnT (Domingo et al., 1998), TpNI (Herranz et al., 2005), TpnCIIb (Herranz et al., 2005),
383 myosin heavy chain (LOC409843) and myosin light chain (LOC409881) (11390828). *nautilus*
384 is a candidate for the equivalent of the vertebrate myogenic regulatory factors (*myoD* and
385 *Myf5*) that act as master control genes in mesoderm to initiate the first steps of somatic muscle
386 development (Abmayr and Keller, 1998; Zammit, 2017). Expression of *nautilus* specifically in
387 cell clusters 12, 24 and 32 indicated that these cell clusters comprised of cells differentiating
388 into somatic muscle. Expression of twist in Drosophila is required earlier in development in
389 mesoderm definition for specification of all muscle types, *twist* was expressed specifically in
390 cell clusters 5, 12, 24, 28 and 32, and was also expressed in cell cluster 60 (unknown identity).
391 Cell clusters 5, 12, 24, 28 and 32 were also associated with differentiating muscle cells based
392 on GO analysis of gene cluster 14 whose gene showed high expression in these cells relative
393 to other cell clusters. The top GO terms for the gene cluster 14 included "striated muscle cell
394 differentiation" (*adj. P value* = 2.02×10^{20}), "muscle structure development" (*adj. P value* =
395 4.12×10^{20}), and "muscle cell differentiation" (*adj. P value* = 8.61×10^{20}).

396

397 *Alimentary canal*: the tissue comprises four major compartments, the foregut, midgut,
398 malpighian tubules and hind gut (Snodgrass, 1910). Genes from gene cluster 12 were highly
399 expressed in cell clusters 8 and 50. The associated gene cluster included alpha-glucosidase
400 I and II shown to be expressed in honey bee ventriculus (Kubota et al., 2004), as well as
401 organic anion transporting polypeptide genes *Oatp33Ea* and *Oatp58Dc* both of which are
402 specific to the *Drosophila* midgut of larva and adult based on the FlyAtlas 2 tissue RNA-Seq
403 database (Leader et al., 2018). Cell cluster 63 had a high expression of genes from gene
404 cluster 18 thought to related to malpighian tubules or pericardial nephrocytes, including *Cubilin*
405 and *Amnionless* which in *Drosophila* mediate protein reabsorption in both malpighian tubules
406 and pericardial nephrocytes (Zhang et al., 2013).

407 Discussion

408 The aim of this study was to generate single cell transcriptomics data for two stages of
409 worker honey bee development. We have generated scRNA-Seq data from a prepupal stage
410 (day 11) and the pupal stage (day 15), these stages being selected to capture cellular diversity
411 immediately before and after the rearrangement of the larval to adult body plan. In
412 holometabolous insects, the larvae and adults have very different body plans enabling them
413 to exploit different resources. Although the larvae of social insects and solitary bees have
414 subsequently evolved to be relatively immobile, this remarkable evolutionary development
415 contributed to holometabolous insects comprising over half of global eukaryotic diversity

416 (Belles, 2017). Despite the importance of metamorphosis in the evolutionary success of
417 insects, the mechanisms governing it are not completely understood. Most is known about
418 regulation by the endocrine system which involves the hormones 20E (20-hydroxyecdysone)
419 and JH (juvenile hormone) (Truman and Riddiford, 2019). Generally, 20E promotes moulting
420 whilst JH inhibits metamorphosis and thus if 20E acts together with JH, moulting results in a
421 juvenile stage and if it acts without JH, it results in metamorphosis (Truman and Riddiford,
422 2019). Activation of the 20E receptor complex results in the up regulation of transcription
423 factors e.g., *HR3, HR4, HR39*, *Broad complex*, *E75* and *FTZ-F1* (King-Jones and Thummel,
424 2005; Nagakawa and Henrich, 2009). Activation of the JH receptor complex results in
425 induction of *Kr-h1*, Kr-h1 subsequently represses transcription of *E93* (Belles and Santos,
426 2014; Urena et al., 2014). If E93 protein levels increase, metamorphosis is triggered. During
427 metamorphosis, tissues can degenerate if they are not present in the adult (e.g. head gland),
428 be remodelled without complete cell replacement (e.g. fat body) or generate a new adult
429 structure (e.g. antenna, eyes, legs and wings develop from undifferentiated cells in imaginal
430 discs) (Tettamanti and Casartelli, 2019).

431 Two strategies have previously been adopted by other researchers in studying
432 development using scRNA-Seq. The first involves scRNA-Seq of whole-organisms and the
433 second, of focussing on individual tissues. Here, we adopt the former approach which has
434 proven useful in the exploration of cell types of model organisms of a similar scale and
435 biological complexity, such as Cnidaria (Sebe-Pedros et al., 2018), *C. elegans* (Packer et al.,
436 2019), and zebrafish (Farnsworth et al., 2020), where the cell diversity is largely unknown.
437 Similar to these studies we have identified the cellular diversity across different lineages and
438 their contribution to each pupal stage. For this study we developed a protocol that can be used
439 to prepare single cells of honey bee worker pupae for scRNA-Seq. Further research could
440 address a wider developmental series and ascertain the efficacy of the protocol as the cuticle
441 toughens in the later pupal stages. It seems unlikely that the protocol would be suitable for a
442 whole adult honey bee due to the presence of a fully developed exoskeleton, however this
443 was not attempted by the authors. The protocol should however be effective for analysis of
444 single cells from a dissected adult brain or other dissected tissues.

445 The cell-to-cell network grouped cells into 63 clusters across which cells from the two
446 stages were differently distributed. Hence, clustering of cells revealed stage-dependent cell
447 types/subtypes i.e. certain cell types were entirely represented by cells from a single stage
448 while other clusters comprised cells from both stages. The majority of cell clusters were
449 entirely comprised of cells from S2, furthermore these cells had a greater number of genes
450 expressed relative to cells from S1. These results suggest an expanding heterogeneity for the
451 types of cells and genes, which define them and reflect the fact that most of the organs of the

452 adult honey bee are present at S2 whilst at S1, a lower number of larval tissues are about to
453 be degenerated, remodelled or replaced. To study the genes that were associated with the
454 cell clusters we developed a novel approach to improve the biological signal representing
455 inter-cell cluster variation. Briefly, this was done by averaging the reads across cells from the
456 same cluster and applying filters on the expression values to address certain technical
457 artefacts within the data including spikes in expression and the variation of lowly expressed
458 genes. The approach enabled the construction of a GCN from scRNA-Seq data, which
459 captures inter-cell type variation while minimising intra-cell type and technical variations. The
460 GCN comprised 32 clusters of coexpressing genes that were associated with a wide range of
461 biology as determined using a combination of GO enrichment and literature mining to identify
462 cell types and tissue-specific biology. Cell types and tissues identified were related to the
463 brain, sensory organs, cuticle, muscle, fat body, blood and alimentary canal. Gene co-
464 expression signatures were identified that were not only unique to cell clusters but also those
465 that were shared across clusters e.g. stage and lineage specific signatures. Some cell clusters
466 would have proved impossible to identify based on using literature for *Apis* only due to the
467 limitations of the available resources as such it was necessary to compare to *Drosophila* where
468 organs are evolutionary conserved and where a database for GO terms are present.

469 Many honey bee tissues were either not detected or not identified in our analysis e.g.,
470 endocrine system, salivary glands, hypopharyngeal glands, oesophagus, honey sac, small
471 intestine, heart, rectum, sting, ovary. This might be because there is insufficient scientific
472 literature relevant to these pupal stages for identification (12 of the 63 clusters remain
473 unidentified) or it might be that the protocol was either too harsh or too gentle to obtain
474 particular cell types. It is surprising that there are noticeably few cells from the ventriculus
475 (mid-gut) despite the relatively large size of this organ in the adult bee, and it therefore seems
476 likely that a harsher or longer digestion might yield more cells from the mid-gut

477 With the lack of a gene expression atlas for the honey bee, this study provides an initial
478 step in determining the cellular heterogeneity, which can only be improved upon by
479 sequencing more samples/cells, cross species comparisons and analysis of gene expression
480 experiments. This study will be of benefit to the construction of more comprehensive gene
481 expression atlases by demonstrating that pupae can be analysed at the single-cell level,
482 which can be potentially extended to larvae and dissected adult organs e.g. brain.
483 Furthermore, the dataset could be used in conducting cross-species comparisons for
484 development, as has been done for Cnidaria (Sebe-Pedros et al., 2018), to study the evolution
485 of certain cell types.

486

Conclusions

In summary, we have demonstrated that a gene expression atlas of the whole honey bee at the level of single cells is possible at prepupal and pupal stages. We have developed approaches from single cell isolation to the analysis of the resultant scRNA-Seq data using GCN. Through this process we have identified several potential cell types and their associated gene signatures which are supported by enrichment analysis, and previous experimental evidence from the literature or databases. The gene lists associated with the cell clusters will be of benefit to future analyses, particularly for transcriptomic studies in whole pupae. Despite the global importance of bees to agriculture, this is the first whole organism RNA expression atlas in Hymenoptera. As a result, the study provides, improved knowledge of transcriptional profiles of many cell types of the worker honey bee at the pupal stage, and functional annotation of its genome.

Author Contributions

AP performed cell preparations from bee pupae, bioinformatics, transcriptomics analysis to assign cell identities, assisted with beekeeping and wrote the manuscript, **AR** and **RF** assisted with experimental design and performed FACS, **BH** and **NH** prepared 10X Genomics libraries and provided advice on experimental strategy, **LM** performed short read Illumina sequencing, **EC** provided advice on manuscript preparation and helped to draft the manuscript, **TF** conceived the idea for the study and managed the project, **MB** performed cell preparations from bee pupae, transcriptomics analysis to assign cell identities, managed beekeeping and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability

The dataset described in this manuscript has been deposited in the National Center for Biotechnology Information BioProject database (BioProject ID: PRJEB45881).

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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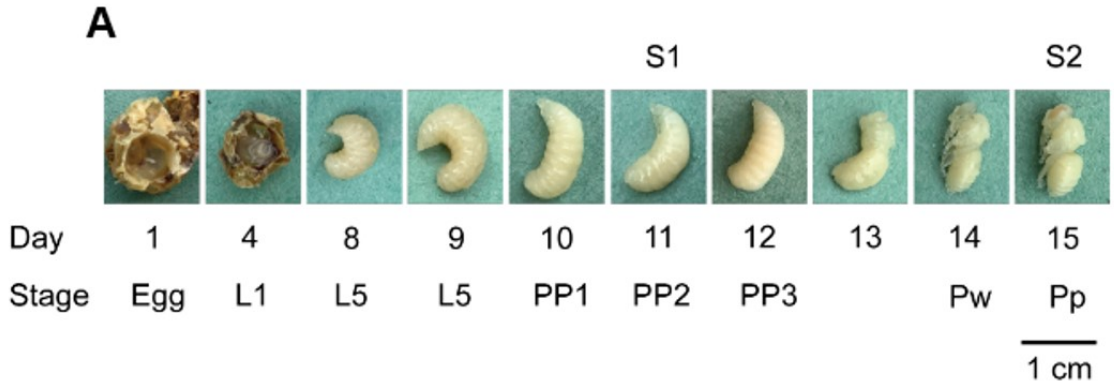
Figures



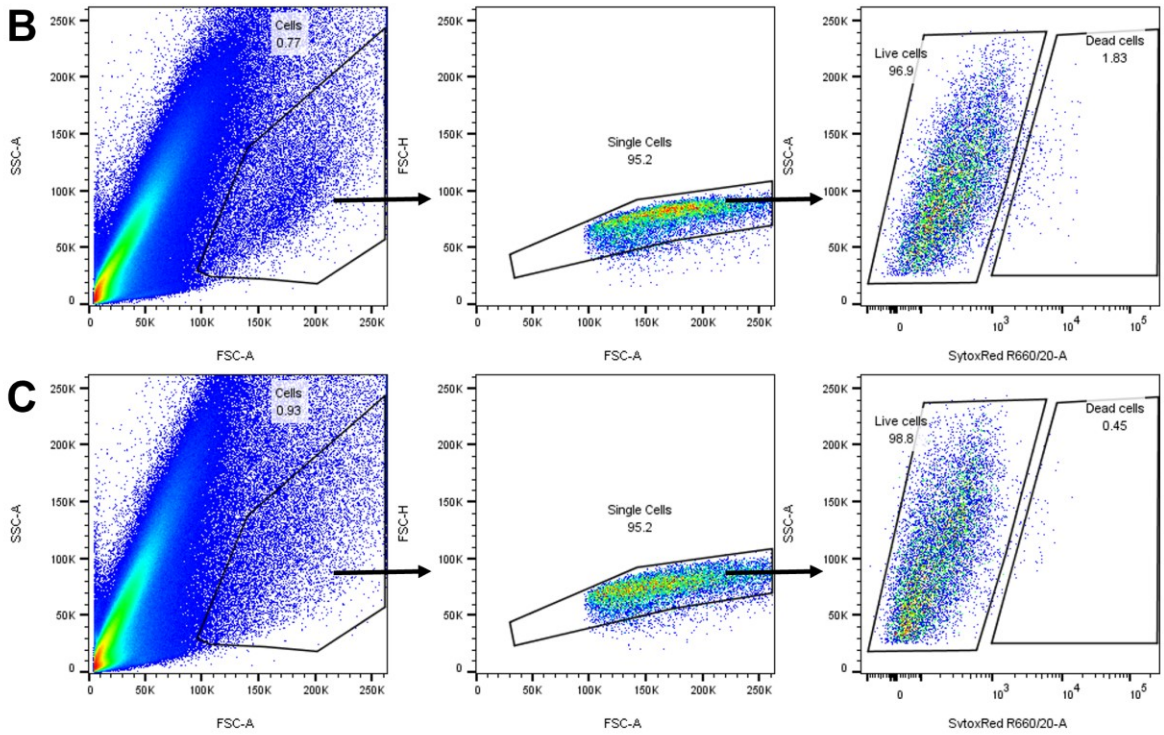


Figure 1. Worker honey bee development and FACS. (A) Development of honey bee worker from egg to Day 15 pupa. Queen bee was trapped on a broodless drawn broodframe in a queen excluder cage for 1 day and removed, samples of eggs, larvae and pupae were taken at one day intervals from frame within excluder cage after queen removal. L1 = 1st larval instar, L5 = 5th larval instar, PP1 = prepupal phase 1, PP2 = prepupal phase 2 , PP3 = prepupal phase 3, Pw= white eyed pupa, Pp = pink eyed pupa. S1 and S2 were stages analysed for single cell transcriptomics. **Representative gating strategy for live single cell sort of stage 1 (B) and stage 2 (C) bee pupae**. The cells gate was defined on size and granularity and then single cells were defined using forward scatter area verses height. Live cells were then sorted by discriminating SytoxRed positive cells.

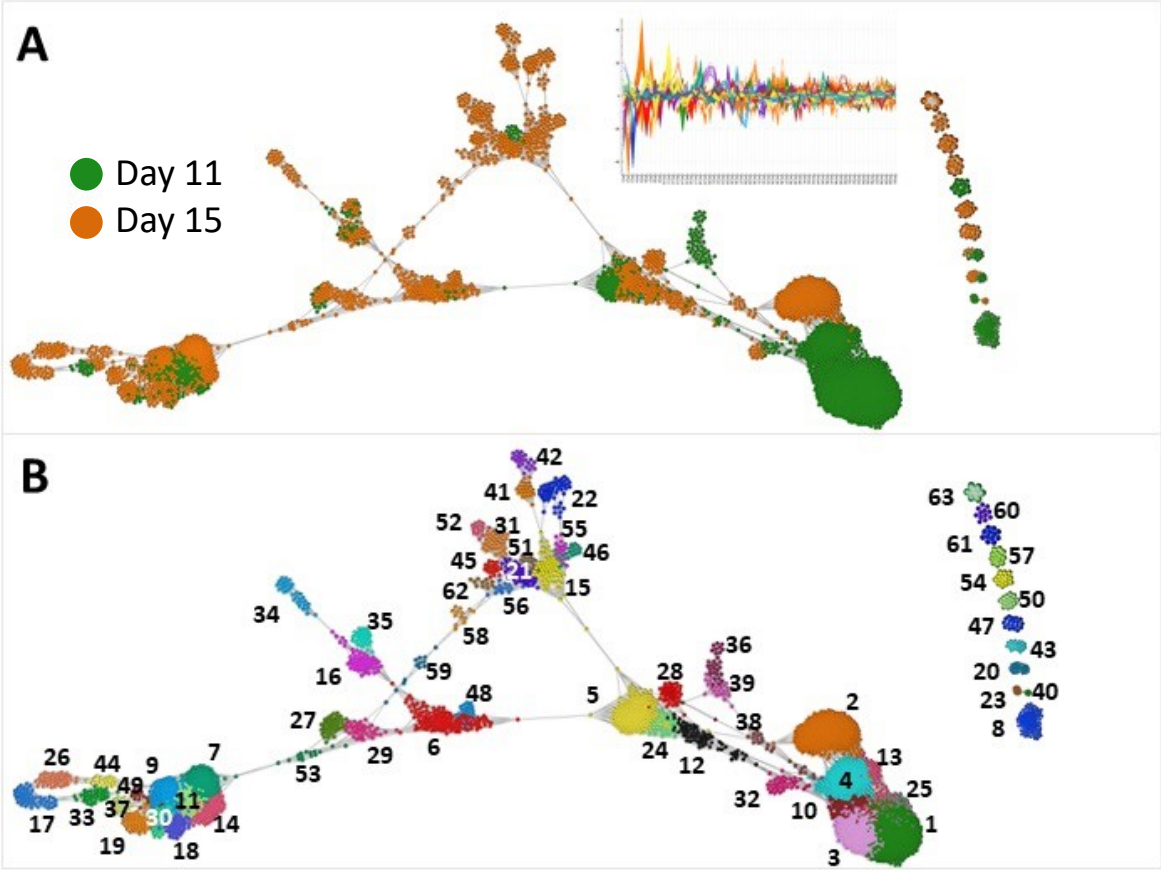


Figure 2. Honey bee cell populations as defined by scRNA-Seq analysis. (A) Cell-to-cell network generated by comparing the 61 most significant PCs for each cell. See insert in **(A)** showing plot of PCA profiles (y axis, each PC signified by colour) for all cells (x axis) in the graph. Each of the node represents an individual cell and the edges the 10 most significant correlations between them *r* threshold > 0.77. The graph is composed of 4,149 cells connected by 31,000 edges. In **(A)** nodes are coloured by the pupal stage from which they were derived. Note the clustering of some cells based on stage, suggesting stage-specific cell populations. In **(B),** nodes are coloured according to their cluster ID, 63 clusters being defined. The clusters disconnected from the central network are positioned on the right. Numbers indicate cluster ID.

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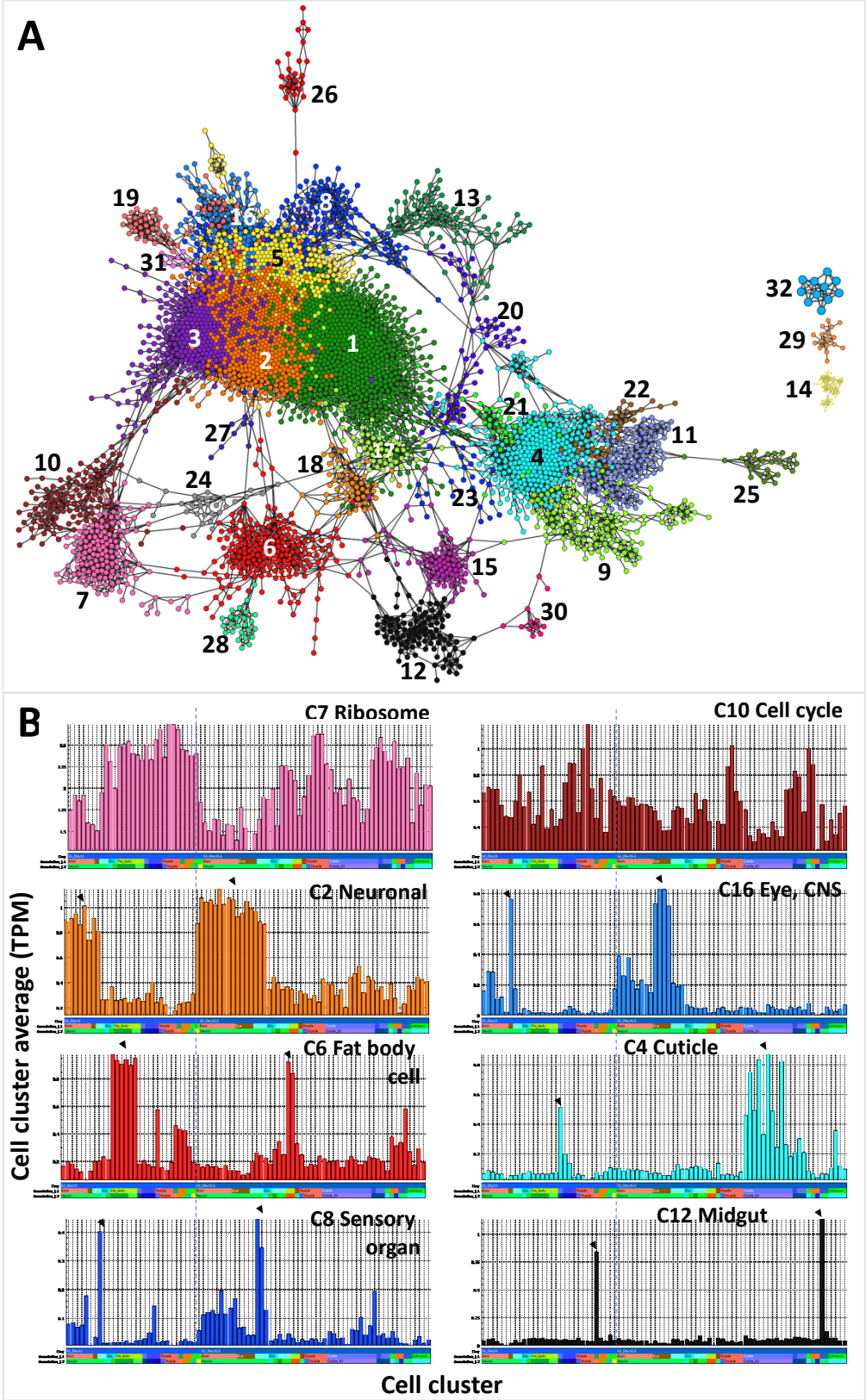

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879 **Figure 3. Gene correlation network analysis of expression profile of genes across cell clusters. (A)**

880 GCN composed of 3,994 nodes (genes) connected by 11,400 edges where *r* threshold > 0.7. Nodes are

881 coloured according to Louvain cluster (granularity 0.65). **(B)** Average expression profile of gene
882 clusters based on each gene's average expression across a cluster of cells. To the left of the dotted
883 line are cell clusters from the day 11 pre-pupa and on the right of the line are cell clusters from the
884 day 15 pupa. Clusters of cells have been grouped based on similarity.

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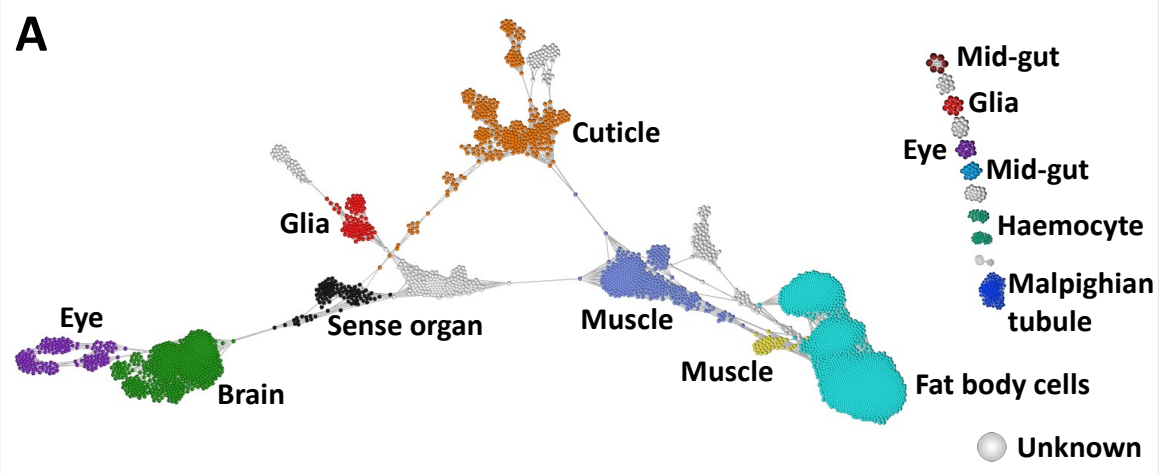

Figure 4. Final assignment of cell identity. The cell-to-cell network is similar to that from figure 3 where each dot represents a cell with similar cells connected to one another. However, it is overlayed with broad level annotation (colour) for the various cell clusters that have been defined based on GCN analysis, Fly Atlas2, Honey Bee Protein Atlas, and literature mining. Clusters where we could not find sufficient supporting evidence are classed as "Unknown" in grey.