

1           **Exploring integument transcriptomes, cuticle ultrastructure, and cuticular hydrocarbons**  
2           **profiles in eusocial and solitary bee species displaying heterochronic adult cuticle maturation**

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32 **Abstract**

33 Differences in the timing of exoskeleton melanization and sclerotization are evident when comparing  
34 eusocial and solitary bees. This cuticular maturation heterochrony may be associated with life style,  
35 considering that eusocial bees remain protected inside the nest for many days after emergence, while  
36 the solitary bees immediately start outside activities. To address this issue, we characterized gene  
37 expression using large-scale RNA sequencing (RNA-seq), and quantified cuticular hydrocarbon (CHC)  
38 through gas chromatography-mass spectrometry in comparative studies of the integument (cuticle plus  
39 its underlying epidermis) of two eusocial and a solitary bee species. In addition, we used transmission  
40 electron microscopy (TEM) for studying the developing cuticle of these and other three bee species  
41 also differing in life style. We found 13,200, 55,209 and 30,161 transcript types in the integument of  
42 the eusocial *Apis mellifera* and *Frieseomelitta varia*, and the solitary *Centris analis*, respectively. In  
43 general, structural cuticle proteins and chitin-related genes were upregulated in pharate-adults and  
44 newly-emerged bees whereas transcripts for odorant binding proteins, cytochrome P450 and  
45 antioxidant proteins were overrepresented in foragers. Consistent with our hypothesis, a distance  
46 correlation analysis based on the differentially expressed genes suggested delayed cuticle maturation in  
47 *A. mellifera* in comparison to the solitary bee. However, this was not confirmed in the comparison with  
48 *F. varia*. The expression profiles of 27 of 119 genes displaying functional attributes related to cuticle  
49 formation/differentiation were positively correlated between *A. mellifera* and *F. varia*, and negatively  
50 or non-correlated with *C. analis*, suggesting roles in cuticular maturation heterochrony. However, we  
51 also found transcript profiles positively correlated between each one of the eusocial species and *C.*  
52 *analisis*. Gene co-expression networks greatly differed between the bee species, but we identified  
53 common gene interactions exclusively between the eusocial species. Except for *F. varia*, the TEM  
54 analysis is consistent with cuticle development timing adapted to the social or solitary life style. In  
55 support to our hypothesis, the absolute quantities of n-alkanes and unsaturated CHCs were significantly  
56 higher in foragers than in the earlier developmental phases of the eusocial bees, but did not  
57 discriminate newly-emerged from foragers in *C. analis*. By highlighting differences in integument gene  
58 expression, cuticle ultrastructure, and CHC profiles between eusocial and solitary bees, our data  
59 provided insights into the process of heterochronic cuticle maturation associated to the way of life.

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63 **Author Summary**

64 From our previous observation that bees with distinct habits of life, eusocial and solitary, exhibit  
65 different degrees of cuticle melanization and sclerotization at the emergence, we decided to analyze the  
66 genetic signatures and ultrastructure of the integument, as well as the CHC profiles that could be  
67 involved in cuticle maturation. The expression patterns of certain genes involved in the  
68 melanization/sclerotization pathway, chitin metabolism, cuticle structure, and also regulators of cuticle  
69 renewal and tanning, in addition to other genes, might be grounded the slow process of cuticle  
70 maturation in the eusocial bees in comparison to the solitary ones. The electron micrographs revealed  
71 differences in the timing of cuticle deposition for the eusocial and solitary species. Among the  
72 identified CHCs, the proportions and quantities of n-alkanes in the developing cuticle are consistent  
73 with the faster cuticular maturation in the solitary bee, thus supporting our hypothesis.

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## 94 Introduction

95 The exoskeleton (cuticle) enables arthropods to exploit a multitude of ecological habitats, and is  
96 central to the evolutionary success and worldwide expansion of insects. It is necessary for muscles  
97 attachment, for protection against predators, injuries, and pathogens [1]. In addition, its thickness is  
98 positively correlated with the resistance to some types of insecticides [2]. The exoskeleton is  
99 periodically shed and a new, larger one is formed, this characterizing the successive molting episodes  
100 that allow for insect growth and development. Its composition is defined by the secretion of products  
101 synthesized by the epidermis as well as by the uptake of molecules from other sources, for instances,  
102 hemolymph [3]. These products are used for cuticle renewal at each molting episode coordinated by  
103 changes in the titer of 20-hydroxyecdysone (20E), the active product of ecdysone hydroxylation. The  
104 Ashburner model postulated to explain 20E-induced chromosomal puffs in the larval salivary glands of  
105 *D. melanogaster* have ultimately led to the knowledge of molecular elements regulating molting and  
106 metamorphosis [4]. When 20E binds to the heterodimeric receptor consisting of EcR (Ecdysone  
107 receptor) and Usp (Ultraspiracle) proteins, its trigger a transcription factor regulatory cascade.  
108 Upstream elements of this cascade respond to the high 20E titer that also induces apolysis and initiates  
109 molting, whereas most downstream elements are only induced by the subsequent decrease in 20E titer.  
110 Binding sites for several of the transcription factors in this cascade were identified in many cuticular  
111 protein genes [5], suggesting that they, and other genes involved in cuticle remodeling [6, 7] are  
112 indirectly regulated by 20E.

113 The exoskeleton comprises an inner procuticle formed by layers of endocuticle and exocuticle,  
114 an outer epicuticle and the superficial envelope. The procuticle consists of a variety of proteins and  
115 chitin, a polymer of the glucose-derived N-acetylglucosamine. Chitin is a major compound in the insect  
116 exoskeleton [8]. Key enzymes in the chitin biosynthetic pathway starting from trehalose are the highly  
117 conserved chitin synthases that catalyze the transformation of UDP-*N*-acetylglucosamine to chitin.  
118 Chitin-modifying enzymes, specifically chitin deacetylases (Cdas), catalyze the conversion of chitin to  
119 chitosan, a polymer of  $\beta$ -1,4-linked d-glucosamine residues. Mutations in Cda genes are lethal to insect  
120 embryos, suggesting that these enzymes play critical roles during development, including the molting  
121 process [9]. Molting involves digestion of the actual cuticle, a process mediated by chitin-degrading en-  
122 zymes, chitinases, which accumulate in the molting fluid [10]. The epicuticle does not contain chitin,  
123 but contains proteins and lipids and is rich in quinones, which are oxidized derivatives of aromatic  
124 compounds [11]. Together with chitin, the structural cuticular proteins constitute the bulk of insect cuti-

125 cle. Based on defining sequence domains, they have been classified into twelve families [12]. Proteins  
126 in the CPR family, with the largest number of members, contain the R&R Consensus [13, 14]. Some  
127 other structural cuticular proteins pertain to the Tweedle (Twdl) class [15], or were classified as Cuticu-  
128 lar Proteins of Low Complexity – Proline-rich (CPLCP), Cuticular Proteins with Forty-four amino acid  
129 residues (CPF), Cuticular proteins analogous to peritrophins (Cpap), Glycine-Rich cuticular Proteins  
130 (GRP), and apidermins, among other classes. Some cuticular proteins, however, do not fill the features  
131 for inclusion in the pre-established classes. The main components of the envelope are the cuticular hy-  
132 drocarbons (CHC) [16] that play roles in chemical communication (unsaturated CHC) [17, 18] and, to-  
133 gether with other lipids, act as a barrier against insect desiccation by preventing water loss (mainly n-  
134 alkanes) [19, 17]. Key enzymes in CHC biosynthetic pathways occurring in the epidermis-associated  
135 oenocytes are the desaturases and elongases [20-22]. We previously determined gene expression pro-  
136 files of six desaturases and ten elongases in the developing integument of *A. mellifera*, and correlated  
137 them with n-alkanes, methyl-alkanes, dimethyl-alkanes, alkenes and alkadienes quantification profiles  
138 [23]. Besides highlighting the CHC composition underlying envelope formation, these data provided  
139 clues to predict the function of these genes in CHC biosynthetic pathways.

140 In addition to chitin, cuticular proteins, CHCs, and other compounds, melanin pigments are  
141 crucial for the exoskeleton formation in insects. The chemical reactions in the core of the melanin  
142 biosynthetic pathway are evolutionary conserved. This pathway comprises the conversion of tyrosine  
143 into 3,4-dihydroxyphenylalanine (dopa) by the action of tyrosine hydroxylase (TH). Dopa is converted  
144 to dopamine, the primary precursor of insect melanin, via a decarboxylation reaction catalyzed by dopa  
145 decarboxylase (Ddc). Dopa or dopamine is further oxidized to dopaquinone or dopaminequinone, and  
146 finally these pigment precursors are converted into dopa-melanin or dopamine-melanin through  
147 reactions catalyzed by dopachrome conversion enzyme, a product of the *yellow* gene, and laccase2.  
148 Alternatively, dopamine is acetylated to N-acetyl-dopamine (NADA), and in conjugation with  $\alpha$ -  
149 alanine originates N- $\beta$ -alanyldopamine (NBAD). Both catechols are precursors for production of  
150 colorless and yellowish sclerotins [24, 25]. Thus, melanization occurs concomitantly to sclerotization  
151 through a shared biosynthetic pathway. Both processes are fundamental for the exoskeleton  
152 development [26], and are developmentally regulated by 20E [27, 28].

153 Among bees, we can distinguish the solitary and eusocial species. In the solitary species, every  
154 female constructs its own nest where it lay eggs, but does not provide care for the eclosed larvae. In  
155 contrast, the social organization is grounded on the division of labor between fertile queens and more

156 or less sterile, or completely sterile, workers that are engaged in nest construction and maintenance,  
157 besides caring for the queen's offspring [29, 30]. The search for genomic signatures of eusociality  
158 evolution in bees has grown since the publication of the *A. mellifera* genome [31] and gained force with  
159 the recent release of two *Bombus* species genomes [32] and the study of Kapheim *et al.* [33] comparing  
160 the genomes of ten bee species.

161 In this context, we draw our attention to the fact that bees greatly vary in the grade of cuticle  
162 melanization/sclerotization at the emergence time (adult ecdysis). In a previous study on the  
163 morphology of the developing adult cuticle [34], we observed that in eusocial bees, but not in the  
164 solitary ones, the process of cuticle melanization/sclerotization leading to cuticle maturation is  
165 extended to the adult stage. After emergence, workers from eusocial species (including the primitively  
166 eusocial bees from Bombini) spend some days performing inside nest activities, and during this period  
167 they stay protected in a safe and provisioned environment [35] where the hygienic behavior provides a  
168 certain level of immunity [36]. In contrast, the newly emerged solitary bees immediately leave the nest.  
169 Therefore, they need a fully mature cuticle to protect them in the external environment. This shift in the  
170 timing of cuticle maturation seems a case of heterochrony, which is defined as a change in the timing of  
171 development of a tissue or anatomical part relative to an ancestor, or between taxa [37]. If this  
172 assumption proves to be true, it can entail a link between the rate of cuticle maturation and the  
173 evolution of sociality in insects.

174 Here, we used the integument (cuticle and its subjacent epidermis) in an approach based on  
175 large-scale RNA sequencing (RNA-seq), transmission electron microscopy (TEM) and gas  
176 chromatography-mass spectrometry (GC/MS) to describe cuticle maturation in two eusocial bee  
177 species, *Apis mellifera* (Apini) and *Frieseomelitta varia* (Meliponini), and a solitary bee species,  
178 *Centris analis* (Centridini), the solitary lifestyle being considered the ancestral condition for bees [38].  
179 TEM was also used for studying the ultrastructure of the cuticle of the primitively eusocial bee,  
180 *Bombus brasiliensis* (Bombini), the facultatively eusocial *Euglossa cordata* (Euglossini), and the  
181 solitary bee, *Tetrapedia diversipes*. This combined approach allowed us to compare the integuments at  
182 the morphological and molecular levels, besides highlighting differences that could be related to the  
183 heterochronic process of cuticle maturation. Among the genes expressed in the integument, we focused  
184 on those involved in the melanization/sclerotization pathway, chitin metabolism, genes encoding  
185 structural cuticular proteins, regulators of cuticle renewal and tanning, desaturase and elongase genes  
186 potentially involved in CHC biosynthesis, circadian clock genes that could determine the rhythm of

187 cuticle layers deposition [39, 40], and genes encoding pigments other than melanin.

188 The comparison of integument transcriptomes of three bee species at developmental points  
189 corresponding to adult cuticle formation, ecdysis, and at a mature age (foragers) gave us back the  
190 discovery of distinct genetic signatures of the integument, and highlighted differences in gene set  
191 expression profiles. The use of TEM and CHC analysis complemented these data by adding new  
192 information on cuticle ultrastructure and chemical profiles of its superficial layer, the envelope.

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## 194 **Results**

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### 196 **Differential gene expression in the integument of *A. mellifera*, *F. varia* and *C. analis* during adult** 197 **cuticle formation/maturation**

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199 We identified the expression of 13,200 genes in the developing integument of *A. mellifera*, and  
200 55,209 and 30,161 contigs in the developing integument of *F. varia* and *C. analis*, respectively (S1  
201 File). The data obtained from the three biological samples of each developmental phase, Pbm (pharate  
202 adult), Ne (newly-emerged) and Fg (forager) of each bee species, in a total of 27 transcriptomes, were  
203 used in Pearson correlation analysis in order to check reproducibility. A hierarchical clustering on  
204 pairwise correlation is shown in S1 Fig. In general, the samples of the same developmental phase  
205 (biological triplicates) joined together, indicating that they are more similar to each other than to  
206 samples of the other developmental phases. As expected, for the three bee species, the least correlated  
207 samples were those originated from the Pbm and Fg integuments. When filtering these data sets for the  
208 genes (DEGs) or contigs (DECs) differentially expressed between the developmental phases, we found  
209 3,184 DEGs for *A. mellifera*, 5,959 DECs for *F. varia* and 2,543 DECs for *C. analis*, representing  
210 24.1%, 10.8%, and 8.4% of the identified genes, respectively. Fig 1 shows the number of genes that  
211 were upregulated in the comparisons between the developmental phases of each of the three bee  
212 species. In *A. mellifera*, 14.8% and 17.8% of the DEGs were upregulated in the Pbm phase in  
213 comparison to the Ne and Fg phases, respectively; 20.9% and 7.8% were upregulated in Ne in  
214 comparison to Pbm and Fg; 24.6% and 10.4% were more expressed in Fg than Pbm and Ne. In *F. varia*,  
215 21.1% and 39.3% of the DECs were upregulated in Pbm compared to the Ne and Fg phases,  
216 respectively; 27.9% and 21.1% DECs were more expressed in Ne than in Pbm and Fg; 39.6% and  
217 16.0% showed higher expression in Fg than in Pbm and Ne. In *C. analis*, the Pbm phase showed higher

218 expression for 39.2% and 31.1% of the DECs in comparison to the Ne and Fg phases; 32.7% and 6.1%  
219 of the DECs were upregulated in Ne in comparison to Pbm and Fg; 31.9% and 3.6% were more  
220 expressed in Fg than in Pbm and Ne. These proportions of upregulated genes significantly vary in the  
221 comparisons of the developmental phases of each bee species and also between the bee species. In  
222 addition, the proportions of genes upregulated in the adult phases (Ne *versus* Fg) were significantly  
223 lower in the solitary *C. analis* than in the eusocial *A. mellifera* and *F. varia* bee species (z test,  $p \leq$   
224 0.001, except for one of the comparisons where  $p = 0.014$ ).

225 To make more comprehensive the RNA-seq analysis of the integument, we searched the Gene  
226 Ontology (GO) functional terms for all *A. mellifera* DEGs and all *F. varia* and *C. analis* DECs. The GO  
227 annotations for Molecular Function, Cellular Component and Biological Process categories are  
228 described in S3 File. We then extracted from this analysis the functional terms more evidently related  
229 to cuticle development (Fig 2). Structural molecule activity, chitin-binding, and chitin metabolic  
230 process were categories overrepresented in the younger phases, i.e., the Pbm and Ne phases of the three  
231 bee species. Structural constituent of cuticle, structural constituent of chitin-based cuticle, and other  
232 cuticular components-related GO categories also included genes more expressed in the Pbm and Ne  
233 integuments of both, or one of the eusocial species. Functional categories related to the epidermis,  
234 which is the tissue responsible for secreting the cuticle, specifically epithelium development, epithelial  
235 cell differentiation/development, cell adhesion, cell junction organization/assembly, among other  
236 categories, were also more represented in the younger Pbm and Ne bees, but only of the eusocial  
237 species. For the three bee species, the DEGs and DECs more related to the functionality of the  
238 integument of newly-emerged (Ne) and forager bees (Fg) (here named older phases for simplification),  
239 were included in the following overrepresented GO terms: fatty acid biosynthetic process, lipid  
240 biosynthetic process, organic acid biosynthetic process, and carboxylic acid biosynthetic process. These  
241 terms and others overrepresented in the older Ne and Fg phases of *F. varia* and *C. analis*, i.e., very-  
242 long-chain fatty acid metabolic process, and fatty acid metabolic process, could be tentatively related to  
243 CHC biosynthetic pathways. For *F. varia* and/or *C. analis*, functional terms related to pigmentation  
244 pathways (pigmentation, pigment metabolic process, pigment biosynthetic process, pigmentation  
245 during development, and terms related to eye pigments), were also significantly more represented in the  
246 Ne and Fg phases. These GO results (Fig 2) evidenced the similarities and differences in terms of  
247 cuticle-related functional attributes between the developmental phases and bee species. Some  
248 functional categories were shared by the three bee species, and a larger number of categories were

249 shared by the two eusocial species than by one of them and *C. analis*.

250 S2 File specifies the genes upregulated between the developmental phases and bee species.  
251 Among the DEGs and DECs, it was clear that those encoding structural cuticular proteins, such as  
252 those in the CPR, Twdl, and Cpap families, and also chitin-related genes with roles in chitin  
253 metabolism, modification and degradation, were upregulated in the Pbm and/or Ne phases of the three  
254 bee species here studied. A series of sequences containing the chitin-binding peritrophin A domain were  
255 similarly overrepresented in the integument of the Pbm and/or Ne phases of *F. varia* and *C. analis*, thus  
256 being candidates to participate as structural proteins or enzymes in cuticle formation. In contrast, genes  
257 encoding odorant-binding proteins that bind to pheromones thus serving as insect chemoreceptors, as  
258 well as genes encoding a variety of CYPs (cytochrome P450), and antioxidant proteins like  
259 glutathione-S-transferase (GST), glutathione peroxidase (GTPx), thioredoxin peroxidase (TPx), and  
260 superoxide dismutase (SOD), were more expressed in the mature integument of foragers of the three  
261 bee species. Transcripts for genes related to the activity of juvenile hormone (JH), which is produced in  
262 a greater quantity in foragers [41], specifically *Krüppel homolog 1* (*Kr-h1*), and *JH-esterase* (*jhe*), were  
263 found in higher levels in the Fg integument of *F. varia* and *C. analis* than in the younger phases;  
264 transcripts for a JH-inducible (JHI-1) protein were overrepresented in the Fg and Ne integuments of *A.*  
265 *mellifera* in comparison to the Pbm integument. The Fg integument of *C. analis* showed a higher  
266 expression of the *ecdysone receptor* (*EcR*) and *seven-up*, an orphan nuclear receptor belonging to the  
267 steroid receptor gene superfamily [42]; *seven-up* is also overexpressed in the Fg integument of *F. varia*.  
268 Defense response genes (*defensin*, *apidaecin*) were also highly expressed in the Fg integument of *A.*  
269 *mellifera* (S2 File). Such developmental differences in gene expression in the integument reflect the  
270 dynamics of cuticle formation and acquisition of its functionality in adult bees

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272 **Distance correlation analysis based on the RNA-seq data is consistent with the earlier cuticle  
273 maturation in the solitary *C. analis* in comparison to the eusocial *A. mellifera***

274 We used the DEGs and DECs in a distance correlation analysis in order to measure the  
275 clustering potential of the studied developmental phases of each bee species (Fig 3). This strategy  
276 allowed us to know for each of the bee species how near, or distant from each other are the Pbm, Ne  
277 and Fg developmental phases in terms of gene expression levels/patterns in the integument. Assuming  
278 that the cuticle of solitary bee species is sufficiently mature at the emergence, the hypothesis  
279 approached here was that the integument samples of the Ne and Fg phases of *C. analis* would cluster

280 together, and separately from the Pbm samples. In contrast, in the eusocial species, the Pbm and Ne  
281 samples would group together, with the Fg samples forming a more distant group. Indeed, the results of  
282 the distance correlation analysis using all the *C. analis* DECs and *A. mellifera* DEGs were consistent  
283 with this hypothesis. In terms of differential gene expression in the integument, the Ne and Fg phases  
284 are nearest to each other in the solitary bee than they are in the eusocial *A. mellifera*. However, in *F.*  
285 *varia*, the distance correlation analysis grouped the Ne and Fg phases in a statistically supported  
286 cluster, in spite of the very distinct cuticle melanization patterns and hardness that they exhibit.

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## 288 **Gene expression profiles in the integument of the eusocial (*A. mellifera* and *F. varia*) and solitary 289 (*C. analis*) bee species**

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291 Heatmaps representing the expression profiles of classes of cuticle-related genes through the  
292 Pbm, Ne and Fg developmental phases were constructed and clearly showed differences between the  
293 bee species (Fig 4).

294 We found in the RNA-seq libraries seven genes involved in the biosynthesis of melanin and  
295 sclerotizing compounds (see a representation of the melanin/sclerotin biosynthetic pathway in Shamim  
296 *et al.* [24]). The genes with roles in the melanization/sclerotization pathway, except for *Dat*, were more  
297 expressed in the younger phases (Pbm and/or Ne) of *A. mellifera*. Similarly, these genes, including *Dat*,  
298 were more expressed in the younger phases of *F. varia*. In contrast, in *C. analis*, the majority of the  
299 genes in this class (*tan*, *Ddc*, *Lac2*, *yellow-y*) did not significantly change their expression levels, *ebony*  
300 was highly expressed in the Ne and Fg phases, and the expression profile of *Dat* also differed from  
301 both eusocial species. *TH* was the only gene in this class showing a significantly higher expression  
302 level in the very same developmental phase (Ne) of the three bee species.

303 Searching for genes related to pigmentation pathways other than the melanin biosynthetic  
304 pathway in the integument RNA-seq libraries, such as those genes involved in pterin, ommochromes,  
305 and heme formation, we found 17 genes in *A. mellifera*, and 18 genes in *F. varia* and also in *C. analis*,  
306 including *cardinal*, *scarlet*, *brown*, *vermillion*, *light*, *sepia*, and *henna* (this one involved in both  
307 biopterin formation, and tyrosine formation for the melanization process), thus indicating that their  
308 products are necessary in the adult cuticle. We also observed that a higher proportion (66.7%) of these  
309 genes displayed higher expression levels in the adults (Ne, Fg, or both phases) of *F. varia* in  
310 comparison to *A. mellifera* (29.4%) and *C. analis* (27.8%).

311        Concerning genes encoding chitin-related enzymes, we found 17, 16 and 33 of these genes in *A. mellifera*, *F. varia* and *C. analis*, respectively. The four *Cda* genes (*Cda4*, *Cda5*, *verm* and *serp*) found  
312        in the eusocial species, and five (*Cda4-like*, *Cda-like-1*, *Cda-like-2*, *verm*, *serp*) of the six *Cda* genes  
313        found in *C. analis* showed the higher expression in the Pbm, Ne, or both developmental phases, the  
314        other *C. analis Cda* gene (*Cda5-like*) showed no significant expression levels variation throughout the  
315        developmental phases. One of the two *ChS* genes found in *A. mellifera* (*kkv*) and *F. varia* (*ChS6*), and  
316        two of the four *ChS* genes found in *C. analis* (*ChS-kkv-like-1*, *ChS6-like1*) were also more expressed in  
317        the Pbm and/or Ne phases whereas the other *ChS* genes of the three bee species did not show  
318        significant expression level variation. Six (*Cht-like2*, *Cht-2 like*, *chitooligosaccharidolytic-domain-like*,  
319        *Cht5*, *chitotriosidase*, *Cht3*) of the eleven *Cht* genes of *A. mellifera*, and four (*Cht-like1*, *Cht-like*,  
320        *chitooligosaccharidolytic-domain-like*, *Cht2-like2*) of the ten *Cht* genes of *F. varia* were also more  
321        expressed in the Pbm and/or Ne phases, the remaining showing no significant variation in expression  
322        levels, except for the chitinase-encoding gene, *Idgf-4*, which is significantly more expressed in *A.*  
323        *mellifera* foragers. In contrast, only a small number (*Cht-like12*, *Cht-like4*, *Cht-like10*, *Cht-like1*) of the  
324        22 *Cht* genes of *C. analis* were more expressed in these phases, the remaining showing no significant  
325        changes in expression levels, except for *Idgf-4*, which is more expressed in foragers.

327        The majority of the CPR genes (encoding cuticle proteins containing the RR1 or RR2  
328        Consensus types) in the eusocial species showed significant variation in expression levels through the  
329        studied developmental phases, the proportions of RR1 and RR2 genes showing variable expression  
330        corresponding to 94.1% and 80.9% in *A. mellifera*, and 66.7% and 70.6% in *F. varia*, respectively. In  
331        contrast, lower proportions of RR1 and RR2 genes in *C. analis*, corresponding to 40% and 35%  
332        respectively, showed significant variation in transcript levels. For the three bee species, most of the  
333        genes showing changing transcript levels, in the range of 75 to 100%, were more expressed in the Pbm  
334        or both Pbm/Ne phases. Interestingly, a few CPR genes were significantly more expressed in the Ne  
335        phase (*AmCPR19*, *AmCPR27*, *AmSgAbd1-like*, *FvUnCPR-1*), or in both Ne and Fg phases (*CaSgAbd1-like*  
336        and *AmUnCPR-RR2-5*), and only a CPR gene, the RR1 motif *AmCPR13* gene, showed a higher  
337        expression exclusively in foragers. Similarly, a higher proportion of the non-RR cuticular protein genes  
338        showed significant transcript levels variation in *A. mellifera* and *F. varia*, 90.6% and 72.2%  
339        respectively, in comparison to *C. analis* (64.5%). These genes were also mostly more expressed in the  
340        Pbm or Pbm/Ne phases of the three bee species. However, like some CPR genes, there were non-RR  
341        genes displaying the highest expression in adults (Ne, Fg or both phases), specifically, *Apd* genes in *F.*

342 *varia* (*FvApd-1*) and *C. analis* (*CaApd-1* and *CaApd-2*), and *Cpap* genes in *C. analis* (*CaUnCpap-3*,  
343 *CaUnCpap-4*, *CaUnCpap-9*, *CaCpap3-e*).

344 For the three studied bee species, a higher proportion of genes encoding elongases (Elo-genes)  
345 and desaturases (Desat-genes) putatively involved in CHC biosynthesis were more expressed in adults  
346 (Ne, Fg, or both phases) than in the Pbm phase. However, in *C. analis*, a higher proportion (66.7%) of  
347 these genes increased significantly their expression levels from the Pbm to the Ne phase in comparison  
348 to *F. varia* (26.7%) and *A. mellifera* (39.1%).

349 A higher proportion of the regulatory genes was significantly more expressed in the Pbm phase  
350 of *A. mellifera* (50%) and *F. varia* (28.6%) than in *C. analis* (4.5%) in which the majority of the genes  
351 (72.7%) did not show significant difference in expression levels between the developmental phases.  
352 Some regulatory genes had a higher expression in adults (Ne, Fg or both phases) of *A. mellifera*  
353 [*Ammirr* (*mirror*), *AmUsp* (*Ultraspiracle*), *AmCCAP* (*Crustacean Cardioactive Peptide*), *AmKr-h1* and  
354 *Amhairy*], *F. varia* (*FvKr-h1*), and *C. analis* (*CaKr-h1*, *Camirr*, *CaE75*, *CaEcR* and *Cahairy*). Two of  
355 the regulatory genes in *A. mellifera*, *AmE75* and *AmMblk* (*Mushroom body large type Kenyon cell*  
356 *specific protein-1* or *E93-like*), which were highly expressed in the younger Pbm phase, were also  
357 highly expressed in the older Fg phase.

358 Four among the seven circadian rhythm genes of *A. mellifera* [*Clk* (*Clock*), *Cry*  
359 (*Cryptochrome*), *Per* (*Period*) and *Tim2* (*Timeless2*)] showed the highest expression in the Pbm phase.  
360 This is in contrast to the majority of the circadian rhythm genes in *F. varia* [*Clk*, *Per*, *Pdp1* (*Par*  
361 *domain protein 1*), *Vri* (*vrielle*), *Tim2*] and *C. analis* (*Per*, *Vri*, *Cry*, *Clk*, *Tim2*), which did not  
362 significantly change their expression levels. The genes *Vri*, *Cyc* (cycle), and *Pdp1* in *A. mellifera*, *Cyc*  
363 in *F. varia*, and *Cyc* and *Pdp1* in *C. analis* showed the highest expression in adults (Ne, Fg or both  
364 phases).

365 In summary, the main differences between the social and solitary bee species were highlighted  
366 in the heatmaps (Fig 4) displaying integument gene expression profiles: **(a)** A higher proportion of  
367 genes involved in the melanization/sclerotization pathway, cuticle formation (RR1, RR2, and non-RR  
368 genes), and regulation (regulatory genes) showed significant transcript levels variation through the  
369 studied developmental phases of *A. mellifera* and *F. varia* in comparison with *C. analis*. Most of these  
370 genes showing transcript levels variation were more expressed in the Pbm or Pbm/Ne phases. In *C.*  
371 *analisis*, the higher proportion of genes displaying no differences in expression levels through the studied  
372 phases, were possibly highly expressed earlier, before the Pbm phase, for faster cuticle formation and

373 maturation, but this assumption requires further investigations; **(b)** The number of chitin-related genes,  
374 higher in *C. analis*, and not their expression patterns, distinguished this species from the eusocial *A.*  
375 *mellifera* and *F. varia*; **(c)** A higher proportion of desaturase and elongase genes putatively involved in  
376 CHC biosynthesis showed significantly increased expression levels at the emergence (Ne phase) of *C.*  
377 *analis* in comparison to the eusocial ones, which is consistent with an accelerated process of cuticle  
378 maturation in the solitary bee.

379 Importantly, all the gene classes here studied included representatives showing increased or  
380 high expression levels in the mature integument of foragers indicating that the mature cuticle is a  
381 dynamic structure requiring structural and regulatory elements for its maintenance.

382

383 **Correlation among expression profiles of genes candidates to play roles in cuticle formation/**  
384 **maturation in the eusocial (*A. mellifera* and *F. varia*) and solitary (*C. analis*) bees**

385

386 We used Pearson's correlation in order to measure the strength of the linear association between  
387 the expression profiles of 119 genes related to cuticle development and maturation shown in Fig 4,  
388 which shared potential orthology relationships between the bee species. A fraction of these ortholog  
389 genes showed non-significantly correlated transcript levels fluctuation among the bee species, thus  
390 highlighting peculiarities in cuticle development for each species. However, 76 orthologs (S1 Table;  
391 Fig 5) displayed expression profiles significantly correlated at least between two of the three bee  
392 species. Importantly, the expression profiles of 21 among these 76 genes were positively correlated  
393 between the eusocial species, and negatively or non-correlated with the solitary bee ( $r \geq 0.6$  and  $p \leq 0.1$ ).  
394 In addition, other six genes, whose transcripts were not identified in *C. analis*, showed expression  
395 profiles positively correlated between the eusocial species. Therefore, these 27 genes are possibly  
396 contributing to differences in the processes of cuticle development and maturation in the eusocial bees  
397 versus the solitary bee. Thus, the expression profiles of genes related to the melanization/sclerotization  
398 pathway (*ebony*, *tan*) and chitin metabolism [*Idgf4-like*, *Cda5* (*Chitin deacetylase 5*),  
399 *chitooligosacchariodolytic-domain-like*], genes encoding cuticular structural proteins containing the  
400 RR1 or RR2 domains (*CPR14*, *CPR17*, *CPR18*, *CPR23*, *CPR25*, *CPR26*), or lacking these domains  
401 (*Apd-3*, *Apd-like*), and also genes in CHC pathways (*Desat-GB40659*, *Elo-GB54401*, *Elo-GB54302*,  
402 *Elo-GB45596*, *Elo-GB46038*), regulators of cuticle development [*Ethr* (*Ecdysis triggering hormone*  
403 *receptor*), *E74*, *Hr4* (*Hormone receptor 4*), *Hr38* (*Hormone receptor 38*), *FTZ-F1* (*Fushi tarazu-factor*)

404 *I*), *ricketts*, *Ptx-1* (bicoid-related *Paired-type homeobox gene D*), circadian rhythm genes (*Tim2*) and a  
405 gene in the non-melanin pigmentation pathways, *ALAS* ( $\delta$ -aminolevulinic acid synthase)], suggest roles  
406 in the differential cuticle development in the solitary versus eusocial bees.

407 Among the above cited 76 orthologs, we also found genes whose expression profiles were  
408 positively correlated between the solitary and eusocial bees. Thus, the following 23 genes shared  
409 expression profiles positively correlated between *A. mellifera* and *C. analis*: *yellow-y* (melanization  
410 /sclerotization pathway), *Cda4* and *ChS-kkv-like1* (chitin metabolism), *SgAbd2-like* and *97Ea-like*  
411 (CPR-RR1 class), *CPR10* (RR2 class), *Twdl(Grp)*, *Cpap3-a*, *Cpap3-b* and *Cpap3-c* (non-RR class),  
412 *Desat-GB48195*, *Desat-GB45034*, *Desat-GB42217*, *Elo-GB51249* and *Elo-GB54404* (CHC pathways),  
413 *Usp*, *CCAPR*, *Mirr* and *hairy* (regulatory genes), *Cyc* (Circadian rhythm), *verm*, *sepia* and *pinta-like*  
414 (other pigmentation biosynthetic pathways than melanin). Similarly, the following 12 genes shared  
415 expression profiles positively correlated between *F. varia* and *C. analis*: *Cda4* and *ChS6* (chitin  
416 metabolism), *Cpap3-c* (non-RR class), *Elo-GB54404* (CHC pathways), *Burs $\beta$* , *CCAP* and *E75*  
417 (regulatory genes), *Cyc* (Circadian rhythm), *verm-like*, *cardinal-like*, *light* and *scarlet* (other  
418 pigmentation biosynthetic pathways than melanin).

419

## 420 **Co-expression networks reconstructed with genes related to cuticle development and maturation, 421 and common interactions between the networks of the eusocial *A. mellifera* and *F. varia* bees**

422

423 The genes related to cuticle formation and maturation in *A. mellifera*, *F. varia*, and *C. analis*  
424 were separately used for co-expression networks reconstruction (S2-S4 Figs). The gene co-expression  
425 networks for the eusocial species, *A. mellifera* and *F. varia*, showed common interactions among  
426 regulatory elements [*FTZ-F1*, *E74*, *Hr4*, *Hr46* (*Hormone receptor 46*)], genes encoding structural  
427 cuticular proteins (CPR14, CPR17, CPR23, CPR24, CPR25, Apd-3 and Apd-like), and encoding the  
428 elongase *Elo-GB54302*, Cdas [*verm* (*vermiform*), *serp* (*serpentine*), *Cda5*], and *Lac2* (Fig 6). However,  
429 by intersecting the gene co-expression networks of the eusocial *A. mellifera* and the solitary *C. analis*,  
430 we found only one common interaction comprising the genes *yellow-y* and *Cpap3-a*. Similarly, only the  
431 interactions between *CPR16* and *Eh-like* (*Eclosion hormone*), and *tan*/*Elo-GB45596* were highlighted  
432 as being common to the eusocial *F. varia* and the solitary *C. analis* after superimposing their respective  
433 gene co-expression networks.

434

435 **Ultrastructure and thickness of the developing adult cuticle shows conspicuous differences among**  
436 **the eusocial, primitively eusocial, facultatively eusocial, and solitary bee models**

437

438 The morphology of the developing adult cuticle is shown for the eusocial *A. mellifera* and *F.*  
439 *varia* bees, for the primitively eusocial *Bombus brasiliensis*, for the facultatively social *Euglossa*  
440 *cordata*, and for two solitary bees, *C. analis* and *T. diversipes* (Fig 7). For *A. mellifera*, there were no  
441 noticeable modifications in cuticle ultrastructure from the pharate-adult phase (Pbm) to 48h after  
442 emergence. Up to this time, only the exocuticle was deposited. At 72h, endocuticle layers became  
443 apparent in the micrographs (Fig 7A). Cuticle ultrastructure was very similar in 96h-aged *A. mellifera*  
444 bees and foragers (Fig 7A). We then measured the thickness of the cuticle in seven time points of *A.*  
445 *mellifera* development (Fig 7A'). As the cuticle measurements in the groups of bees aging 0h to 96h  
446 post-emergence, and in the group of foragers, did not show a normal distribution (Shapiro-Wilk  
447 normality test,  $p = 0.0074$ ) we used the Kruskal-Wallis test associated with the *post hoc* Conover-Iman  
448 test and Bonferroni correction to compare the sample collection data. Foragers have a significantly  
449 thicker cuticle in comparison to the earlier developmental phases, i.e., the Pbm phase, and bees at 0h,  
450 24h and 48h after emergence (Fig 7A'). At 72h and 96h post-emergence, cuticle measurements values  
451 did not significantly differ from foragers. Differently, the cuticle of the eusocial *F. varia* showed very  
452 little variation in morphology (Fig 7B), and no significant variation in thickness (Fig 7B') from the  
453 Pbm phase to the forager time. For the solitary species, *C. analis*, we observed remarkable differences  
454 in cuticle ultrastructure (Fig 7C) and thickness (Fig 7C') between the Pbm and Ne phases, whereas the  
455 cuticles of the Ne and Fg phases were very similar. Pore canals are abundant in the Pbm cuticle of *C.*  
456 *analis*. At the Ne and Fg phases, the *C. analis* cuticle can be described as a succession of lamellae, the  
457 most superficial ones, i.e., those first deposited, became thicker and reached a higher degree of  
458 differentiation (Fig 7C). Like *C. analis*, the cuticle of *B. brasiliensis* (Fig 7D, 7D'), *E. cordata* (Fig 7E,  
459 7E'), and *T. diversipes* (Fig 7F, 7F'), did not show noticeable ultrastructural changes, or statistically  
460 significant thickness differences, from the emergence (Ne phase) to the forager time (Fg phase).

461 Together, these data indicate that cuticle deposition in the solitary species, *C. analis* and *T.*  
462 *diversipes*, and the primitively and facultatively eusocial species, *B. brasiliensis* and *E. cordata*,  
463 respectively, is completed or almost completed at the time of adult emergence. In contrast, in *A.*  
464 *mellifera*, the endocuticle was deposited only after the emergence. Surprisingly, the cuticle of the  
465 eusocial *F. varia* species did not undergo significant variation in ultrastructure and thickness from the

466 Pbm to the Fg phases, although a great increase in pigmentation and sclerotization has been clearly  
467 noticed in *in vivo* observations.

468

469 **Cuticular n-alkanes mark the earlier cuticle maturation in the solitary *C. analis* compared to the**  
470 **eusocial *A. mellifera* and *F. varia* bee species**

471

472 The CHC composition of the superficial cuticle layer, the envelope, was determined for *A. mellifera*, *F. varia* and *C. analis* as another strategy potentially able to uncover differences that could be  
473 associated to the cuticle maturation heterochrony. The proportion of CHCs in the chromatograms, the  
474 significance level of each peak and the contribution of these peaks for discriminating the  
475 developmental phases of the eusocial and solitary species are shown in S4 File. The Euclidean distance  
476 clustering analysis applied to the total CHC quantification data clearly discriminated the Fg phase from  
477 the earlier Pbm and Ne phases in the eusocial bees, *A. mellifera* and *F. varia*, as well as in the solitary  
478 *C. analis* (S5 Fig). Total CHC quantification data grouped together the Pbm and Ne samples of *A. mellifera* (AU=100; BT=100), *F. varia* (AU= 100; BT=100), and *C. analis* (AU=96; BT=88). For *F. varia*, the group including Ne samples showed AU=95 and BT=87, which is a moderate to high BT  
481 value usually associated with Bayesian posterior probabilities  $\geq 95\%$  [43]. The same was verified for  
482 the *F. varia* Pbm samples (AU=94; BT=87). For the two eusocial species, the Fg samples grouped with  
483 maximal AU (100) and BT (100) values. For *C. analis*, however, these values were significantly lower  
484 (AU=78; BT=57) (S5 Fig).

486 When we analyzed separately the CHC classes, n-alkanes discriminated the *A. mellifera*  
487 foragers (Fg) (AU=94; BT=84) from the Pbm and Ne developmental phases, which were clustered  
488 together (AU=85; BT=77). As in *A. mellifera*, n-alkanes also discriminated the *F. varia* foragers (Fg) as  
489 a separate group (AU=97; BT=76), and the Ne and Pbm phases were clustered together (AU=98;  
490 BT=77). However, the n-alkanes did not significantly distinguish the developmental phases of *C. analis* (S5 Fig).

492 The unsaturated CHCs data from *A. mellifera* did not give us back a strong support for  
493 distinguishing the developmental phases. Although all the Ne samples and the majority of the Pbm  
494 samples have been grouped with a high AU value (99%), the BT=1 value was low. Three of the *A. mellifera*  
495 foragers (Fg) escaped from the main cluster formed by twelve foragers (AU=96; BT=23). In  
496 contrast, the unsaturated CHCs discriminated each of the developmental phases of *F. varia*. The groups

497 of Pbm samples (AU=99; BT=93) and Ne samples (AU=99; BT=94) were maintained together in a  
498 larger cluster (AU=99; BT=98), and separately from the group of Fg samples (AU=99; BT=97). This  
499 CHC class clustered together the Pbm and Ne samples of *C. analis* (AU=96; BT=80). The Fg samples  
500 of *C. analis* were separated into two main clusters, respectively supported by AU=94; BT=70 and  
501 AU=93, BT=83 (S5 Fig).

502 Branched CHCs from *A. mellifera* clearly clustered the Fg samples (AU=97; BT=94). The Ne  
503 and Pbm phases were joined together in a single well-supported group (AU=100; BT=100). In *F. varia*,  
504 separation of Fg from the earlier phases was not clear: three of the fifteen Fg samples joined to the  
505 group encompassing the Pbm and Ne samples, this group being supported by 98% AU, but showing a  
506 low BT value (BT=3). The *F. varia* forager samples were also clustered with low BT values. In the  
507 solitary *C. analis*, the branched CHCs clustered six of the seven Fg samples into a single group (AU=  
508 97; BT=72), and all the Ne samples plus two of the four Pbm samples were clustered together in  
509 another group supported by AU=99, but presenting a low BT value (BT=39) (S5 Fig).

510 These data on the Euclidean distance based on the relative quantification of CHCs was  
511 contrasted with the results on the absolute quantification of CHCs (CHC  $\mu\text{g}$  per bee) (Table 1; S4 File).  
512 Table 1 shows that Fg bees of the eusocial species have significantly higher quantities of n-alkanes than  
513 the Ne and Pbm bees, which is not true for *C. analis*. In addition, absolute quantification of unsaturated  
514 CHCs also distinguished the foragers from the earlier developmental phases of *A. mellifera*, but not of  
515 *C. analis*. For *F. varia*, the mass of unsaturated compounds could not be quantified due to their very  
516 low quantities.

517 In summary, the Euclidean distance analysis based on the relative quantifications of n-alkanes,  
518 as well as the absolute quantifications of n-alkanes and unsaturated CHCs, were consistent with the  
519 hypothesis of interdependence between cuticle maturation timing and the eusocial/solitary ways of life.  
520 These analyses distinguished the foragers from the younger bees, but only in *A. mellifera* and *F. varia*,  
521 this being interpreted as the cuticle achieving its complete maturation tardily in the eusocial species,  
522 whereas the solitary bee emerges with an already mature cuticle.

523  
524

525 **Table 1. Absolute quantification of n-alkanes and unsaturated CHCs in the cuticle of eusocial and**  
526 **solitary bee species.** Developmental phases are indicated: Pbm (pharate-adults), Ne (newly emerged  
527 bees), Fg (foragers). Means and standard deviations (STD) of 3 samples (N=3) per developmental

528 phase. Different lowercase letters in the Sig (statistical significance) column indicate difference  
529 between the developmental phases of each species.

N-alkanes				
<i>A. mellifera</i>				
Phase	Mean	±	STD	Sig.
Pbm	9.35965938	±	2.89275421	a
Ne	7.36272669	±	1.44535498	a
Fg	18.2360314	±	4.35877417	b
<i>F. varia</i>				
Phase	Mean	±	STD	Sig.
Pbm	1.63324436	±	0.15390427	a
Ne	3.40142407	±	1.35387231	a
Fg	9.28337077	±	3.03839358	b
<i>C. analis</i>				
Phase	Mean	±	STD	Sig.
Pbm	6.56549413	±	1.62457012	a
Ne	14.8349947	±	0.32610609	b
Fg	13.3273848	±	5.07830924	ab
Unsaturated CHC				
<i>A. mellifera</i>				
Phase	Mean	±	STD	Sig.
Pbm	0.60042039	±	0.17210242	a
Ne	0.92421769	±	0.09047864	a
Fg	6.5543118	±	2.38207067	b
<i>C. analis</i>				
Phase	Mean	±	STD	Sig.
Pbm	9.24954719	±	2.48578756	a
Ne	19.242	±	2.40516304	ab
Fg	28.3380901	±	11.855003	b

Standard deviation (STD). Different red letters in significance (Sig.) column represent statistical significance (ANOVA associated to Tukey's HSD post hoc test) between developmental phases of a species.

531

532

533 **Discussion**

534

535 The RNA-seq analysis revealed the set of genes expressed in the integument of three bee

536 species, and also the changes in gene expression as the adult cuticle is deposited and differentiates in a  
537 mature and fully functional cuticle. For *A. mellifera*, for which we have the sequenced genome, the  
538 genes expressed in the integument represented 95.07% of the genes in the released genome assembly  
539 version 4.5. Similar proportions will likely be found for *F. varia* and *C. analis* in the near future, after  
540 the sequencing of their respective genomes. Selected genes with potential roles in cuticle formation and  
541 maturation were characterized in terms of differential expression profiles. Co-expression networks  
542 were reconstructed. In parallel, we examined the ultrastructure of the developing adult cuticle of bee  
543 species. Furthermore, the CHC composition of the envelope, the less known cuticle layer, was also  
544 characterized. Our data expanded the knowledge on the insect integument. It is our expectation that the  
545 obtained data provide a valuable resource for future studies on exoskeleton formation and maturation in  
546 arthropods.

547

548 **Expression profiles of cuticle-related genes may significantly differ during adult cuticle  
549 formation/maturation, and among bee species.**

550

551 Genes involved in adult cuticle formation in *A. mellifera* in general show higher expression  
552 soon after the ecdysteroid titer peak that signalizes pupal cuticle apolysis and the beginning of the  
553 pharate-adult stage [44, 45]. Consistently, the majority of the integument genes showing expression  
554 levels variation in the three bee species, and identified as playing roles in cuticle melanization/sclerotiza-  
555 zation, cuticle structure (RR1, RR2, and non-RR genes), and regulation of the molting events (regula-  
556 tory genes), displayed a higher expression in pharate-adults (Pbm phase), sometimes extending their  
557 higher expression up to the emergence time (Ne phase). However, we found genes, including those re-  
558 lated to melanization/sclerotization and other pigmentation pathways, and also genes related to chitin  
559 metabolism, and structural cuticle protein genes, which showed the highest expression later, at emer-  
560 gence (Ne phase), and even in foragers (Fg phase), suggesting that their products are incorporated into  
561 the mature cuticle. Moreover, all transcripts identified in higher quantities during cuticle formation in  
562 pharate-adults were also identified in the newly emerged and forager bees, although in lower quantities.  
563 Their products may be involved in adult cuticle maintenance. Our gene expression findings indicate  
564 that the structure of the mature cuticle entails a dynamism, which has been up to now mainly character-  
565 ized in studies on CHC composition of its most superficial layer, the envelope [23, this work].

566 Among the genes identified in the RNA-seq analysis of the integument, we focused on classes

567 of genes playing roles in cuticle formation and maturation, such as those below discriminated.

568

569 ***Genes related to cuticle pigmentation and sclerotization***

570 The expression patterns of the first gene in the pigmentation/sclerotization biosynthetic  
571 pathway, *TH*, were positively correlated between *A. mellifera*, *F. varia* and *C. analis*, and apparently,  
572 *TH* does not contribute to the differential timing of cuticle pigmentation among them. Lower levels of  
573 *TH* transcripts were verified for the forager bees of the three bee species, which is consistent with the  
574 reported reduction in *TH* transcripts levels in *T. castaneum* [46, 47] and *Diacamma* sp [48] following  
575 the emergence. However, the expression patterns of *ebony* and *tan*, whose protein products act in a  
576 reversible reaction between dopamine and NBAD sclerotin [49], were positively correlated exclusively  
577 between the eusocial species, thus differentiating these species from the solitary one. The expression  
578 profiles of the remaining genes in the melanization/sclerotization pathway, including the *Lac2* gene  
579 previously characterized in *A. mellifera* [50], did not show such correlation patterns. Interestingly, *Dat*  
580 showed significantly increased expression in the mature cuticle of *A. mellifera* foragers, which is an  
581 uncommon pattern for genes in the melanization/sclerotization pathway.

582 We also observed that in general, the genes involved in the biosynthesis of other pigments  
583 except melanin displayed a higher expression levels in adults (Ne, Fg, or both phases) of *F. varia*,  
584 which may be tentatively interpreted as these genes playing roles in the process of post-ecdysial cuticle  
585 pigmentation in this bee species. Two of these genes, *cardinal* and *scarlet*, are both necessary for  
586 ommochromes formation in *B. mori* [51], and are associated to the formation of red and brown  
587 pigments [52]. The expression profiles of *light*, which is required for pigment granules formation [53],  
588 were positively correlated in *F. varia* and *C. analis*, and might be related to the brownish and reddish  
589 color pattern typical of the cuticle of these two species. The expression profiles of the gene encoding  
590 *ALAS*, which catalyzes the first enzymatic step in heme biosynthesis, were positively correlated  
591 exclusively between the eusocial species, *F. varia* and *A. mellifera*. *ALAS* might be involved in  
592 detoxification, as suggested for *D. melanogaster* [54, 55], and in prevent dehydration [56].  
593 Interestingly, in contrast to the eusocial bees, the expression of *ALAS* is higher in the Pbm phase of *C.*  
594 *analis*, which may suggests that mechanisms of protection against cuticle dehydration develop  
595 anticipatedly in the solitary species.

596

597 ***Genes involved in chitin synthesis, modification and degradation***

598 In insects, *Cht*, *Cda*, and *ChS* genes have been described as highly expressed during cuticle  
599 renewal at the pharate-adult development [8, 57-62]. This was also observed in the bee species here  
600 studied, but with variations: the expression of a putative chitinase, *Idgf4-like*, increased in newly-  
601 emerged *C. analis*, and like reported for *T. castaneum* [63], this may be important for the transition to  
602 the adult stage. In *A. mellifera* and *F. varia*, the expression of *Idgf4-like* is high in foragers, supporting  
603 roles in the mature adult cuticle. Therefore, the decay in the expression of chitinase genes in adult  
604 insects seems not a standard pattern. Concerning the *Cda* genes, in *Drosophila*, they have a strict  
605 relationship with the mechanical properties of the exoskeleton [64], and this might be true for the *Cda*  
606 genes expressed in the integument of the bee species. The other class of chitin-related genes encodes  
607 ChS enzymes, which catalyze the last step in the chitin biosynthetic pathway and have been implied in  
608 the synthesis of epidermal cuticle in *T. castaneum* [65]. A *ChS* gene, *CS-1*, also called *krotzkopf*  
609 *verkehrt* (*kkv*), is required for procuticle formation, stabilization of the epicuticle, and attachment of the  
610 cuticle to the epidermis in *D. melanogaster* [66]. We found a *kkv* gene in *A. mellifera* (*Amkkv*) and three  
611 potential orthologs in *C. analis* (*CaChS-kkv-like 1*, *CaChS-kkv-like 2*, *CaChS-kkv-like 3*); this gene was  
612 not identified in the *F. varia* integument transcriptome.

613

#### 614 ***Genes encoding structural cuticular proteins***

615 The large number of different cuticular protein genes found in insect genomes suggested that  
616 their products display redundant and complementary functions [67]. A variable number of genes encode  
617 the different classes of structural cuticular proteins in the three bee species and other hymenopterans  
618 (S2 Table). Thirty-two CPR genes had been previously identified in *A. mellifera* [12]. We detected  
619 other six CPR genes in our RNA-seq analysis of the *A. mellifera* integument, and also 32 and 35 CPR  
620 genes in the integument of *F. varia* and *C. analis*, respectively. In addition to have roles as structural  
621 proteins in the horizontally arrayed cuticular laminae, the function of some CPR proteins in *T.*  
622 *castaneum* was associated to the formation and organization of the pore canals vertically extended  
623 across the cuticle [68, 69]. This finding and the variety of CPR genes identified up to now suggest that  
624 distinct and additional functions are yet to be discovered for members of the CPR protein class.

625 Like the class of CPR proteins, *Twdl* proteins are structural cuticular components that  
626 effectively bind chitin, as demonstrated in *Bombyx mori* [70]. Two *Twdl* genes were previously  
627 characterized in the thoracic integument of *A. mellifera* [44], and now in the abdominal integument,  
628 thus indicating that *Twdl* proteins participate of both rigid (thoracic) and more flexible (abdominal)

629 cuticles. Like *A. mellifera*, *C. analis* has two *Twdl* genes, but we identified only one in *F. varia*.

630 Two CPLCP-encoding genes as reported in Willis [12] were herein confirmed in *A. mellifera*.  
631 Genes in this family were identified in insect genomes in general and are very enriched in mosquito  
632 genomes [71]. Based on sequence homology, we could not identify *CPLCP* transcripts in the *F. varia*  
633 and *C. analis* abdominal integument.

634 CPF proteins were associated to the outer cuticle layers of *A. gambiae* and, apparently, do not  
635 bind chitin [72]. Three *CPF* genes were previously reported for *A. mellifera* [12] and one of them,  
636 *AmCPF1*, was validated in the thoracic integument through microarray analysis [45]. Here we found  
637 *CPF1* and *CPF2* transcripts in the abdominal integument of *A. mellifera*, and in addition, transcripts for  
638 two other CPF proteins, *AmUnCPF1* and *AmUnCPF2*. We also identified one *CPF* gene in *F. varia* and  
639 one in *C. analis*.

640 *Apd* genes seem exclusive of hymenopterans and three of these genes were previously identified  
641 in *A. mellifera* [73]. Their transcript levels in the thoracic integument were higher in pharate-adults  
642 compared to earlier developmental phases [45]. Here, we detected one more *Apd* gene in *A. mellifera*,  
643 *AmApd-like*, and three *Apd* genes in *F. varia* as well as in *C. analis*.

644 C<sub>p</sub>ap proteins are essential for the correct formation of the cuticular exoskeleton and elytra in *T.*  
645 *castaneum* [74]. In our RNA-seq analysis, we identified transcripts of three *Cpap1* genes (encoding  
646 C<sub>p</sub>ap proteins containing one chitin-binding domain) in *A. mellifera* and two *Cpap1* genes in *F. varia*,  
647 and also verified that the *C. analis* integument is very enriched in *Cpap1* transcripts (n=12), and also in  
648 *Cpap* transcripts (n=11) that we could not classify as encoding C<sub>p</sub>ap1 or C<sub>p</sub>ap3 (containing three  
649 chitin-binding domains). The number of *Cpap3* genes (5 genes) in *A. mellifera* [12] is here confirmed,  
650 and two and seven *Cpap3* genes were found in the *F. varia* and *C. analis* integument transcriptomes,  
651 respectively. It is important to observe that the genes originally named as *Am-C* and *Am-D* by Soares *et*  
652 *al.* [45] were here renamed as *AmCpap3-c* and *AmCpap3-d*.

653 The genes, *umpy* (*dp*), *knk* (*knickkopf*) and *Rtv* (*Retroactive*) have also been identified as  
654 encoding cuticular proteins. In *D. melanogaster*, *dp* play roles in cuticle formation [12]. We detected  
655 transcripts for *dp* in the abdominal integument of *A. mellifera*, but not in the integument of the other  
656 two bee species. The genes *knk* and *Rtv* are both involved in cuticle stabilization in *Drosophila* [75]. In  
657 *T. castaneum*, *Rtv* activity is essential for localization of the Knk protein, facilitating its transport to the  
658 cuticle [76, 77]. The co-expression of *Rtv* and *knk* in *A. mellifera*, as shown in the reconstructed co-  
659 expression network, supports interaction of their respective products, as verified in *T. castaneum*. We

660 also found *knk* transcripts in *C. analis* integument transcriptome, but not in *F. varia*. *Rtv* transcripts  
661 were not detected in the integument of these two bee species.

662

### 663 ***Genes encoding desaturases and elongases potentially involved in CHC biosynthesis***

664 CHC biosynthesis occurs in the epidermis-associated oenocytes [20] through biosynthetic  
665 pathways where desaturase and elongase enzymes have essential roles. Previously, we characterized the  
666 gene expression profiles of six desaturases and ten elongases in the developing integument of *A.*  
667 *mellifera* [23]. Our RNA-seq data confirmed these findings, besides identifying three more desaturase  
668 genes and other four genes encoding elongases potentially involved in CHC biosynthesis for deposition  
669 in the cuticular envelope. For *A. mellifera*, *F. varia* and *C. analis*, a higher proportion of the  
670 differentially expressed desaturase and elongase genes showed increased expression in the adults (Ne  
671 and/or Fg phases), and only for the eusocial species there were genes more expressed in the pharate-  
672 adults (Pbm phase). Among the desaturase and elongase genes, we highlight the expression profiles of  
673 *Desat-GB40659*, *Elo-GB54401*, *Elo-GB54302*, *Elo-GB45596* and *Elo-GB46038* orthologs, all showing  
674 positive correlation exclusively between the eusocial species.

675

### 676 ***Genes of the ecdysone signaling cascade regulating cuticle formation and ecdysis in the*** 677 ***integument***

678 We detected in the integument the expression of genes that are part of the signaling cascade  
679 underlying insect molting and ecdysis, such as *EcR*, *Usp*, *E74*, *E75*, *FTZ-F1*, *CCAP*, *CCAPR*  
680 (*Crustacean Cardioactive Peptide Receptor*), *Eth* (*Ecdysis triggering hormone*), *Ethr*, and *Eh* [78].  
681 Importantly, transcripts for these regulators were also detected in greater or lesser levels after ecdysis,  
682 in the integument of adult bees. *Usp*, which together with *EcR* forms the nuclear receptor complex that  
683 binds 20E and regulates the expression of a cascade of ecdysone-responsive genes, showed the higher  
684 expression in *A. mellifera* foragers. This is here tentatively related to the elevated JH titer at this phase  
685 of *A. mellifera* worker life [41] once *Usp* also has been proposed as a mediator of JH action [79].

686 *CCAP*, *hairy*, *mirr*, and *Kr-h1* in *A. mellifera*, *CCAP*, *Kr-h1*, and *Met* (*Methoprene-tolerant*) in  
687 *F. varia*, and *Kr-h1*, *E75*, *EcR*, *hairy*, and *mirr* in *C. analis* showed increased expression levels at the  
688 Ne and/or Fg phases. The roles of these genes in adult bees, evidently dissociated from the molting  
689 events and metamorphosis, are yet to be determined. *Kr-h1* is a direct JH-response gene. *Met*, the JH  
690 receptor, has roles in the crosstalk of JH and 20E signaling pathways, which are critical in the

691 regulation of insect metamorphosis [80]. Since *Met*, and also *hairy*, mediate the action of JH on gene  
692 regulation [81], they certainly are needed in adult bees where JH has important physiological roles. The  
693 *mirr* gene encodes a homeodomain transcription factor with roles in *Drosophila* oogenesis [82]. To our  
694 knowledge, its role in the integument has not yet been studied.

695 Some of the identified regulatory genes have been described as playing roles in cuticular  
696 melanization, as an example, the *Abdominal B* (*Abd-B*) Hox gene, which regulates *yellow* in the  
697 pigmentation/sclerotization pathway in *Drosophila* [83]. *Hairy*, which is a pair-rule gene in *Drosophila*  
698 embryos [84], may be involved in the polarity of abdominal segment melanization. The heterodimeric  
699 neuropeptide bursicon, composed by the gene products Bursα and Bursβ, is responsible for the  
700 regulation of the laccase2-encoding gene, and is crucial for the melanization/sclerotization of the newly  
701 formed cuticle [85, 86]. Bursicon interacts with the target tissue through its receptor, the product of the  
702 *rickets* gene, whose transcripts were also identified in our RNA-seq analysis of the integument of the  
703 three bee species.

704

705 **Searching for clues linking cuticle maturation heterochrony to eusocial or solitary life  
706 styles in the RNA-seq analysis**

707

708 Our RNA-seq analyses were used to discover active genes in the integument of three bee  
709 species and, in addition, we looked for differences in gene expression profiles that could be linked to  
710 the heterochronic cuticle maturation dependent on the social/solitary ways of life. The following main  
711 findings highlighted differences in integument gene expression distinguishing the eusocial *A. mellifera*  
712 and *F. varia* from the solitary *C. analis*: **(a)** In contrast to the eusocial species, a smaller proportion of  
713 the genes differentially expressed in the integument was upregulated in *C. analis* foragers in  
714 comparison to the newly emerged bees, which is consistent with the cuticle of the solitary bee reaching  
715 maturity already at the emergence time; **(b)** The GO analysis including all the integument genes  
716 displaying orthology relationship with *Drosophila* genes highlighted functional categories that were  
717 mainly shared by both eusocial species in detriment of the solitary *C. analis*; **(c)** The Euclidean distance  
718 analysis based on the set of differentially expressed genes clearly separated the *A. mellifera* foragers  
719 from the newly-emerged, whereas in *C. analis* these bee groups were clustered, thus suggesting a  
720 greater similarity between the integument of newly-emerged and foragers of *C. analis*; **(d)** In contrast  
721 to the eusocial species, most of the genes for melanization/sclerotization, genes encoding RR1, RR2, or

722 non-RR structural proteins, and also regulatory genes, did not show significant expression level  
723 variations in *C. analis*. Such differential fluctuation in transcript levels during development may have  
724 possibly contributed to the molecular heterochrony of cuticle maturation associated with bee life style.  
725 In addition, consistent with the comparatively earlier cuticle maturation process in the solitary bee, we  
726 found a higher proportion of CHC biosynthesis-related genes (desaturase and elongase genes) with  
727 significantly increased expression levels at the emergence (Ne phase) of *C. analis* in comparison to the  
728 eusocial bees. Furthermore, correlation analysis showed that a fraction of cuticle-related genes  
729 displayed congruent expression profiles between the eusocial species, but not with the solitary one; (e)  
730 By superposing the integument gene co-expression networks constructed for the three bee species, we  
731 found common interactions for the eusocial species, which were not seem when we compared these  
732 species with the solitary one.

733 Taken together, the comparative approach of the RNA-seq data highlighted suitable gene  
734 expression signatures related to adult cuticle formation and maturation in the bee species, in addition of  
735 revealing differences in gene expression that may possibly be involved in cuticle maturation  
736 heterochrony. Yet, this process may have entailed changes in the expression profiles of regulators of  
737 molting and metamorphosis.

738

739 **Abdominal adult cuticle deposition timing and its ultrastructure exhibit marked differences**  
740 **between the bee species**

741

742 Cuticle ultrastructure and thickness did not significantly vary between the pharate adults (Pbm  
743 phase), newly-emerged (Ne) and foragers (Fg) of *F. varia*, as evidenced by TEM analysis. This was an  
744 unexpected result, considering that at the emergence time, *F. varia* workers visibly show an immature  
745 cuticle, i.e., incompletely pigmented and sclerotized. Therefore, the evident intensification of cuticular  
746 pigmentation and sclerotization in *F. varia* in the subsequent days after the emergence, which is  
747 necessary for flight and task performances outside the nest, do not imply in changes in abdominal  
748 cuticle thickness. It is possible, however, that thickness measurements taken from cuticular regions  
749 other than the abdominal, could evidence a different result, considering that regions of the insect body  
750 may diverge in the number of cuticle layers [87] and, consequently, in cuticle thickness.

751 In contrast, in *A. mellifera*, cuticle deposition is extended through the initial adult stage. Only in  
752 the honeybee we could identify post-ecdysiologically-deposited cuticle layers. Both, the pre- and post-

753 ecdysiolytic-deposited cuticle layers, or laminae, form the procuticle, which corresponds to the largest  
754 portion of the cuticle in insects in general. The term exocuticle has been used synonymously with pre-  
755 ecdysiolytic cuticle, whereas those layers deposited post-ecdysiolytic form the endocuticle. However, there is  
756 some divergence concerning these concepts [88]. In beetles, for example, up to three endocuticle layers  
757 are already present in specific areas of the body surface at the time of the adult ecdysis [87]. In  
758 *Sarcophaga bullata* flies, deposition of endocuticle occurs before the adult ecdysis [89].

759 As expected, the solitary bees, *C. analis* and *T. diversipes*, and even the primitively eusocial *B.*  
760 *brasiliensis* and the facultatively eusocial *E. cordata*, showed a fully deposited cuticle as soon as they  
761 emerge, and newly-emerged and forager bees in each of these species displayed similar cuticle  
762 ultrastructure, pigmentation and sclerotization. The rapid cuticle maturation in *E. cordata* is consistent  
763 with its nesting biology and social structure. *E. cordata* nests are founded by a single female that build  
764 up until ten brood cells. The offspring will leave the nest immediately after the emergence for founding  
765 new nests. However, daughters may return to the maternal nest, thus resulting in a facultatively social  
766 organization with a dominant female (the mother) and its subordinate daughters. There are also nests  
767 formed by sisters' females or even by unrelated females, the oldest one showing dominance over the  
768 youngest. The dominant female produces all the offspring and rarely leaves the nest, whereas the  
769 subordinates assume the tasks of nest provisioning and maintenance, and they also lay trophic eggs that  
770 are eaten by the dominant [90-92]. Such female associations may have preceded the highly eusocial  
771 way of life [93]. Therefore, in *E. cordata*, as well as in the truly solitary bees, *C. analis* and *T.*  
772 *diversipes*, rapid cuticle maturation is the condition for the immediate exit from the nest after  
773 emergence.

774 This situation is somewhat diverse for the primitively eusocial *Bombus*. In *B. brasiliensis*, as  
775 demonstrated here, the final adult cuticle ultrastructure and thickness are achieved at the emergence.  
776 This would allow the workers start foraging soon, as reported for *B. atratus* workers that may leave the  
777 nest as soon as at the emergence day (0 day). However, workers of this species may start foraging later,  
778 at the age of 10-20 days [94], thus similar to the eusocial bees. Moreover, younger workers in the genus  
779 *Bombus* have, in general, incompletely pigmented cuticle and hairs, denoting that cuticle maturity was  
780 not yet completely achieved. Such characteristics that seem intermediary to the eusocial and solitary  
781 condition may be inherent to the primitively eusocial species, but this requires further investigation.  
782 Studies correlating the grade of cuticle pigmentation with the age of starting foraging among  
783 primitively eusocial bee species should clarify this issue.

784 Our TEM analysis and thickness measurements showed that in the same abdominal segment,  
785 cuticle ultrastructure greatly differs between the bee species, not only in the number of the adjacently  
786 arranged chitin/protein sheets (laminae), but also in the morphology of the most superficial layers.  
787 Except for *F. varia*, these results are consistent with a cuticle development timing adapted to the life  
788 style, as observed for the highly eusocial *A. mellifera*, the facultatively eusocial *E. cordata*, the  
789 primitively eusocial *B. brasiliensis*, and the solitary *C. analis* and *T. diversipes* bees.

790 Considering that the timing of cuticle deposition is peculiar to bee species, and that cuticle de-  
791 position rhythm in *Drosophila* is regulated by a peripheral circadian oscillator in the epidermal cells,  
792 which requires the expression of the clock genes *Per*, *Tim2*, *Cyc*, and *Clk* [39], and also that a *Cry*  
793 clock gene regulates the rhythm of cuticle deposition in the bean bug *Riptortus pedestris* [40], we com-  
794 pared the expression of seven circadian rhythm genes (*Per*, *Tim2*, *Cyc*, *Clk*, *Cry*, *Vri* and *Pdp1*) in the  
795 developing integument of *A. mellifera*, *F. varia* and *C. analis*. Consistent with the differences in the  
796 timing of cuticle deposition, the expression profiles of *Clk* in *A. mellifera*, *F. varia*, and *C. analis* were  
797 negatively or non-correlated. Similarly, the expression profiles of *Cry* in *A. mellifera* and *C. analis*  
798 were negatively correlated (*Cry* was not identified in *F. varia*), as well as the expression of *Per*. It is  
799 likely that *AmPer* has roles in adult cuticle organization. Interaction of *AmPer* and other genes involved  
800 in cuticle formation was specifically observed in *A. mellifera*, whose sequenced genome gives more  
801 support for gene co-expression network reconstruction. In *A. mellifera*, *Per* was co-expressed with the  
802 *knk* gene, which in *T. castaneum* was associated with stabilization of the cuticular laminae [95]. Both  
803 genes were co-expressed with structural cuticular protein genes such as *AmCpap3-a*, *AmTwdl*(*Grp*-  
804 *Glycine-rich protein*), *AmUnCPR-RR2-2*, *AmCPR26*, *Am49Ah-like* and *AmSgAbd2-like*, and also with  
805 *Amyellow-y*, a gene in the yellow family, involved in cuticle pigmentation [96]. The expression profiles  
806 of another clock gene, *Tim2*, were positively correlated between the eusocial species, with a marked de-  
807 crease in expression levels at the emergence, suggesting roles in the final step of adult cuticle formation  
808 in these bees. The *Pdp1* gene encodes a basic leucine zipper transcription factor and is expressed at  
809 high levels in the epidermis and other tissues of *Drosophila* embryos. *Pdp1* is an essential clock gene  
810 linked to the circadian rhythm. It is a regulator of *Clk* and other clock genes, such as *Tim*, *Per*, and *Pdf*  
811 (*Pigment dispersing factor*), a neuropeptide controlling circadian behavioral rhythms [97, 98]. *Pdp1*  
812 seems an important gene in the *C. analis* integument since it is connected with nine structural cuticle  
813 protein genes, three chitin-related genes, and two desaturase encoding genes in the co-expression net-  
814 work. However, it is significantly more expressed after the emergence, when the cuticle of the solitary

815 bee is already formed, thus virtually excluding a role in cuticle laminae deposition rhythm. Differently  
816 from *C. analis*, *Pdp1* was not co-expressed in the networks reconstructed with the *A. mellifera* and *F.*  
817 *varia* genes involved in cuticle formation and maturation. Some of the cuticular genes were also co-ex-  
818 pressed with *Cyc* in the integument of *A. mellifera* and *C. analis*.

819

## 820 **Cuticular n-alkanes as markers of cuticle maturity in bees**

821

822 N-alkanes are structural lipids in the insect cuticle [99, 17], where they compose the envelope  
823 [100]. The absolute quantities of n-alkanes and unsaturated CHCs were significantly higher in the  
824 foragers than in the earlier developmental phases of the eusocial *A. mellifera* and *F. varia* species. The  
825 n-alkanes detected in higher proportions in *A. mellifera* foragers than in the newly-emerged were  $C_{23}$ ,  
826  $C_{24}$ ,  $C_{25}$ ,  $C_{26}$ ,  $C_{27}$ ,  $C_{29}$ ,  $C_{31}$ , and  $C_{33}$ , the  $C_{25}$  and  $C_{27}$  n-alkanes presenting the highest proportions. The  
827 analysis of the individual CHC peaks obtained from *F. varia* also showed higher proportions of  $C_{27}$  and  
828  $C_{29}$ , besides a higher proportion of  $C_{22}$ , in the foragers. All these n-alkanes, except  $C_{27}$  and  $C_{33}$ , were also  
829 proportionally increased in foragers than in newly-emerged bees of the eusocial *Melipona marginata*  
830 [101]. These data are consistent with previous reports on higher levels of n-alkanes in *A. mellifera*  
831 foragers [102] and in foragers of an ant species, *Pogonomyrmex barbatus* [103]. In contrast, the  
832 proportions and absolute quantities of n-alkanes did not differentiate foragers from the newly-emerged  
833 in *C. analis*. Together, these findings may be interpreted as the solitary bee displaying an accelerated  
834 process of cuticle maturation in comparison to the eusocial ones. N-alkanes may be markers of cuticle  
835 structure maturation. Long-chain alkanes are thought to increase cuticle waterproofing [104, 103],  
836 suggesting that this essential ability for the performance of extra-nidal activities was acquired earlier in  
837 the development of *C. analis*. At the adult emergence, the solitary bee already has the chemical profile  
838 needed for a prompt interaction with the environment outside the nest. Consistently, the levels of n-  
839 alkanes also did not significantly differ between young and old females of the solitary leafcutter bee  
840 species, *Megachile rotundata* [105].

841

## 842 **Conclusions**

843

844 Using RNA-seq analysis of the integument of two eusocial bee species, *A. mellifera* and *F.*  
845 *varia*, and a solitary bee, *C. analis*, we identified genes involved in cuticle (exoskeleton) formation and

846 maturation. The expression profiles of these genes were determined at three developmental time points  
847 corresponding to adult cuticle deposition/differentiation at the pharate-adult stage, newly-ecdysed  
848 cuticle, and fully developed cuticle of forager bees. TEM analysis of the cuticle at these time points,  
849 including other bee species, and CHC profiles determination were performed in addition to the  
850 transcriptome analysis. Together, these experimental approaches provided novel data on integument  
851 developmet. We also searched for clues in integument gene expression, structure, and CHC profiles that  
852 could be consistent with the premise that eusociality might have entailed heterochronic changes in  
853 cuticle development, resulting in faster cuticle maturation in the solitary bee, thus allowing flight and  
854 forager activities immediately after emergence, and in slow cuticle maturation in the eusocial bees,  
855 which benefit from the protected nest environment for a period of time after the emergence. This study  
856 expands our understanding on the molecular biology and structure of the developing integument,  
857 besides highlighting differences in the process of cuticle maturation related to the eusocial/solitary  
858 behaviors.

859

## 860 Materials and Methods

### 861 Sample collection

862 We collected workers of *A. mellifera* (Africanized) and *F. varia* from colonies maintained in the  
863 Experimental Apiary of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo,  
864 Ribeirão Preto, SP, Brazil. Trap-nests to collect samples of the solitary species *C. analis* and *T.*  
865 *diversipes* were made [106] and placed in the Experimental Apiary area. Additional bee species (*B.*  
866 *brasiliensis*, *E. cordata* and *T. diversipes*) were obtained from donations (see acknowledgments  
867 section).

868 We used females from three developmental phases: pharate adults in process of cuticle  
869 pigmentation (Pbm phase), newly emerged (Ne) adults and foragers. Carrying pollen bees from the  
870 solitary and social species, and building-nest females from the solitary species, were identified as  
871 foragers (Fg). The *B. brasiliensis*, *E. cordata* and *T. diversipes* species were exclusively used for cuticle  
872 morphology studies through TEM. In this case, we used the Ne and Fg phases.

### 873 RNA extraction and sequencing

874 For each developmental phase (Pbm, Ne and Fg) of *A. mellifera* and *F. varia*, we prepared three  
875 independent samples, each made with five abdominal integuments. For the corresponding

876 developmental phases of *C. analis*, we prepared three independent samples, each containing three  
877 abdominal integuments. The RNA extractions were made using TRIzol® reagent (Invitrogen)  
878 following manufacturer's instruction. The extracted RNAs (2 µg/per sample) were sent to a facility  
879 (Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida, Universidade Estadual  
880 de Campinas, Campinas, Brazil) to access sample quality through a 2100 Bioanalyzer and for library  
881 preparation (TruSeq™ RNA - Illumina®) and RNA sequencing in an Illumina HiSeq 2500 equipment  
882 (paired-end reads, 2 x 100 bp read length). We obtained an average of 30 million reads per sample, with  
883 90% of the bases showing quality scores > Q30. The RNA-seq data is deposited at the National Center  
884 for Biotechnology Information (NCBI) database under the BioProject ID PRJNA490324.

885 **Adapters trimming and quality check**

886 We used the software Scythe v. 0.991 (<https://github.com/vsbuffalo/scythe>) for trimming 3'  
887 standard Illumina adapter sequence. We followed a Cutadapt v. 1.4.1 [107] trimming at 5' ends of the  
888 reads, and we filtered reads with Phred quality > 20. The trimmed sequences were filtered using the  
889 software PRINSEQ-lite v. 0.20.3 [108] and sequence quality was evaluated through the software  
890 FastQC v. 0.11.2 [109].

891 **Transcriptome assembly and gene expression**

892 We aligned the high quality reads from *A. mellifera* against its genome v. 4.5 using the software  
893 TopHat v. 2.0.9 [110]. The *A. mellifera* aligned sequences were quantified and the developmental  
894 phases compared using the software Cufflinks v. 2.1.1 [111]. The extension Cuffmerge integrated the  
895 reads to the mapping results and the tool Cuffdiff checked the expression levels for each sample and the  
896 significance of comparisons. CuffmeRbund R package v. 2.8.2 allowed us to access all this information  
897 [112].

898 For *F. varia* and *C. analis*, we used the software Trinity (trinityrnaseq\_r2014717) [113, 114].  
899 The N50 contig length (smallest contig length for which the sum of fewest contigs corresponds to 50%  
900 or more of the assembly [115]) of all transcripts of *F. varia* was 2372 and of *C. analis* was 2440.  
901 Orthology search was performed through the software InParanoid 8 [116]. We only accepted those  
902 transcripts with higher similarity with *A. mellifera* than to *Drosophila melanogaster*. Statistical  
903 evaluation of these data was done with the R software v. 3.1.2, using the packages R DESeq2 v. 1.6.3  
904 [117] and edgeR v. 3.8.6 [118]. We considered as differentially expressed between developmental  
905 phases, those contigs with significant results for both R packages.

906 All heat maps were designed using the function heatmap.2 from gplots R package [119]. For all  
907 groups of genes, we measured the clustering potential of the samples for each phase and species. For  
908 this approach, we used the R package pvclust v. 1.3.2 [120] based on correlation distances, with a  
909 complete linkage method, and 10,000 bootstrap replication. We used unbiased p values (AU) and  
910 bootstrap values as measurements of clusters' significance. Clusters showing AU > 95% were  
911 considered statistically significant [120].

912 **Molecular and functional characterization of differentially expressed genes**

913 For the analysis of gene expression in *A. mellifera*, we filtered the differentially expressed genes  
914 using the following thresholds: q-value < 0.05; Log 2 Fold Change  $\leq$  -1 or  $\geq$  1 and Fragments Per  
915 Kilobase of transcript per Million mapped reads (FPKM)  $\geq$  5. In the case of the other two bee species,  
916 *F. varia* and *C. analis*, we used the parameters cited in the previous section. For the Gene Ontology  
917 (GO) enrichment analysis we used the *A. mellifera* gene IDs to look for *D. melanogaster* orthologues in  
918 Fly Base, through the support of the online softwares g:Profiler (<http://biit.cs.ut.ee/gprofiler/gorth.cgi>),  
919 and g:Orth function [121, 122]. The same was done for *F. varia* and *C. analis* but using *A. mellifera*  
920 orthologues. We filtered the *Drosophila* IDs to avoid ID repetition and used them to generate an input  
921 list for the software DAVID v. 6.7 (<http://david.abcc.ncifcrf.gov>) [123, 124], used to perform the Gene  
922 Ontology analysis. The annotated functions belonged to Biological Process (BioP), Cellular  
923 Components (CC) and Molecular Function (MF) categories. Structural cuticular protein encoding genes  
924 were classified in accordance with the software CutProtFam-Pred ([http://aias.biol.uoa.gr/CutProtFam-  
925 Pred/home.php](http://aias.biol.uoa.gr/CutProtFam-Pred/home.php)) [125]. Venn diagrams were plotted with the online version of the software jvenn [126]  
926 (<<http://bioinfo.genotoul.fr/jvenn/example.html>>).

927 **Transcription factor binding sites search with TRANSFAC®**

928 Transcription factors whose binding sites could be enriched in specific groups of *A. mellifera*  
929 gene models were searched using TRANSFAC® [127] against insects database. For this enrichment  
930 analysis, we searched the 5' UTR regions covering -3,000 bases relative to the transcription start sites  
931 of the genes involved in sclerotization/melanization processes, chitin metabolism, CHC biosynthetic  
932 pathways, regulation of cuticle formation and maturation, circadian rhythm, and non-melanization  
933 pigmentation pathways (see gene IDs S1 File). We excluded the genes with 5' UTR < 500 bases. We  
934 used the *A. mellifera* genes in this analysis once it is the only among the species here studied with an  
935 available reference genome. For the transcription factor FTZ-F1 binding sites, we used the  
936 TRANSFAC database (DROME\$FTZF1\_01, DROME\$FTZF1\_02, and DROME\$FTZF1\_03 from *D.*

937 *melanogaster*, and BOMMO\$CPR92\_01, and BOMMO\$CPR92\_02 from *B. mori*) to generate a  
938 positional matrix. Here, we only highlighted those transcription factor binding sites (TFBS), which  
939 could be relevant for insect cuticle formation and maturation (see **Discussion section**).

#### 940 **Gene co-expression networks**

941 The networks were plotted based on the correlation of gene expression in the integument of the  
942 analyzed bee species. We used the software Cytoscape v. 3.3.0 [128] – for Linux, and its plugin  
943 ExpressionCorrelation App v. 1.1.0. We only accepted correlations  $\geq +0.95$  or  $\leq -0.95$  and  $p \leq 0.05$ .

#### 944 **Transmission electron microscopy (TEM)**

945 We dissected the integument from the right anterior region of the third abdominal tergite of the  
946 studied bee species. The ultrastructure of the integument was compared between species and between  
947 the developmental phases (Pbm, Ne and Fg) using 11 *A. mellifera* integument pieces (4 from Pbm, 3  
948 from Ne, and 4 from Fg phases), 9 integuments from *F. varia* (3 Pbm, 3 Ne, 3 Fg), 11 integuments from  
949 *C. analis* (4 Pbm, 4 Ne, 3 Fg), 4 integuments from *B. brasiliensis* (2 Ne, 2 Fg), 6 integuments from *E.*  
950 *cordata* (3 Ne, 3 Fg), and 6 integuments from *T. diversipes* (3 Ne, and 3 Fg). The Ne and Fg phases of  
951 *B. brasiliensis* were recognized based on the grade of body pigmentation criterion (less intense in the  
952 Ne bees) and for the Fg phase we also examined the wings in the search for erosion signals that could  
953 indicate intense foraging activity. The integument samples were fixed in 5% glutaraldehyde in  
954 cacodylate buffer 0.1 M, pH 7.2, during 2 h under shaking, washed 3X in the cacodylate buffer, fixed in  
955 osmium tetroxide 1% diluted in phosphate buffer 0.1M, pH 7.2, dehydrated in acetone and propylene  
956 oxide and embedded in resin. We used uranyl acetate for enhancing image contrast. The ultrathin  
957 sections were examined in a Jeol-Jem-100cx-II Electron Microscope and the software ImageJ v. 10.2  
958 [129] was used to measure integument thickness. Measurements were compared among the  
959 developmental phases and species using Analysis of Variance (ANOVA) associated with the Tukey's  
960 Honestly Significance Difference (Tukey's HSD) post hoc test in R software v. 3.1.2, except for the *A.*  
961 *mellifera* and *B. brasiliensis* data. As for *A. mellifera* the data did not present a normal distribution  
962 (Shapiro-Wilk normality test) we used the Kruskal-Wallis test associated with the *post hoc* Conover-  
963 Iman test and Bonferroni correction, and for *B. brasiliensis* we used the Student's t-test [130].

#### 964 **Cuticular hydrocarbon profiles**

965 The quantification of CHCs was based on their peak area in each chromatogram. For the  
966 analysis of relative peak area, we collected 15 bees per developmental phase for each of the highly  
967 eusocial species, while for *C. analis* we obtained four Pbm-staged bees, seven Ne and seven Fg bees.

968 The *F. varia* hind legs were removed to avoid resin contamination. Except for this species, we bathed  
969 each sample in 1.5 ml of n-hexane 95% (Mallinckrodt Chemicals) for 1 min and 30 s to extract the  
970 CHCs. Due to the small size of *F. varia* species, we used 500  $\mu$ l of n-hexane to extract the CHCs [131].  
971 The extracts were dried under  $N_2$  flow and resuspended in 160  $\mu$ l of n-hexane (100  $\mu$ l for *F. varia*  
972 extracts) before running the analysis. CHC identification was made in a Gas Chromatograph / Mass  
973 Spectrometer (GC-MS) system (Shimadzu GCMS model QP2010), equipped with a 30 m DB-5MS  
974 column using helium as the carrier gas (1 ml/min), through electronic ionization (EI) mode. CHC  
975 relative quantification and normalization of the peak areas were performed following Falcón *et al.* [23]  
976 description. We also compared each developmental phase considering the classes of CHC (n-alkanes,  
977 unsaturated, and branched alkanes), repeating the normalization process for each case.

978 In order to verify differences between the developmental phases and between the bee species,  
979 we performed a clustering approach as described in the section **Transcriptome assembly and gene**  
980 **expression**, but using the Euclidean distance instead of the correlation distance. We also verified the  
981 compounds that better explained the detected differences. With this purpose, we performed a Principal  
982 Component Analysis (PCA) using the R software. The variation of each CHC peak area between the  
983 developmental phases was accessed through a Tukey's HSD test in R software.

984 Additionally, we calculated the absolute quantities of n-alkanes and unsaturated CHC per bee  
985 for the species *A. mellifera*, *F. varia*, and *C. analis*. An analytical curve [132] was built to establish the  
986 correlation between the quantities of the used standards and the CHCs. It is described by the equation:  
987  $y=ax+b$ , where  $y$  is the known amount of the standard,  $x$  is the quantity of the unknown CHC,  $a$  is the  
988 peak area of the standard, and  $b$  is the area of the intercepted background. We prepared the curve based  
989 on the n-alkanes standards  $C_{23}$ ,  $C_{25}$ , and  $C_{32}$  (Alltech Corporation) and using 1.25, 2.5, 5, 10, 15 and 20  
990  $\mu$ g/ml of each alkane. To the standard solutions and to each sample, we added 100  $\mu$ l of the internal  
991 standard  $\alpha$ -colestane (6.25  $\mu$ g/ $\mu$ l) (Sigma-Aldrich) under the previously cited chromatography  
992 conditions. The values of the correlation curve ( $R$ ) between CHCs and standards were  $\geq 0.99$ . After  
993 curve preparation, CHCs were quantified in three independent samples, each prepared with individual  
994 bees, for each developmental phase and species. Due to the reduced body size of *F. varia*, we used  
995 pools of three bees for each one of the three independent samples per developmental phase and the  
996 obtained CHC values were corrected accordingly. To calculate the concentrations of compounds up to  
997  $C_{24}$ , we used the curve of  $C_{23}$ ; for compounds from  $C_{25}$  up to  $C_{29}$ , we used the  $C_{25}$  curve; and for  
998 compounds larger than  $C_{29}$  up to  $C_{35}$  we used the curve of  $C_{32}$ . We followed an ANOVA associated with

999 the *post hoc* Tukey's HSD test to compare the absolute quantity of each CHC (n-alkanes and  
1000 unsaturated) between developmental phases. The absolute quantities of the *F. varia* unsaturated  
1001 compounds were not calculated once their quantities are very low.

1002

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1011

1012 **References**

- 1014 1. Hopkins TL, Kramer KJ. Insect cuticle sclerotization. *Annul Rev Entomol.* 1992;37: 273-302.
- 1015 2. Wood OR, Hanrahan S, Coetzee M, Koekemoer LL, Brooke BD. Cuticle thickening associated with  
1016 pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Parasit Vectors.* 2010;3: 67.
- 1017 3. Csikós G, Molnár K, Borhegyi NH, Talián GC, Sass M. Insect cuticle, an in vivo model of protein  
1018 trafficking. *J Cell Sci.* 1999;112: 2113-2124.
- 1019 4. Hill RJ, Billas IML, Bonneton F, Graham LD, Lawrence MC. Ecdysone receptors: from the Ash-  
1020 burner model to structural biology. *Annu Rev Entomol.* 2013;58: 251–271.
- 1021 5. Ali MS, Iwanaga M., Kawasaki H. Ecdysone-responsive transcriptional regulation determines the  
1022 temporal expression of cuticular protein genes in wing discs of *Bombyx mori*. *Gene* 2013;512: 337-  
1023 347.
- 1024 6. Hiruma K, Riddiford LM. The coordination of the sequential appearance of MHR4 and dopa decar-  
1025 boxylase during the decline of the ecdysteroid titer at the end of the molt. *Mol Cell Endocrinol.*  
1026 2007;276: 71-79.
- 1027 7. Gu J, Huang L-X, Gong Y-J, Zheng S-C, Liu L, Huang L-H, et al. De novo characterization of tran-  
1028 scriptome and gene expression dynamics in epidermis during the larval-pupal metamorphosis of  
1029 common cutworm. *Insect Biochem Mol Biol.* 2013;43: 794-808.
- 1030 8. Kramer JK, Dziadik-Turner C, Koga D. Chitin metabolism in insects. In: Kerkut GA, Gilbert LI,  
1031 editors. *Comprehensive insect physiology, biochemistry and pharmacology.* Oxford: Pergamon  
1032
- 1033
- 1034
- 1035
- 1036
- 1037
- 1038

1039 Press; 1985. pp. 75-115.

1040

1041 9. Dixit R, Arakane Y, Specht CA, Richard C, Kramer KJ, Beeman RW, et al. Domain organization and  
1042 phylogenetic analysis of proteins from the chitin deacetylase gene family of *Tribolium castaneum*  
1043 and three other species of insects. Insect Biochem Mol Biol. 2008;38: 440–451.

1044

1045 10. Merzendorfer H, Zimoch L. Chitin metabolism in insects: structure, function and regulation of  
1046 chitin synthases and chitinases. J Exp Biol. 2003;206: 4393-4412.

1047

1048 11. Locke M, Krishnan N. The distribution of phenoloxidases and polyphenols during cuticle  
1049 formation. Tissue Cell. 1971;3: 103-126.

1050

1051 12. Willis JH. Structural cuticular proteins from arthropods: annotation, nomenclature and sequence  
1052 characteristics in the genomic era. Insect Biochem Mol Biol. 2010;40: 189-204.

1053

1054 13. Rebers JE, Riddiford LM. Structure and expression of a *Manduca sexta* larval cuticle gene  
1055 homologous to *Drosophila* cuticle genes. J Mol Biol. 1988;203: 411-423.

1056

1057 14. Andersen SO. Amino acid sequence studies on endocuticular proteins from the desert locust,  
1058 *Schistocerca gregaria*. Insect Biochem Mol Biol. 1998;28: 421-434.

1059

1060 15. Guan X, Middlebrooks BW, Alexander S, Wasserman SA. Mutation of TweedleD, a member of an  
1061 unconventional cuticle protein family, alters body shape in *Drosophila*. Proc Natl Acad Sci.  
1062 2006;103: 16794–16799.

1063

1064 16. Hepburn HR. Structure of integument. In: Kerkut GA, Gilbert LI, editors. Comprehensive insect  
1065 physiology, biochemistry and pharmacology. Oxford: Pergamon Press; 1985. pp. 1-58.

1066

1067 17. Gibbs AG. Lipid melting and cuticular permeability: new insights into an old problem. J Insect  
1068 Physiol. 2002;48: 391-400.

1069

1070 18. Howard RW, Blomquist GJ. Ecological, behavioral and biochemical aspects of insect hydrocarbons.  
1071 Annu Rev Entomol. 2005;50: 371-393.

1072

1073 19. Edney EB. Water balance in desert arthropods. Science. 1967;156: 1059-1065.

1074

1075 20. Piek T. Synthesis of wax in the honeybee (*Apis mellifera* L.). J Insect Physiol. 1964;10: 563-572.

1076

1077 21. Blomquist GJ, Dillwith JW. Cuticular lipids. In: Kerkut GA, Gilbert LI, editors. Comprehensive In-  
1078 sect Physiology, Biochemistry and Pharmacology. Oxford: Pergamon Press; 1985. vol. 3, pp. 117-  
1079 154.

1080

1081 22. Blomquist GJ, Jurenka R, Schal C, Tittiger C. Pheromone production: biochemistry and molecular  
1082 biology. In: Gilbert LI, editor. Insect Endocrinology. New York: Elsevier; 2012. pp. 523-567.

1083

1084 23. Falcón T, Ferreira-Caliman MJ, Nunes FMF, Tanaka ED, Nascimento FS, Bitondi MMG.  
1085 Exoskeleton formation in *Apis mellifera*: cuticular hydrocarbons profiles and expression of

1086 desaturase and elongase genes during pupal and adult development. Insect Biochem Mol Biol.  
1087 2014;50: 68-81.

1088

1089 24. Shamim G, Ranjan SK, Pandey DM, Ramani R. Biochemistry and biosynthesis of insect pigments.  
1090 Eur J Entomol. 2014;111: 149-164.

1091

1092 25. Solano F. Melanins: skin pigments and much more – types, structural models, biological functions,  
1093 and formation routes. New J Sci. 2014: 1-28. Article ID 498276.  
1094 <http://dx.doi.org/10.1155/2014/498276>.

1095

1096 26. Andersen SO. Insect cuticular sclerotization: a review. Insect Biochem Mol Biol. 2010;40: 166-178.

1097

1098 27. Hiruma K, Riddiford LM, Hopkins TL, Morgan TD. Roles of dopa decarboxylase and  
1099 phenoloxidase in the melanization of the tobacco hornworm and their control by 20-  
1100 hydroxyecdysone. J Comp Physiol B. 1985;155: 659-669.

1101

1102 28. Zufelato MS, Bitondi MMG, Simões ZLP, Hartfelder K. The juvenile hormone analog pyriproxyfen  
1103 affects ecdysteroid-dependent cuticle melanization and shifts the pupal ecdysteroid peak in the  
1104 honey bee (*Apis mellifera*). Arthropod Struct Dev. 2000;19: 111-119.

1105

1106 29. Michener CD. The social behavior of the bees. Massachusetts: Harvard University Press; 1974.

1107

1108 30. Wilson EO. Sociobiology. The New Synthesis. Cambridge: Harvard University Press; 1975.

1109

1110 31. Honeybee Genome Sequencing Consortium. Insights into social insects from the genome of the  
1111 honeybee *Apis mellifera*. Nature. 2006;443: 931-949.

1112

1113 32. Sadd BM, Barribeau SM, Bloch G, de Graaf DC, Dearden P, Elsik CG, et al. The genomes of two  
1114 key bumblebee species with primitive eusocial organization. Genome Biol. 2015;16: 76. DOI:  
1115 [10.1186/s13059-015-0623-3](https://doi.org/10.1186/s13059-015-0623-3).

1116

1117 33. Kapheim KM, Pan H, Li C, Salzberg SL, Puiu D, Magoc T, et al. Genomic signatures of  
1118 evolutionary transitions from solitary to group living. Science. 2015;348: 1139-1143. DOI: [10.1126/science.aaa4788](https://doi.org/10.1126/science.aaa4788).

1119

1120

1121 34. Elias-Neto M, Nascimento ALO, Bonetti AM, Nascimento FS, Mateus S, Garófalo CA, Bitondi  
1122 MMG. Heterochrony of cuticular differentiation in eusocial corbiculate bees. Apidologie. 2013;45:  
1123 397-408.

1124

1125 35. Hansell MH. The ecological impact of animal nests and burrows. Funct Ecol. 1993;7: 5-12.

1126

1127 36. Evans JD, Spivak M. Socialized medicine: individual and communal disease barriers in honey bees.  
1128 J Invertebr Pathol. 2010;103: S62-S72.

1129

1130 37. West-Eberhard MJ. Developmental plasticity and the origin of species differences. Proc Natl Acad  
1131 Sci. 2005;102: 6543-6549.

1132

1133 38. Wilson EO, Hölldobler B. Eusociality: origin and consequences. *Proc Natl Acad Sci.* 2005;205:  
1134 13367-13371.

1135

1136 39. Ito C, Goto SG, Shiga S, Tomioka K, Numata H. Peripheral circadian clock for the cuticle  
1137 deposition rhythm in *Drosophila melanogaster*. *Proc Natl Acad Sci.* 2008;105: 8446-8451.

1138

1139 40. Ikeno T, Katagiri C, Numata H, Goto SG. Causal involvement of mammalian-type cryptochrome in  
1140 the circadian cuticle deposition rhythm in the bean bug *Riptortus pedestris*. *Insect Mol Biol.*  
1141 2011;20: 409-415.

1142

1143 41. Huang ZY, Robinson GE, Tobe SS, Yagi KJ, Strambi C, Strambi A, et al. Hormonal regulation of  
1144 behavioural development in the honey bee is based on changes in the rate of juvenile hormone  
1145 biosynthesis. *J Insect Physiol.* 1991;37: 733-741.

1146

1147 42. Mlodzik M, Hiromi Y, Weber U, Goodman CS, Rubin GM. The *Drosophila seven-up* gene, a mem-  
1148 ber of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell.* 1990;60: 211-  
1149 224.

1150

1151 43. Zander RH. Minimal values for reliability of bootstrap and jackknife proportions, decay index, and  
1152 Bayesian posterior probability. *PhyloInformatics.* 2004;2: 1-13.

1153

1154 44. Soares MPM, Silva-Torres FA, Elias-Neto M, Nunes FMF, Simões ZLP, Bitondi MMG.  
1155 Ecdysteroid-dependent expression of the *Tweedle* and *Peroxidase* genes during adult cuticle  
1156 formation in the honey bee, *Apis mellifera*. *PloS One.* 2011;6: e20513.

1157

1158 45. Soares MPM, Barchuk AR, Simões ACQ, Cristina AS, Freitas FCP, Canhos LL, et al. Genes  
1159 involved in thoracic exoskeleton formation during the pupal-to-adult molt in a social insect model,  
1160 *Apis mellifera*. *BMC Genomics.* 2013;14: 576.

1161

1162 46. Arakane Y, Lomakin J, Beeman RW, Muthukrishnan S, Gehrke SH, Kanost MR. Molecular and  
1163 functional analyses of amino acids decarboxylases involved in cuticle tanning in *Tribolium*  
1164 *castaneum*. *J Biol Chem* 2009a; 284: 16584-16594.

1165

1166 47. Gorman MJ, Arakane Y. Tyrosine hydroxylase is required for cuticle sclerotization and  
1167 pigmentation in *Tribolium castaneum*. *Insect Biochem Mol Biol.* 2010;40: 267-273.

1168

1169 48. Miyazaki S, Okada Y, Miyakawa H, Tokuda G, Cornette R, Koshikawa S, et al. Sexually dimorphic  
1170 body color is regulated by sex-specific expression of *Yellow* gene in ponerine ant, *Diacamma* sp.  
1171 *PLoS One.* 2014;9: e92875.

1172

1173 49. Wright TR F. The genetics of biogenic amine metabolism, sclerotization, and melanization in  
1174 *Drosophila melanogaster*. *Adv Genet.* 1987; 24: 127-222.

1175

1176 50. Elias-Neto M, Soares MPM, Simões ZLP, Hartfelder K, Bitondi MMG. Developmental  
1177 characterization, function and regulation of a Laccase2 encoding gene in the honeybee, *Apis*  
1178 *mellifera* (Hymenoptera, Apinae). *Insect Biochem Mol Biol.* 2010;40: 241-251.

1179

1180 51. Osanai-Futahashi M, Tatematsu KI, Futahashi R, Narukawa J, Takasu Y, Kayukawa T, et al.  
1181 Positional cloning of a *Bombyx* pink-eyed white egg locus reveals the major role of *cardinal* in  
1182 ommochrome synthesis. *Heredity*. 2016;116: 135-145.

1183

1184 52. Sugumaran M. Complexities of cuticular pigmentation in insects. *Pigment Cell Melanoma Res.*  
1185 2009;22: 523-525.

1186

1187 53. Lloyd V, Ramaswami M, Krämer H. Not just pretty eyes: *Drosophila* eye-colour mutations and  
1188 lysosomal delivery. *Trends Cell Biol.* 1998;8: 257-259.

1189

1190 54. Hamza I, Dailey HA. One ring to rule them all: trafficking of heme and heme synthesis  
1191 intermediates in the metazoans. *Biochim Biophys Acta*. 2012;1823: 1617-1632.

1192

1193 55. Stubenhaus BM, Dustin JP, Neverett ER, Beaudry MS, Nadeau LE, Burk-McCoy E, et al. Light-  
1194 induced depigmentation in planarians models the pathophysiology of acute porphyrias. *eLife*.  
1195 2016;5: e14175.

1196

1197 56. Shaik KS, Meyer F, Vázquez AV, Flötenmeyer M, Cerdán ME, Moussian B. δ-Aminolevulinate  
1198 synthase is required for apical transcellular barrier formation in the skin of the *Drosophila* larva.  
1199 *Eur J Cell Biol.* 2012;91: 204-215.

1200

1201 57. Xi Y, Pan PL, Ye YY, Yu B, Zhang CX. Chitin deacetylase family genes in the brown planthopper,  
1202 *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Mol Biol.* 2014;23: 695-705.

1203

1204 58. Arakane Y, Hogenkamp DG, Zhu YC, Kramer KJ, Specht CA, Beeman RW, et al. Characterization  
1205 of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage  
1206 in one of the genes during development. *Insect Biochem Mol Biol.* 2004;34: 291-304.

1207

1208 59. Arakane Y, Dixit R, Begum K, Park Y, Specht CA, Merzendorfer H, et al. Analysis of functions of  
1209 the chitin deacetylase gene family in *Tribolium castaneum*. *Insect Biochem Mol Biol.* 2009b;39:  
1210 355-365.

1211

1212 60. Tellam RL, Vuocolo T, Johnson SE, Jarmey J, Pearson RD. Insect chitin synthase: cDNA sequence,  
1213 gene organization and expression. *Eur J Biochem.* 2000;267: 6025-6042.

1214

1215 61. Gagou ME, Kapsetaki M, Turberg A, Kafetzopoulos D. Stage-specific expression of the chitin  
1216 synthase *DmeChSA* and *DmeChSB* genes during the onset of *Drosophila* metamorphosis. *Insect*  
1217 *Biochem Mol Biol.* 2002;32: 141-146.

1218

1219 62. Ampasala DR, Zheng S, Zhang D, Ladd T, Doucet D, Krell PJ, et al. An epidermis-specific chitin  
1220 synthase cDNA in *Choristoneura fumiferana*: cloning, characterization, developmental and  
1221 hormonal-regulated expression. *Arch Insect Biochem Physiol.* 2011;76: 83-96.

1222

1223 63. Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S. Functional specialization among  
1224 insect chitinase family genes revealed by RNA interference. *Proc Natl Acad Sci.* 2008;105: 6650-  
1225 6655.

1226

1227 64. Luschnig S, Bätz T, Armbruster K, Krasnow MA. *serpentine* and *vermiform* encode matrix proteins  
1228 with chitin binding and deacetylation domains that limit tracheal tube length in *Drosophila*. *Curr*  
1229 *Biol.* 2006;16: 186-194.

1230

1231 65. Arakane Y, Muthukrishnan S, Kramer KJ, Specht CA, Tomoyasu Y, Lorezen MD, et al. The  
1232 *Tribolium* chitin synthase genes *TcCHS1* and *TcCHS2* are specialized for synthesis of epidermal  
1233 cuticle and midgut peritrophic matrix. *Insect Mol Biol.* 2005;14: 53-463.

1234

1235 66. Moussian B, Schwarz H, Bartoszewski S, Nüsslein-Volhard C. Involvement of chitin in exoskeleton  
1236 morphogenesis in *Drosophila melanogaster*. *J Morphol.* 2005;264: 117-130.

1237

1238 67. Pan PL, Ye YX, Lou YH, Lu JB, Cheng C, Shen Y, et al. A comprehensive omics analysis and  
1239 functional survey of cuticular proteins in the brown planthopper. *Proc Natl Acad Sci.* 2018;115:  
1240 5175-5180.

1241

1242 68. Noh MY, Kramer KJ, Muthukrishnam S, Kanost MR, Beeman RW, Arakane A. Two major cuticular  
1243 proteins are required for assembly of horizontal laminae and vertical pore canals in rigid cuticle of  
1244 *Tribolium castaneum*. *Insect Biochem Mol Biol.* 2014;53: 22-29.

1245

1246 69. Noh MY, Muthukrishnam S, Kramer KJ, Arakane Y. *Tribolium castaneum* RR-1 cuticular protein  
1247 TcCPR4 is required for formation of pore canals in rigid cuticle. *PLoS Genet.* 2015;11: e1004963.

1248

1249 70. Tang L, Liang J, Zhan Z, Xiang Z, He N. Identification of the chitin-binding proteins from the lar-  
1250 val proteins of silkworm, *Bombyx mori*. *Insect Biochem Mol Biol.* 2010;40: 228-234.

1251

1252 71. Cornman RS, Willis JH. Annotation and analysis of low-complexity protein families of *Anopheles*  
1253 *gambiae* that are associated with cuticle. *Insect Mol Biol.* 2009;18: 607-622.

1254

1255 72. Togawa T, Dunn WA, Emmons AC, Willis J. CPF and CPFL, two related gene families encoding  
1256 cuticular proteins of *Anopheles gambiae* and other insects. *Insect Biochem Mol Biol.* 2007;37: 675-  
1257 688.

1258

1259 73. Kucharski R, Maleszka J, Maleszka R. Novel cuticular proteins revealed by the honey bee genome.  
1260 *Insect Biochem Mol Biol.* 2007;37: 128-134.

1261

1262 74. Jasrapuria S, Specht CA, Kramer KJ, Beeman RW, Muthukrishnan S. Gene families of cuticular  
1263 proteins analogous to peritrophins (CPAPs) in *Tribolium castaneum* have diverse functions. *PLoS*  
1264 *One* 2012;7: e49844

1265

1266 75. Moussian B, Tång E, Tonning A, Helms S, Schwarz H, Nüsslein-Volhard C, et al. *Drosophila*  
1267 Knickkopf and Retroactive are needed for epithelial tube growth and cuticle differentiation through  
1268 their specific requirement for chitin filament organization. *Development.* 2006;133: 163-171.

1269

1270 76. Chaudhari SS, Arakane Y, Specht CA, Moussian B, Boyle DL, Park Y, et al. Knickkopf protein  
1271 protects and organizes chitin in the newly synthesized insect exoskeleton. *Proc Natl Acad Sci.*  
1272 2011;108: 17028-17033.

1273

1274 77. Chaudhari SS, Arakane Y, Specht CA, Moussian B, Kramer KJ, Muthukrishnan S. et al. Retroactive  
1275 maintains cuticle integrity by promoting trafficking of Knickkopf into the procuticle of *Tribolium*  
1276 *castaneum*. PLoS Genet. 2013;9: e1003268.

1277

1278 78. Zitnam D, Adams ME. Neuroendocrine regulation of ecdysis. In LI Gilbert, editor. San Diego:  
1279 Elsevier Academic Press; 2012. pp. 253-309.

1280

1281 79. Jones G, Sharp PA. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc Natl  
1282 Acad Sci. 1997;94: 13499-13503.

1283

1284 80. Jindra M, Uhlirova M, Charles J-P, Hill RJ. Genetic evidence for function of the bHLH-PAS protein  
1285 Gce/Met as a juvenile hormone receptor. PLoS Genetics. 2015;11: e1005394.

1286

1287 81. Saha TT, Shin SW, Dou W, Roy S, Zhao B, Hou Y, et al. Hairy and Groucho mediate the action of  
1288 juvenile hormone receptor Methoprene-tolerant in gene repression. Proc Natl Acad Sci. 2016;7:  
1289 E735-E743.

1290

1291 82. Zhao D, Woolner S, Bownes M. The Mirror transcription factor links signalling pathways in  
1292 *Drosophila* oogenesis. Dev Genes Evol. 2000;210: 449-457

1293

1294 83. Jeong S, Rokas A, Carroll SB. Regulation of body pigmentation by the Abdominal-B Hox protein  
1295 and its gain and loss in *Drosophila* evolution. Cell. 2006;125: 1387-1399.

1296

1297 84. Carroll SB, Laughon A, Thalley BS. Expression, function, and regulation of the hairy segmentation  
1298 protein in the *Drosophila* embryo. Genes Dev. 1988;2, 883-890.

1299

1300 85. Song Q. Bursicon, a neuropeptide hormone that controls cuticle tanning and wing expansion. In:  
1301 Gilbert LI, editor. Insect Endocrinology. New York: Academic Press; 2012. pp. 93-105.

1302

1303 86. Costa CP, Elias-Neto M, Falcon T, Dallacqua RP, Martins JR, Bitondi MMG. RNAi-mediated  
1304 functional analysis of bursicon genes related to adult cuticle formation and tanning in the honeybee,  
1305 *Apis mellifera*. PLoS One . 2016;11: e0167421.

1306

1307 87. Zelazny B, Neville AC. Endocuticle layer formation controlled by non-circadian clocks in beetles.  
1308 Insect Physiol. 1972;18: 1967-1979.

1309

1310 88. Andersen SO, Hojrup P, Roepstorff P. Insect cuticular proteins. Insect Biochem Mol Biol. 1995;25:  
1311 153-176.

1312

1313 89. Whitten J. Coordinated development in the fly foot: sequential cuticle secretion. J Morphol.  
1314 1969;127: 73-104.

1315

1316 90. Garófalo CA. Social structure of *Euglossa cordata* nests (Hymenoptera: Apidae: Euglossini).  
1317 Entomol Gen. 1985;11: 77-83.

1318

1319 91. Augusto SC, Garófalo CA. Comportamento das fêmeas nas associações formadas em ninhos de  
1320 *Euglossa cordata* (Hymenoptera; Apidae; Euglossini). In: Encontro sobre Abelhas, Ribeirão Preto,

1321 SP, Brazil; 1994. pp. 171-181.

1322

1323 **92.** Freiria GA, Garófalo CA, Del Lama MA. The primitively social behavior of *Euglossa cordata* (Hymenoptera, Apidae, Euglossini): a view from the perspective of kin selection theory and models of reproductive skew. *Apidologie*. 2017;48: 523-532. Doi: 10.1007/s13592-017-0496-4

1324

1325

1326

1327 **93.** Cardinal S, Danforth BN. The antiquity and evolutionary history of social behavior in bees. *PloS One*. 2011;6: e21086.

1328

1329

1330 **94.** Silva-Matos EV, Garófalo CA. Worker life tables, survivorship, and longevity in colonies of *Bombus (Fervidobombus) atratus* (Hymenoptera: Apidae). *Rev Biol Tropical*. 2000;48: 657-664.

1331

1332

1333 **95.** Chaudhari SS, Moussian B, Specht CA, Arakane Y, Kramer KJ, Beeman RW, et al. Functional specialization among members of Knickkopf family of proteins in insect cuticle organization. *PLoS Genet*. 2014;10: e1004537.

1334

1335

1336

1337 **96.** Hinaux H, Bachem K, Battistara M, Rossi M, Xin Y, Jaenichen R, et al. Revisiting the developmental and cellular role of the pigmentation gene *yellow* in *Drosophila* using a tagged allele. *Dev Biol*. 2018;438: 111-123

1338

1339

1340

1341 **97.** Reddy KL, Rovani MK, Wohlwill A, Katzen A, Storti RV. The *Drosophila* Par domain protein I gene, *Pdp1*, is a regulator of larval growth, mitosis and endoreplication. *Dev Biol*. 2006;289: 100-114.

1342

1343

1344

1345 **98.** Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, Hardin PE, et al. *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell*. 2003;112: 329-341.

1346

1347

1348 **99.** Gibbs A, Pomonis JG. Physical properties of insect cuticular hydrocarbons: the effects of chain length, methyl-branching and unsaturation. *Comp Biochem Physiol*. 1995;112B: 243-249.

1349

1350

1351 **100.** Lockey KH. Lipids of the insect cuticle: origin, composition and function. *Comp Biochem Physiol Part B: Comp Biochem*. 1988;89: 595-645.

1352

1353

1354 **101.** Ferreira-Caliman MJ, Nascimento FS, Turatti IC, Lopes NP, Zucchi R. The cuticular hydrocarbons profiles in the stingless bee *Melipona marginata* reflect task-related differences. *J Insect Physiol*. 2010;56: 800-804.

1355

1356

1357

1358 **102.** Kather R, Drijfhout FP, Martin SJ. Task group differences in cuticular lipids in the honey bee *Apis mellifera*. *J Chem Ecol*. 2011;37: 205-212.

1359

1360

1361 **103.** Wagner D, Brown MJF, Broun P, Cuevas W, Moses LE, Chao DL, et al. Task-related differences in the cuticular hydrocarbon composition of the harvester ants, *Pogonomyrmex barbatus*. *J Chem Ecol*. 1998;24: 2021-2037.

1362

1363

1364

1365 **104.** Gibbs AG. Water-proofing properties of cuticular lipids. *Amer Zool*. 1998;38: 471-482.

1366

1367 **105.** Paulmier I, Bagnères AG, Afonso CMM, Dusticier G, Rivière G, Clément JL. Alkenes as sexual

1368 pheromone in the alfalfa leaf-cutter bee *Megachile rotundata*. J Chem Ecol. 1999;25: 471-490.  
1369

1370 **106.** Jesus BMV, Garófalo CA. Nesting behaviour of *Centris (Heterocentris) analis* (Fabricius) in  
1371 southeastern Brazil (Hymenoptera, Apidae, Centridini). Apidologie. 2000;31: 503-515.  
1372

1373 **107.** Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads.  
1374 EMBnet.journal. 2011;17: 10-12.  
1375

1376 **108.** Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets.  
1377 Bioinformatics. 2011;27: 863-864.  
1378

1379 **109.** Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. Available  
1380 from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.  
1381

1382 **110.** Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq.  
1383 Bioinformatics. 2009;25: 1105-1111.  
1384

1385 **111.** Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript  
1386 assembly and abundance estimation from RNA-Seq reveals thousands of new transcripts and  
1387 switching among isoforms. Nature Biotechnol. 2010;28: 511-515.  
1388

1389 **112.** Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript  
1390 expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protoc. 2012;7:  
1391 562-578.  
1392

1393 **113.** Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length  
1394 transcriptome assembly from RNA-seq data without a reference genome. Nature Biotechnol.  
1395 2011;15: 644-652.  
1396

1397 **114.** Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, et al. De novo transcript  
1398 sequence reconstruction from RNA-seq using the Trinity platform for reference generation and  
1399 analysis. Nature Protoc. 2013;8: 1494-1512.  
1400

1401 **115.** Miller JR, Koren S, Sutton G. Assembly algorithms for the next-generation sequencing data.  
1402 Genomics. 2010;95: 325-327.  
1403

1404 **116.** Sonnhammer ELL, Östlund G. InParanoid 8: ontology analysis between 273 proteomes, mostly  
1405 eukaryotic. Nucleic Acids Res. 2014;43: D234-D239.  
1406

1407 **117.** Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq  
1408 data with DESeq2. Genome Biol. 2014;15: 550.  
1409

1410 **118.** Robinson MD, Smyth GK. Small sample estimation of negative binomial dispersion, with  
1411 applications to SAGE data. Biostatistics. 2008;9: 321-332.  
1412

1413 **119.** Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, et al. gplots: various  
1414 R programming tools for plotting data. R package version 2.16.0. 2015. Available from:

1415 http://CRAN.R-project.org/package=gplots  
1416  
1417 **120.** Suzuki R, Shimodaira H. Pvclust: an R package for assessing the uncertainty in hierarchical  
1418 clustering. *Bioinformatics*. 2006;22: 1540-1542.  
1419  
1420 **121.** Reimand J, Kull M, Peterson H, Hansen J, Vilo J. g:Profiler - a web-based toolset for functional  
1421 profiling of gene lists from large-scale experiments. *Nucleic Acids Res.* 2007;35: W193-W200.  
1422  
1423 **122.** Reimand J, Arak T, Vilo J. g:Profiler - a web server for functional interpretation of gene lists (2011  
1424 update). *Nucleic Acids Res.* 2011;39: W307-W315.  
1425  
1426 **123.** Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists  
1427 using DAVID Bioinformatics Resources. *Nature Protocols*. 2009a;4: 44-57.  
1428  
1429 **124.** Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the  
1430 comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009b;37: 1-13.  
1431  
1432 **125.** Ioannidou ZS, Theodoropoulou MC, Papandreou NC, Willis JH, Hamodrakas SJ. CutProtFam-  
1433 Pred: detection and classification of putative structural cuticular proteins from sequence alone,  
1434 based on profile hidden Markov models. *Insect Biochem Mol Biol*. 2014;52: 51-59.  
1435  
1436 **126.** Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer.  
1437 *BMC Bioinformatics*. 2014;15. DOI: 10.1186/1471-2105-15-293.  
1438  
1439 **127.** Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, et al. TRANSFAC: an integrated  
1440 system for gene expression regulation. *Nucleic Acids Res.* 2000;28: 316-319.  
1441  
1442 **128.** Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software  
1443 environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003;13:  
1444 2498-2504.  
1445  
1446 **129.** Abramoff MD, Magalhães PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Internat.*  
1447 2004;11: 36-42.  
1448  
1449 **130.** Fay DS, Gerow K. A biologist's guide to statistical thinking and analysis. In: Hobert O, editor.  
1450 *Wormbook*. 2013. DOI: doi/10.1895/wormbook.1.159.1 .  
1451  
1452 **131.** Nunes TM, Nascimento FS, Turatti IC, Lopes NP, Zucchi R. Nestmate recognition in a stingless  
1453 bee: does the similarity of chemical cues determine guard acceptance? *Anim Behav*. 2008;75: 1165-  
1454 1171.  
1455  
1456 **132.** Analytical Methods Committee. Internal quality control of analytical data. *Analyst* 1995;120: 29-  
1457 34.  
1458  
1459  
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1461 **Figures**

1462 **Fig 1. Venn diagrams constructed with the genes and contigs differentially expressed in the**  
1463 **integument of the developmental phases of (A) *A. mellifera*, (B) *F. varia*, and (C) *C. analis*.** The  
1464 number of genes upregulated in each pairwise comparison is indicated. Pbm: pharate adults; Ne:  
1465 newly-emerged bees; Fg: foragers.

1466 **Fig 2. Gene Ontology (GO) functional terms attributed to integument genes during adult cuticle**  
1467 **development and maturation.** The functional terms more represented in the Pbm and Ne phases than  
1468 in the Fg phase are indicated as Younger>Fg, and those more represented in the Ne and Fg phases than  
1469 in the Pbm phase are reported as Older>Pbm. The green box includes GO terms related to the cuticle-  
1470 producing tissue, the epidermis. Purple box: GO terms associated to structural components of the  
1471 cuticle. Black box: GO terms potentially associated to CHC biosynthetic pathways. Yellow box: GO  
1472 terms related to pigments and pigmentation.

1473 **Fig 3. Distance correlation analysis between developmental phases based on the expression of**  
1474 **DEGs and DECs.** (A) *A. mellifera*; (B) *F. varia*; (C) *C. analis*. Red values (BP): bootstrap support.  
1475 Green values (AU): cluster support. Arrows point to significant clusters (AU > 95%). Branch edges are  
1476 shown in gray. Pbm = pharate adults. Ne = newly emerged bees. Fg = foragers.

1477 **Fig 4. Representative heatmaps of gene expression profiles through the Pbm (pharate-adult), Ne**  
1478 **(newly-emerged), and Fg (forager) developmental phases of (A) *A. mellifera*, (B) *F. varia*, and (C)**  
1479 ***C. analis*.** Genes were grouped according to their potential function in adult cuticle formation and  
1480 maturation. Different lowercase letters on the heatmaps means statistically significant difference (see  
1481 Materials and Methods) in the expression levels between the developmental phases of each bee species.

1482 **Fig 5. Ortholog genes showing significantly correlated expression profiles at least between two of**  
1483 **the three bee species, *A. mellifera*, *F. varia* and *C. analis*.** Expression profiles of *ebony*, *tan*, *Idgf4-*  
1484 *like*, *Cda5*, *chitooligosaccharidolytic-domain-like*, *CPR14*, *CPR17*, *CPR25*, *CPR26*, *Apd-like*, *Elo-*  
1485 *GB54302*, *Elo-GB54401*, *Elo-GB45596*, *Ethr*, *E74*, *Hr4*, *Hr38*, *FTZ-F1*, *rickets*, *Tim2*, and *ALAS* were  
1486 positively correlated between the eusocial bee species, and negatively or non-correlated with the  
1487 solitary bee. Expression profiles of *CPR18*, *CPR23*, *Apd-3*, *Desat-GB40659*, *Elo-GB46038*, and *Ptx-1*  
1488 were positively correlated between the eusocial species, the basal line in the graphic representations  
1489 indicating undetected orthologs in *C. analis*. Pbm: pharate adults, Ne: newly emerged, and Fg:  
1490 foragers. Color key at the bottom of figure.

1491 **Fig 6. Overlapping interactions in the gene co-expression networks reconstructed with *A.***

1492 ***mellifera* (S2 Fig) and *F. varia* (S3 Fig) genes related to cuticle formation and maturation.**  
1493 **Fig 7. Ultrastructure and thickness of the developing and mature adult cuticle of bees differing in**  
1494 **ways of life. (A) *A. mellifera* (eusocial), (B) *F. varia* (eusocial), (C) *C. analis* (solitary), (D) *B.***  
1495 ***brasiliensis* (primitively eusocial), (E) *E. cordata* ( facultatively eusocial), and (F) *T. diversipes***  
1496 **(solitary).** Developmental phases are indicated: Pbm (pharate-adult); Ne (newly-emerged); 0h, 24h,  
1497 48h, 72h, and 96h after adult emergence; Fg (forager). The cuticle/epidermis junction was used to align  
1498 the cuticle images. Means and standard deviations of cuticle thickness measurements are represented in  
1499 red. The number of cuticle samples measured (N) is indicated for the Pbm, Ne and Fg phases of each  
1500 bee species. (A'- F') Cuticle thickness measurements ( $\mu$ m) for the corresponding bee species. Different  
1501 lowercase letters indicate significant statistical difference between the developmental phases of each  
1502 species.

1503

#### 1504 **Supporting information**

1505 **S1 Fig.** Correlation heatmaps based on the RNA-seq data obtained from the integument of the pharate  
1506 adults (Pbm), newly-emerged (Ne) and foragers (Fg) of the three bee species: (A) *A. mellifera*, (B) *F.*  
1507 *varia* and (C) *C. analis*. The numbers 1, 2 and 3 following the Pbm, Ne, and Fg abbreviations indicate  
1508 the independent samples of each developmental phase.

1509 **S2 Fig. Gene co-expression networks in the integument of *A. mellifera* for adult cuticle formation**  
1510 **and maturation.** The genes are indicated in the nodes, and the edges represent significant correlation  
1511 among genes.

1512 **S3 Fig. Gene co-expression networks in the integument of *F. varia* for adult cuticle formation and**  
1513 **maturation.** The genes are indicated in the nodes, and the edges represent significant correlation  
1514 among genes.

1515 **S4 Fig. Gene co-expression networks in the integument of *C. analis* for adult cuticle formation**  
1516 **and maturation.** The genes are indicated in the nodes, and the edges represent significant correlation  
1517 among genes.

1518 **S5 Fig. Distances between the developmental phases of *A. mellifera*, *F. varia* and *C. analis* based**  
1519 **on Euclidean distance analysis of total CHCs, n-alkanes, unsaturated CHCs, and branched CHCs**  
1520 **relative quantifications.** Red boxes indicate significant clusters with 95% of confidence. Arrows  
1521 indicate significantly supported clusters. AU clusters' support (red values); BP bootstrap support (green  
1522 values); Branches' edges (gray values). Pharate-adults (Pbm), newly emerged (Ne), and forager (Fg)

1523 bees.

1524

1525 **S1 Table. Ortholog genes displaying significantly correlated expression profiles.** Comparisons of  
1526 gene expression levels through the pharate-adult (Pbm), newly-emerged (Ne) and forager (Fg)  
1527 developmental phases of *A. mellifera*, *F. varia*, and *C. analis*. Blue: significant positive correlation.  
1528 Red: significant negative correlation. (-): undetectable gene expression.

1529 **S2 Table – Number of genes encoding the different classes of structural cuticular proteins in**  
1530 **hymenopterans.**

1531

1532 **S1 File. Genes identified in the RNA-seq analysis of the integument of *A. mellifera*, *F. varia* and *C.***  
1533 ***analis*.**

1534 **S2 File. Genes upregulated in the comparisons of the developmental phases of each bee species,**  
1535 ***A. mellifera*, *F. varia* and *C. analis*.** Pharate-adult (Pbm), newly-emerged (Ne) and forager (Fg)  
1536 developmental phases. The bee species and developmental phases compared are specified in the  
1537 inferior margin of each table in this File. For example: Amel\_Pbm>Ne means the list of *A. mellifera*  
1538 genes upregulated in Pbm in comparison to Ne.

1539 **S3 File. Gene Ontology (GO) functional analysis of the differentially expressed genes.** The bee  
1540 species and developmental phases compared are specified in the inferior margin of each table in this  
1541 File. For example: Amel\_Pbm\_Ne>Fg\_ID means the bees' gene IDs and Fly Base gene IDs from  
1542 higher expressed genes at younger developmental phases; and Amel\_Ne\_Fg>Pbm\_GO means the Gene  
1543 Ontology of the higher expressed genes at older developmental phase based on their fly orthologues  
1544 IDs.

1545 **S4 File. Cuticular hydrocarbon (CHC) profiles determined for the developmental phases of *A.***  
1546 ***mellifera*, *F. varia*, and *C. analis*, variable contribution (total and per comparison) and mass**  
1547 **quantification.** Pharate-adult (Pbm), newly-emerged (Ne) and forager (Fg) developmental phases.

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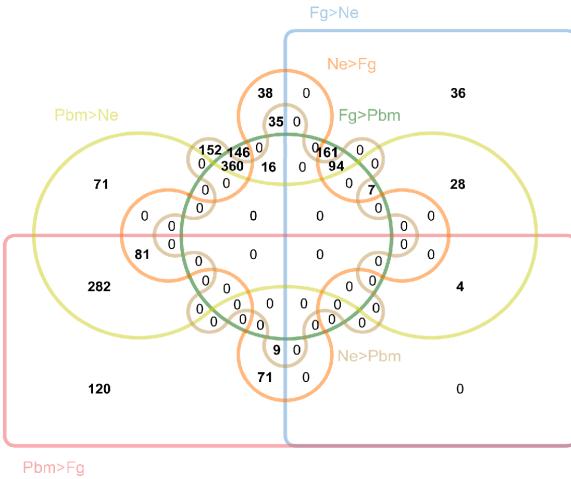
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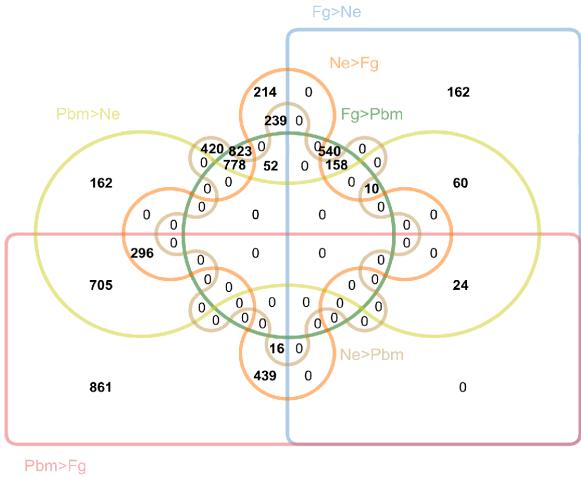
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1554 Figure 1

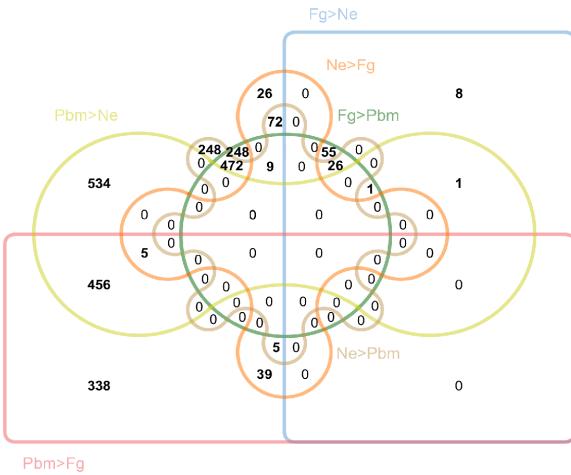
A.



B.



C.



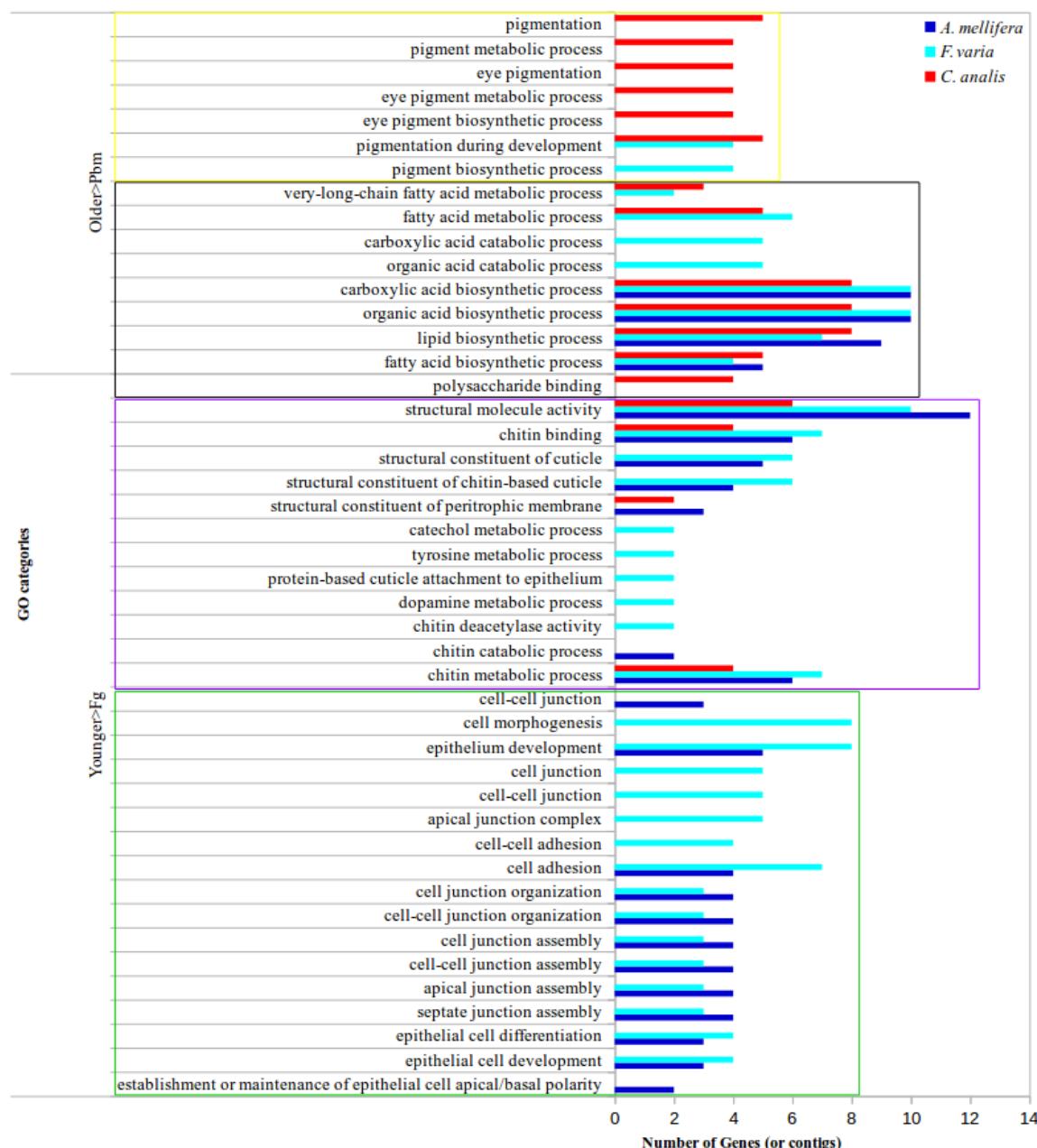
1556 Figure 2

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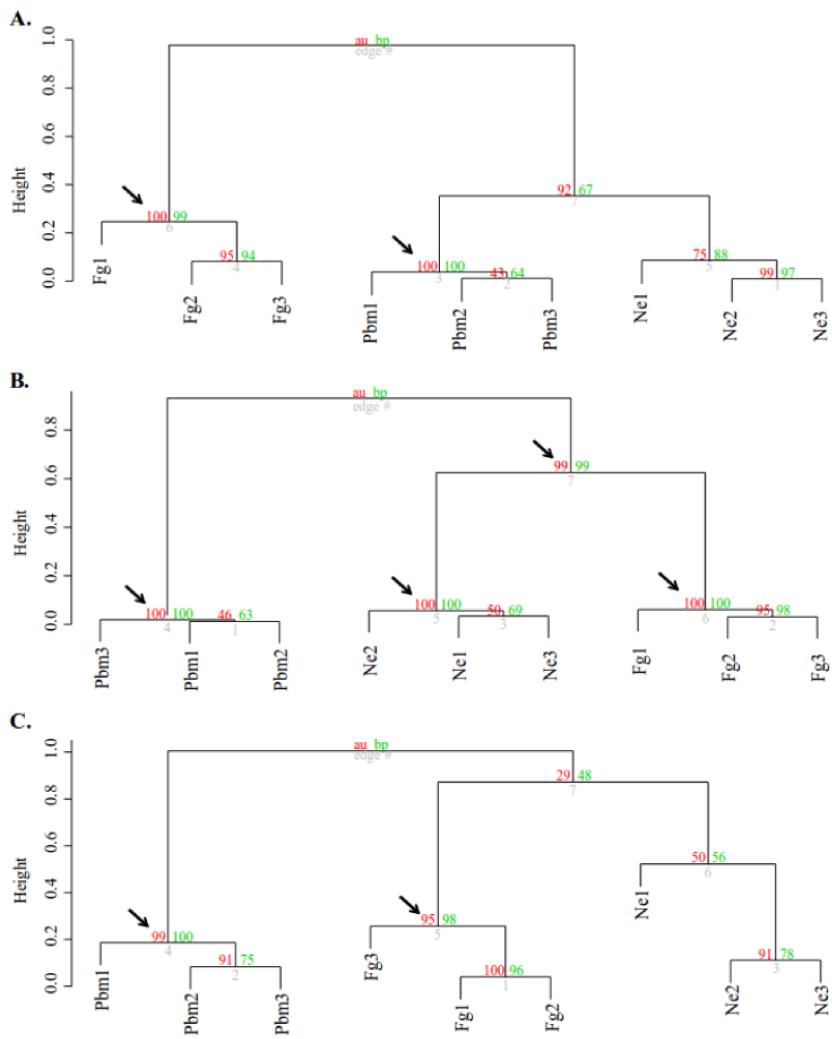
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1563 Figure 3

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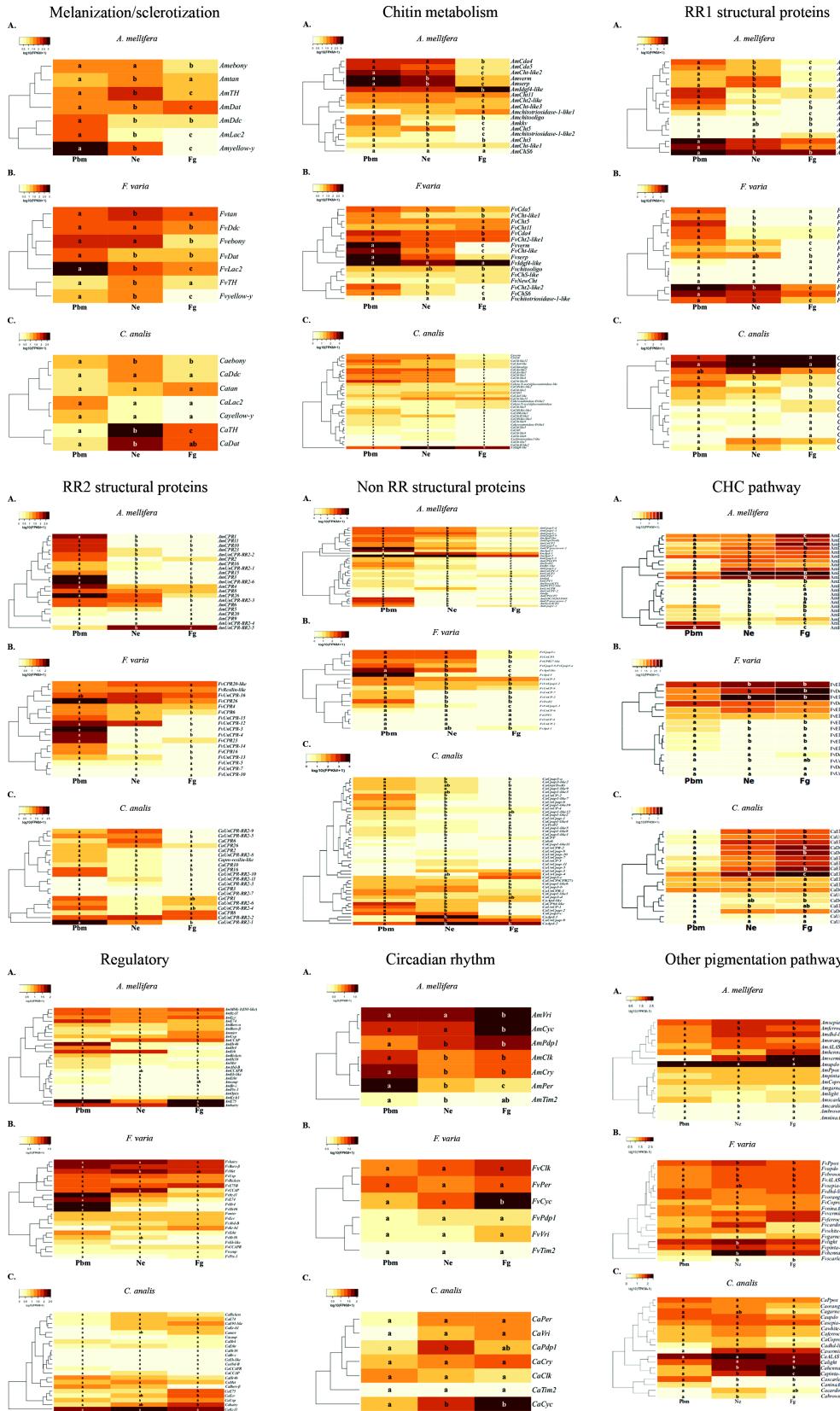
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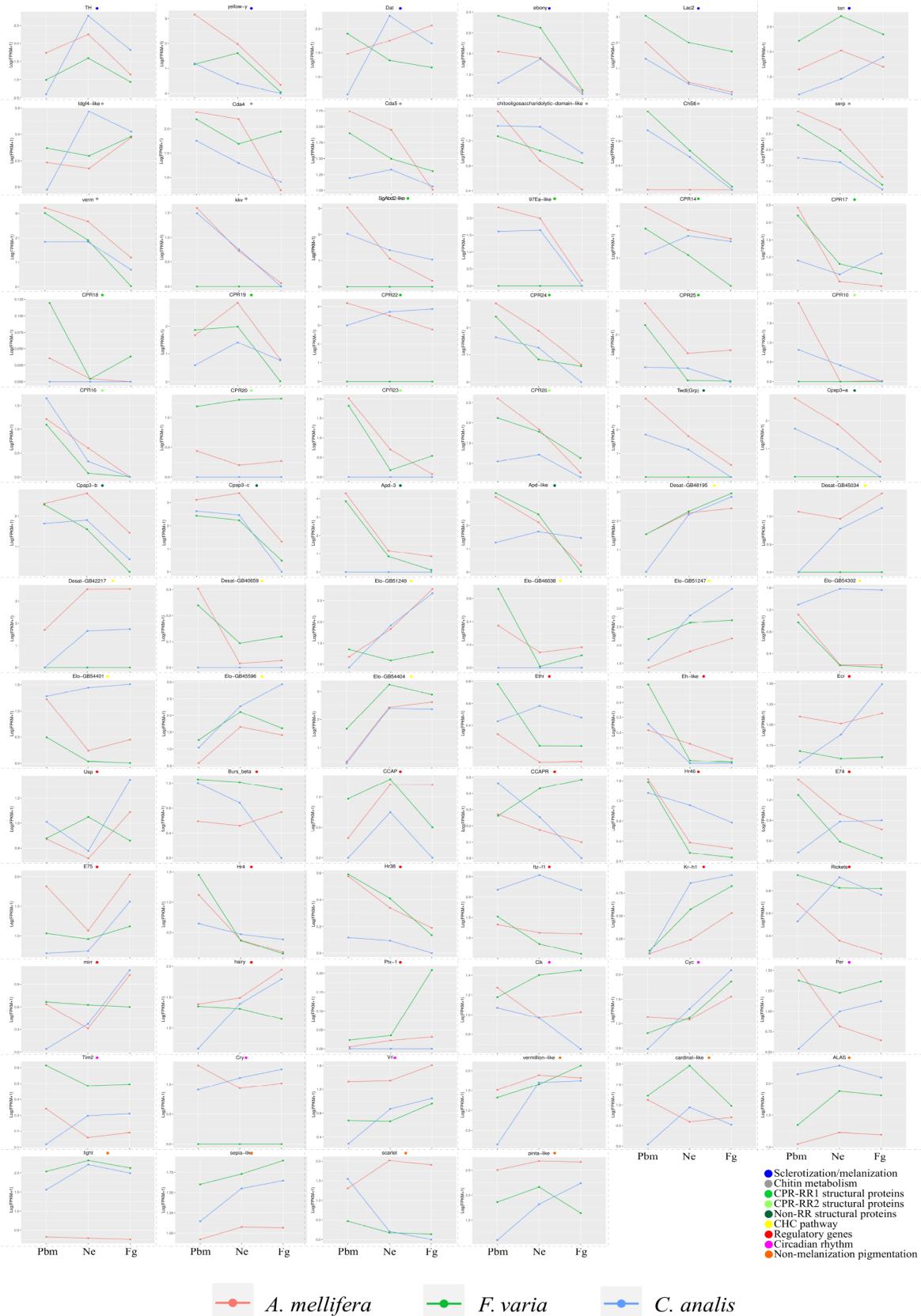
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1575 Figure 4



1577 Figure 5

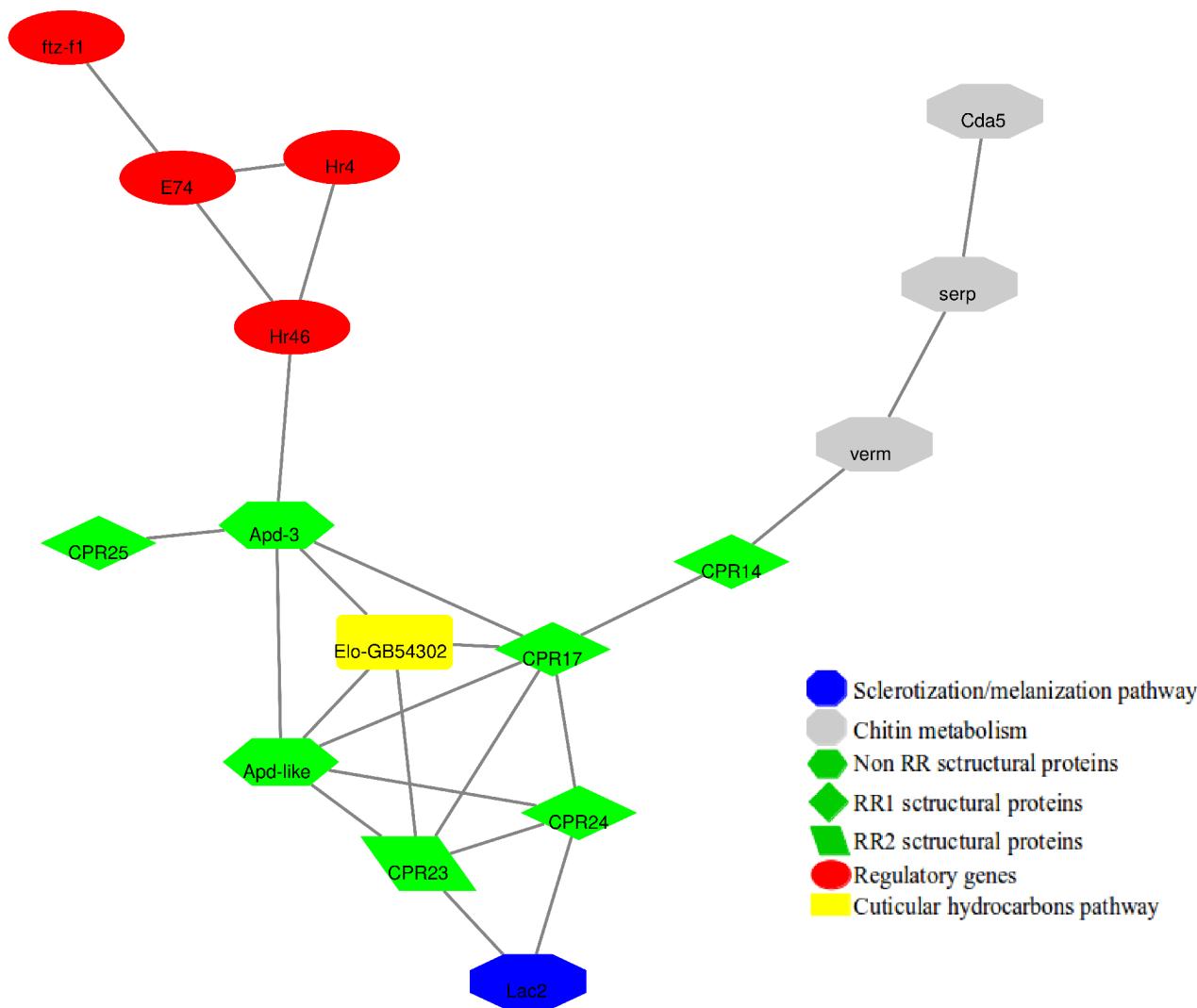


*A. mellifera*

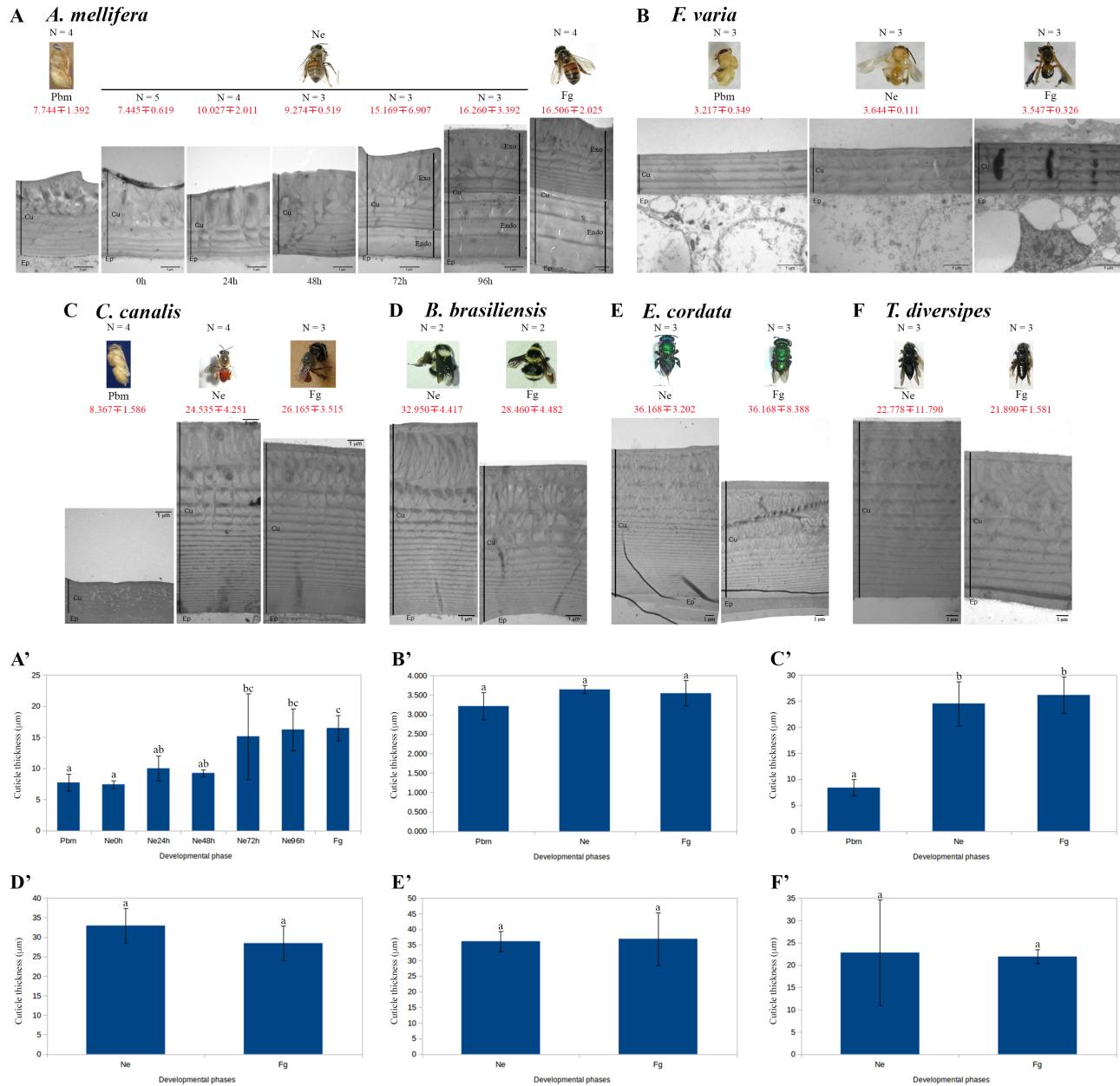
*F. varia*

*C. analis*

1579 Figure 6



1590 Figure 7



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