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1 # Longitudinal survey reveals delayed effects of forager gene
2 # expression on stingless bee colony health

3

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25 **Keywords:** stingless bee, *Melipona quadrifasciata*, bee health, colony collapse, sublethal effects.

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31 **Abstract**

32

33 Bee populations are declining globally due to different environmental stressors, such as pathogens,
34 malnutrition, and agrochemicals. Brazil is the home of hundreds of stingless bee species, some of
35 them now considered endangered, though very little is known about the impact of disease on native
36 bees. In Southern Brazil the endangered stingless bee *Melipona quadrifasciata* is affected by an
37 annual syndrome that causes sudden death of workers, eventually leading colonies to collapse.
38 Although novel viruses were found in foragers from diseased colonies, none has been consistently
39 implicated in the outbreak. Here we used transcriptomics in combination with an integrative
40 longitudinal survey on managed colonies to identify predictors for preventing *M. quadrifasciata* colony
41 failures. We found that key genes related to xenobiotic metabolization, nutrition and immune
42 responses are downregulated in foragers from colonies that became diseased three months later. The
43 period that preceded the outbreak was marked by pronounced forager weight loss as well as
44 behavioural changes. Our findings support the proposition that worldwide bee mortality is influenced
45 by a combination of diverse sublethal factors, and increase awareness of the long-term effects of
46 genetic diversity erosion in stingless bee species, which enhances their vulnerability to environmental
47 stressors.

48

49 **Introduction**

50

51 The worldwide decrease in bee populations observed in the last decade is a matter of great
52 concern. Some colony losses are explained by the presence of pathogens and other infectious agents
53 [1,2], but several additional interacting stressors, such as habitat loss, malnutrition, agrochemicals and
54 colony management practices are known to reduce the fitness of bee populations [3]. Theoretical
55 studies indicate that although multifactorial stresses may cause colony failure, it is most likely a result
56 of a critical level of stress from the accumulation of sublethal factors [4].
57 One of the intrinsic properties of sublethal factors is their delayed effect on organismal fitness.
58 In eusocial bees, where individual fitness is achieved indirectly by cooperation, sublethal factors
59 characteristically compromise the ability of non-reproductive females to perform their regular tasks [5].
60 For example, honey bee workers reared in pollen-stressed colonies show normal development, but as

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61 adults are less likely to waggle dance, and precisely inform food location [6]. Exposure of larvae to
62 sublethal doses of pesticides induces multiple changes in gene expression, leading to developmental
63 and adult behavioural changes that weaken bee colonies [5,7]. Besides interacting with each other,
64 environmental stressors are modulated by endogenous factors that are ultimately linked to the bee's
65 genetic background [8]. Some bee management practices, including colony translocation, may lead to
66 genetic homogenization, loss of genetic diversity, and the breakdown of local adaptations, finally
67 impacting their capacity to respond to stressors [9,10].
68 The stingless bee *M. quadrifasciata* is one of the most extensively managed species and the
69 object of intensive trading in Southern Brazil, where its nests virtually disappeared from nature [11]. In
70 this region, the practice of colony division has been performed for decades and became more
71 common with trade intensification [12], which is now threatened by a syndrome that annually occurs in
72 late summer, often leading to colony collapse [13,14]. Some worker bees from affected colonies show
73 neurologic symptoms, such as tremors and paralysis, suggesting the implication of viruses in the
74 syndrome. However, in spite of having found novel viruses associated with symptomatic bees, such as
75 dicistroviruses, which cause similar symptoms in *A. mellifera* [15], no virus [14] or other pathogen [13]
76 was found consistently associated with bees from diseased colonies. Here we report an integrative
77 and longitudinal study designed to uncover the causes underlying the annual syndrome of *M.*
78 *quadrifasciata*. In addition to exploratory transcriptomic analyses, a temporal survey was conducted on
79 three pairs of mother-daughter colonies kept in two separate localities, in order to evaluate the
80 contribution of both genetic and environmental factors to the syndrome manifestation, by measuring
81 individual- and colony-level traits.
82
83 **Materials and Methods**
84
85 **(a) Transcriptome analyses**
86 Three *M. quadrifasciata* colonies were sampled for exploratory transcriptomic analyses aiming
87 to identify differentially expressed genes (DEGs) during the syndrome, and to select candidate genes
88 for further analyses. Two colonies (D1 and D2) presented symptoms during the yearly outbreak, while
89 the third (H) remained healthy and was sampled after the outbreak period. RNA of three pooled bees
90 from each colony was purified by polyA-tail selection, followed by library construction using TruSeq

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91 Stranded mRNA Library Prep Kit (Illumina, USA). Single-end sequencing (read length = 150 nt) was
92 performed on an Illumina NextSeq instrument. Low quality reads were removed with Trimmomatic
93 v.0.36 [16], and mapped onto *M. quadrifasciata* genome (GenBank: GCA_001276565.1) with GSNAP
94 v. 2018-07-04 [17]. Gene expression was estimated with the *depth* command from Samtools v.1.3.1
95 [18], and DEGs were recovered with custom Perl scripts, after a normalization step (Available at
96 https://github.com/liliancaesar/Publication_scripts/tree/main/2020_Longitudinal_survey/Transcriptome
97 _analyses). Functional enrichment analysis was performed with g:Profiler [19]. Detailed information on
98 the samples, nucleic acid extraction and transcriptome analyses are provided as electronic
99 supplementary material (text S1). Three DEGs known for their roles in bee health were selected for
100 RT-qPCR assays.
101
102 **(b) Monitoring bee colonies and forager relative gene expression**
103 To monitor changes of key biological features, observations were made in *M. quadrifasciata*
104 mother-daughter (MD) colonies under semi-controlled conditions during six months in two localities.
105 Three colonies (named BP1, BP2 and BP3) were kept in a small agricultural property where
106 agrochemicals are regularly used. Daughter colonies obtained by division from each BP colony
107 (named PA1, PA2 and PA3), were translocated to the vicinity of a secondary forest, located about 70
108 km away from BP. Colonies were equipped with a datalogger device (ONSET, Brazil) to record within-
109 hive temperature and humidity every six hours. Downstream analyses were conducted with the daily
110 lowest temperature (t) and the highest humidity (h), or with a variable called delta (Δ = maximum value
111 - minimum value). Aliquots of pollen stored inside colonies were collected every month from the
112 experimental hives or from nearby colonies in each meliponary. Around 500 pollen grains were
113 identified at the family, genus, or species level, using reference material from pollen libraries and the
114 literature (see electronic supplementary material, text S1). Every month five foragers (figure 1A) were
115 collected, weighted, and stored at -80°C until RNA extraction for RT-qPCR.
116 Three DEGs were selected for monitoring forager bee expression patterns during the survey,
117 namely putative mitochondrial *cytochrome P450* (*CYP450;* WN51_04136), *phenoloxidase* (*PO;*
118 WN51_02761) and *apolipophorin* (*ApoLp*; WN51_14077). Primers based on each respective gene
119 sequence were designed with Primer3 from Geneious R11 [20] (electronic supplementary material,
120 table S1). Actin (*act*) and 40S ribosomal protein S5 (*rps5*) were used as references for gene

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121 expression normalization [21–23]. StepOnePlus Real-Time PCR System (Applied Biosystems, USA)
122 was used for the RT-qPCR assays, and primer amplification efficiency was calculated with qBASE+
123 software [24] from the slope of a five-point 1:10 serial dilution of calibrator cDNA samples.
124 Experimental setup of qPCR involved sample maximization method, three technical replicates for each
125 sample, and inter-run calibrator samples [24]. The default of qBASE+ pipeline was used to calculate
126 relative gene expression, and presented in the form of calibrated normalized relative quantities
127 (CNRQ) [24]. Detailed information on sample processing and RT-qPCR quantifications is provided as
128 electronic supplementary data (text S1).
129
130 **(c) Statistical analyses**
131 All analyses were conducted in R version 3.6.3 [25] and available at
132 https://github.com/liliancaesar/Publication_scripts/tree/main/2020_Longitudinal_survey/Statistical_anal
133 yses. Data were tested for their fit to normality and variance homogeneity using Shapiro's and
134 Bartlett's tests ($P < 0.05$), respectively. When applicable, package MASS [26] was used for Box-Cox
135 transformation of non-normal and non-homogeneous data. Temporal differences in forager weight
136 were assessed by one-way ANOVA using "month" as factor. Monthly differences of daily variations in
137 colony temperature and humidity (Δt and Δh) were tested with Kruskal-Wallis. To test whether
138 unhealthy colonies were on average cooler and/or more humid, the March daily lowest temperatures
139 and highest humidities were analysed by Mann-Whitney. The package Laercio [27] was used for mean
140 comparisons with Tukey's test, and PMCMRplus [28] for Nemenyi's test. Forager CNRQ differences
141 for *CYP450, PO and ApoLp* were evaluated by one-way ANOVA using "month" and "colony" as factors
142 separately. Taking into account that the syndrome occurs in March, and that colonies have been
143 monitored from January until April, a two-way ANOVA using either "colony", "MD colonies", "health
144 status" or "intensity" combined with "month" as factors was also performed, enabling us to identify
145 gene expression effects in specific periods during the course of our survey. Pearson's correlation
146 coefficients among all traits were calculated with package Hmisc [29].
147
148 **Results**
149
150 **(a) Differentially expressed genes in diseased colonies**

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151 Transcriptome sequencing of D1, D2 and H foragers yielded 99,766,936, 102,034,731 and
152 135,982,124 single-end high quality-trimmed reads, respectively. From all reads, 87-99% mapped
153 against the *M. quadrifasciata* genome. A total of 558 DEGs were found comparing bees from healthy
154 and both diseased colonies, with 493 downregulated in foragers from diseased colonies (figure 1B;
155 electronic supplementary material, table S2). Membrane components (GO:0016020 and GO:0016020)
156 are significantly enriched in DEGs (figure 1C; electronic supplementary material, table S3), from which
157 the vast majority is downregulated, representing major deficits in bees affected by the syndrome. To
158 our knowledge, most of the highly differentially expressed genes were never directly implicated in bee
159 health. Therefore, three DEGs homologous to genes commonly found differentially expressed in
160 unhealthy bees from other species were chosen to quantify temporal variations in gene expression in
161 foragers under semi-controlled conditions using RT-qPCR, *i.e.*, *CYP450*, *PO* and *ApoLp* (electronic
162 supplementary material, table S4). *CYP450* and *ApoLp* are both downregulated in our transcriptomes
163 from diseased colonies, but curiously *PO* showed inconsistent patterns of differential expression in D1
164 and to D2 relative to H.
165
166 **(b) Outcomes of the survey**
167 Among the six colonies monitored monthly in our study, four manifested signs of the syndrome
168 in March 2019. Colonies BP2 and BP3 manifested the strongest signs of disease, with high mortality
169 of workers, and some bees showing tremors or paralysis. Their respective daughter colonies PA2 and
170 PA3 showed less intense signs and were characterized as mildly diseased. We rule out the possibility
171 that bees died due to lethal doses of agrochemicals, since residue analyses did not indicate
172 contamination by agrochemical compounds in bees from diseased colonies (data not shown). MD
173 colony pair BP1 and PA1 did not manifest health alterations.
174
175 **(c) Temporal variation in gene expression**
176 There was a significant temporal variation in the transcription of *CYP450* ($P = 0.009$), with
177 peaks in March in both foragers from healthy and diseased colonies, and in January only in healthy
178 ones (figure 1D). Monthly variations in *CYP450, PO* and *ApoLp* gene expression showed similar
179 interactions with the colony health status ($P = 0.0103$, 0.0152 and < 0.0005, respectively), and
180 foragers from colonies that remained healthy during the outbreak period showed the highest

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181 expression of these three genes in January (figure 1D).

182

183 **(d) Changes in worker bee nutrition and colony microclimate**

184 We found a marked reduction in forager weight from January until March ($P < 0.0005$; figure
185 2A), with the lowest average weight reached during the syndrome outbreak (March). From January to
186 February we observed a sudden change in the pollen stored by worker bees, *i.e.,* its composition
187 shifted from Myrtaceae (as *Eucalyptus* sp.) to Fabaceae (mainly *Mimosa bimucronata;* figure 2B). Bee
188 weight reduction was accompanied by a reduction in the internal temperature of colonies ($r = 0.63$, $P =$
189 0.0088), and increase in humidity ($r = -0.58$, $P = 0.0179$; electronic supplementary material, figure S1).
190 This pattern was more pronounced in colonies that became diseased during the outbreak, *i.e.*, MD
191 colonies 2 (BP2 and PA2; $P < 0.0005$). Furthermore, temperature was lowest ($P < 0.0005$) and
192 humidity was highest ($P < 0.0005$) inside diseased colonies when the syndrome symptoms were first
193 observed in March (figure 2C). The highest differences in daily temperature and humidity within
194 colonies occurred from December to March ($P < 0.0005$; figure 2D).

195

196 **Discussion**

197

198 Our study revealed that both genetic and environmental factors influence the annual *M.*
199 *quadrifasciata* syndrome. Mother-daughter colonies, exhibiting an average genetic relatedness of
200 0.375 [30], showed similar health status during the outbreak, despite having been kept in different
201 localities. We suspect that the routine practice of colony division, associated with the concomitant loss
202 of wild nests, may have reduced the genetic diversity of *M. quadrifasciata* populations. Reduced
203 genetic diversity may have affected stingless bees to properly respond to environmental stresses in
204 the form of sublethal effects such as pesticides, pathogens, and limited food resources, predisposing
205 them to disease. We found that symptoms of worker bees were stronger in two affected colonies
206 located nearby an agricultural setting. Thus, two lines of evidence allow us to identify some of the
207 mechanisms involved in a higher susceptibility to disease, and eventually colony failure, possibly
208 through the impairment of forager responses to interacting stressors.

209 Firstly, our exploratory transcriptomic analyses showed that components of the cellular
210 membrane, representing the layer that directly communicates with the environment, are enriched with

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211 DEGs that are downregulated in foragers from colonies affected by disease. Furthermore, enrichment
212 analysis also indicated a slight over representation of lipid metabolic processes and oxidoreductase
213 molecular functions, which were previously shown to respond to pesticides in honey bees [7].
214 Secondly, foragers from our surveyed colonies affected by the syndrome in March expressed
215 significantly less *CYP450, ApoLp and PO* in January. Bees rely on CYP450s for xenobiotic
216 detoxification, which determines their sensitivity to agrochemicals [31]. ApoLp is a major component of
217 the honey bee royal jelly [32] and is responsible for lipid transport, being mostly involved in innate
218 immunity [33]. PO is an enzyme responsible for activating melanogenesis, an important defense
219 mechanism of insects, and considered as an indicator of health condition strongly influenced by diet
220 [34]. Thus, it is not surprising that the expression patterns of these three genes (specially *ApoLp* and
221 *PO*) are so remarkably similar in our survey, suggesting the combined involvement of three
222 physiological processes in the *M. quadrifasciata* syndrome, *i.e.*, xenobiotic detoxification, immunity
223 and nutrition.

224 Agrochemicals are known to reduce ApoLp levels in honey bees [35], and increase their
225 demand for food [36]. Pollen stress in turn may lead to developmental and behavioural impairment [6].
226 Moreover, January is characterized by a high density of pollinators in general, including *A. mellifera*,
227 creating opportunities for getting in contact with a larger diversity of pathogens. In our study,
228 pronounced forager weight loss occurred between January and March, as well as a shift in the pollen
229 stored by worker bees from Myrtaceae to mostly *Mimosa bimucronata* between January and February.
230 Such a shift may result from the opportunity to forage *Mimosa,* that starts to bloom in February, and
231 from competitive exclusion, since *M. quadrifasciata* apparently competes with *A. mellifera* for
232 Myrtaceae flowers [37]. Interestingly, two months after *M. quadrifasciata* initiated storing *M.*
233 *bimucronata,* a gradual recovery of forager weight was noticed. Colony recovery after the outbreak
234 period is also suggested by a better performance of worker bees in controlling their nest
235 microenvironment, as observed by the reduced differences in colony daily temperature and humidity in
236 April and May.

237 Unfortunately, we cannot rule out that the differences we observed in forager gene expression
238 are due to age differences. *Melipona* spp. development takes around 40 days from egg to adult [38],
239 thus *M. quadrifasciata* foragers sampled during the outbreak in March were immatures in January.
240 However, age polyethism in bees is known to be a labile feature, since forager differentiation from

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241 nurses might be accelerated if the colony weakens [39]. Conducting invasive studies with stingless
242 bees is not straightforward. Their colonies are much smaller than honey bees, semi-wild, and present
243 a number of significant challenges for conducting experiments avoiding the side effects of excessive
244 manipulation. Although four of the six colonies surveyed in our study manifested some degree of the
245 syndrome symptoms, none of them collapsed during the outbreak period. We think that the annual
246 collapses reported for *M. quadrifasciata* colonies in Southern Brazil could result from positive density
247 dependence influenced by the combination of diverse sublethal factors [4,40]. The complexity of
248 causes behind worldwide colony collapses demand efforts to sustain pollination services. Based on
249 our findings, actions such as limiting the use of agrochemicals in the vicinity of managed colonies and
250 providing abundant natural polyfloral resources through the conservation of native forests could help
251 prevent the annual *M. quadrifasciata* syndrome. Furthermore, it warrants a better understanding of the
252 impact of management practices on stingless bee genetic diversity and fitness.
253

254 **Acknowledgments**

255 We thank the beekeeper Evald Gossler for making his meliponary available for the survey,
256 and for collaborating throughout the whole survey. We also thank the beekeeper Gilson Maas for
257 providing bee samples for our study.
258

259 **Funding**

260 Funding was provided by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
261 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (FAPERGS/CNPq 12/2014 –
262 PRONEX, protocol #19694.341.13831.26012015, CNPq/MCTIC/IBAMA/ Associação ABELHA
263 #400597/2018-7, and CNPq PQ #302121/2017-0). LC was supported by Coordenação de
264 Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Finance Code 001).
265
266

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373 **Figure legends:**

374

375 **Figure 1:** (A) *Melipona quadrifasciata* worker bee (Fototeca Cristiano Menezes,
376 http://www.splink.org.br/search?lang=en&collectioncode=FCM). (B) Venn diagram showing
377 differentially expressed genes (DEGs) up or downregulated in foragers from diseased colonies (D1 or
378 D2). (C) Functional overview of DEGs based on gene ontology annotation ($P < 1$), with the asterisk
379 indicating GO terms enriched in the DEGs ($P < 0.05$). Bars are sorted according to biological
380 processes (BP), molecular function (MF) and cellular component (CC). (D) Barplot comparing relative
381 expression (CNRQ) of *CYP450*, *PO* and *ApoLp* in foragers from colonies that remained healthy (H),
382 with those that showed signs of disease (D) during the outbreak period**.** Bars indicate the standard
383 error; uppercase letters indicate significant differences among months, and lowercase letters indicate
384 significant differences between healthy and unhealthy colonies within months, according to Tukey's
385 test ($P < 0.05$).

386

387 **Figure 2:** (A) Boxplot showing the monthly variation in average forager weight. (B) Barplot showing
388 the monthly variation in stored pollen, revealing a switch between January and February. (C) Line plot
389 of the minimum daily temperature and maximum daily humidity in March, in which the colors identify
390 colonies that remained healthy (shades of green) or became diseased (shades of purple) during the
391 outbreak period (horizontal bars). (D) Barplot showing the average daily difference in maximum *vs.*
392 minimum colony temperature (Δt) and humidity (Δh). Vertical bars indicate the standard error;
393 lowercase letters represent significant differences according to (A) Tukey's test or (D) Nemenyi's test
394 ($P < 0.05$).

Figure 1

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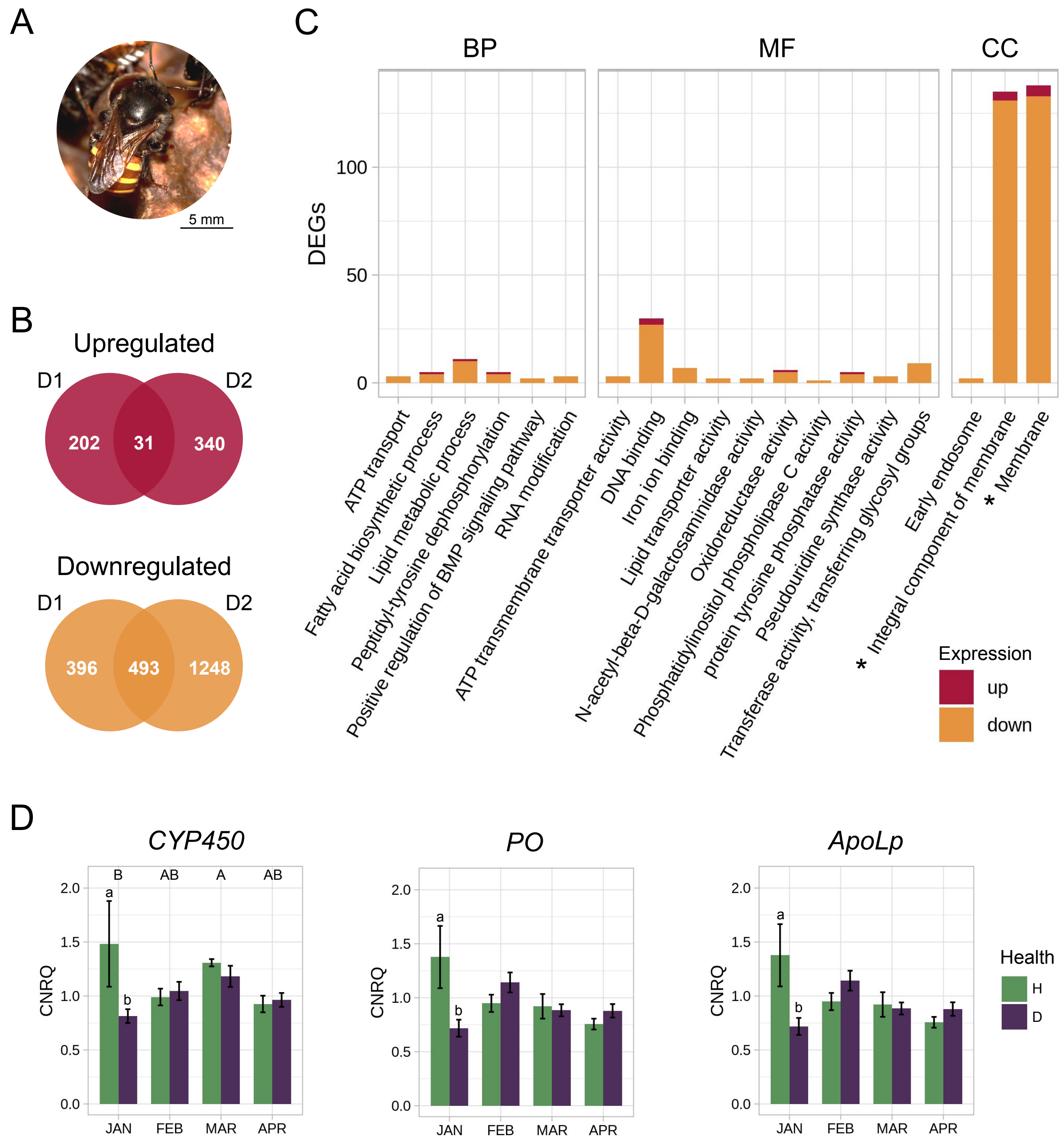

Figure 2

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