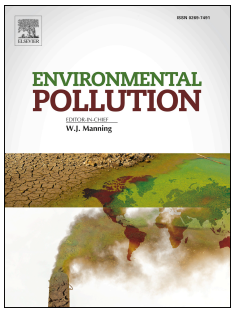

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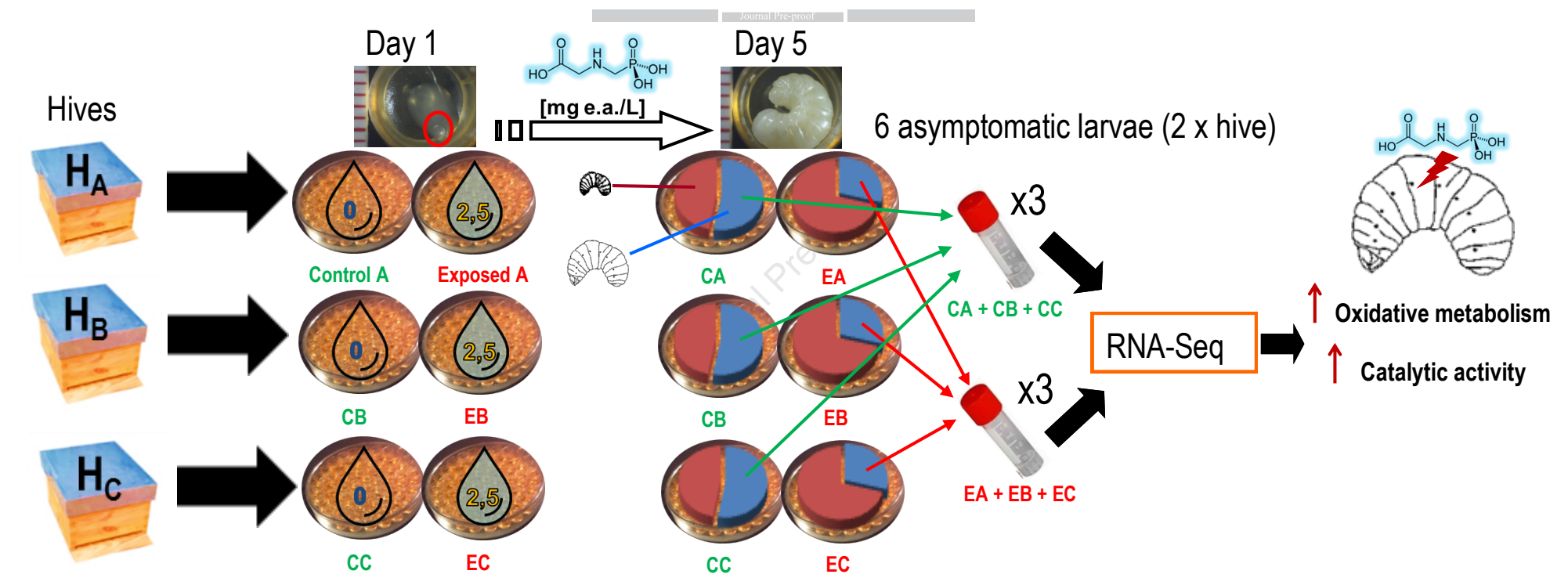

Chronic exposure to glyphosate induces transcriptional changes in honey bee larva: a toxicogenomic study

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Abstract

The honey bee *Apis mellifera* is the most abundant managed pollinator in diverse crops worldwide. Consequently, it is exposed to a plethora of environmental stressors, among which are the agrochemicals. In agroecosystems, the herbicide glyphosate (GLY) is one of the most applied. In laboratory assessments, GLY affects the honey bee larval development by delaying its moulting, among other negative effects. However, it is still unknown how GLY affects larval physiology when there are no observable signs of toxicity. We carried out a longitudinal experimental design using the *in vitro* rearing procedure. Larvae were fed with food containing or not a sub-lethal dose of GLY in chronic exposure (120 h). Individuals without observable signs of toxicity were sampled and their gene expression profile was analyzed with a transcriptomic approach to compare between treatments. Even though 29% of larvae were asymptomatic in the exposed group, they showed transcriptional changes in several genes after the GLY chronic intake. A total of 19 transcripts were found to be differentially expressed in the RNA-Seq experiment, mainly linked with defensive response and intermediary metabolism processes. Furthermore, the enriched functional categories in the transcriptome of the exposed asymptomatic larvae were linked with enzymes with catalytic and redox activity. Our results suggest an enhanced catabolism and oxidative metabolism in honey bee larvae as a consequence of the sub-lethal exposure to GLY, even in the absence of observable symptoms.

Keywords: *Apis mellifera*, RNA-Seq, *in vitro* rearing, transcriptomic, energy metabolism

1. Introduction

Animal pollination is a crucial ecosystem service for biosphere provided mainly by different species of bees and other insects. Nevertheless, in most crops worldwide, the honey bee *Apis mellifera* is the most used pollen vector (IPBES 2016). This generalist pollinator is managed by pollination services placing beehives in agricultural settings. Consequently, honey bees are exposed to different environmental stressors such as landscape fragmentation, large-scale monocultures, extreme climate conditions, pathogens, parasites and especially exposure to agrochemicals (Foley et al. 2005; Potts et al. 2010). In croplands and surroundings, several routes of acute or chronic exposure to agrochemicals occur, including the contact with spray drift, residues in vegetation and dust (IPBES 2016, Krupke *et al.* 2012, Peruzzo *et al.* 2008). Once the food incomes into the beehive, there is a rapid distribution of contaminated pollen, nectar and water among nestmates (Thompson *et al.* 2014, Orantes-Bermejo *et al.* 2010, Blasco *et al.* 2003). All these concomitant factors in sublethal exposure and in addition to their cumulative biological response make the honey bee exposome (Traynor *et al.* 2016, Miller and Jones 2013). This concept places the toxic exogenous agents in a broader context in which they interact with the inner physiology of each animal, including its diet, behaviour and endogenous agents (e.g., metabolites and microbiota). A maladaptive biological response of some individuals could affect the colony survival. In this context, the honey bee becomes a suitable sentinel species for pollinator community (Gerhardt 2002, Pham-Delegue *et al.* 2002, Bromenshenk *et al.* 1985), especially those individuals under development within beehives, given that they are much more vulnerable to environmental challenges (Wu *et al.* 2011). The interaction between environment and individual's physiology in chronic adaptation can lead to a high allostatic load and subclinical diseases (Juster *et al.* 2010). Therefore, the disturbed development of honey bees could cause a long-term negative effect on the pollination service in commercial crops.

One of the most applied agrochemicals in agriculture landscapes worldwide is the active ingredient so-called glyphosate [N-(phosphonomethyl)glycine], henceforth: GLY (Benbrook 2016, Duke and Powles 2008, Giesy *et al.* 2000). This biocide chemical takes part in a wide range of herbicide formulations with broad-spectrum action. GLY became

extensively and intensively applied since the late 1990s due to the technological advances in genetically modified crops and no-till farming (Benbrook 2016, Duke and Powles 2008). This molecule inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), part of the shikimate pathway, which exists only in higher plants, algae and bacteria (Duke and Powles 2008). The disruption of the aromatic amino acid biosynthesis is the main mechanism for toxicity of GLY. However, different studies have shown detrimental effects of GLY on the development and growth in a wide variety of animals (Seide *et al.* 2018, Dutra *et al.* 2011, Paganelli *et al.* 2010, Cauble and Wagner 2005, Marc *et al.* 2004, Tate *et al.* 1997). Indeed, a previous work reported an increased prevalence of delayed moulting in honey bee larvae exposed to GLY (Vázquez *et al.* 2018). This effect was observed mainly as reduced growth and prolonged duration of early larval stadia. In addition, other studies showed that GLY acts as a stressor during the larval development of *A. mellifera* under *in vitro* rearing by modulating some immune/detoxifying genes (e.g., antibacterial proteins and some cytochrome P450 monooxygenases) and inducing high levels of cell apoptosis in the gut epithelium (Gregorc *et al.* 2012, Gregorc and Ellis 2011). Although there is no a clear molecular mechanism of action, recent researches proved the disruption of gut microbiota in honey bees after GLY ingestion (Blot *et al.* 2019, Dai *et al.* 2018, Motta *et al.* 2018). This result is consistent with the biocide action of GLY in bacteria, but its direct or indirect connection with delayed growth or the occurrence of other harmful effects in bees is still unknown (Farina *et al.* 2019). After chronic exposure to GLY, young worker honey bees reared in the laboratory showed impaired associative learning, reduced sucrose sensitivity and depleted antioxidants associated with carotenoid–retinoid system (Goñalons and Farina 2018, Helmer *et al.* 2015, Herbert *et al.* 2014). The accumulated evidence suggests that chronic exposure to GLY is not harmless for bees; however, little is known about its consequences on brood physiology after the intake of contaminated food (El Agrebi *et al.* 2019, Berg *et al.* 2018, Chamkasem and Vargo 2017, Rubio *et al.* 2014). Thus, a toxicogenomic approach could provide a better understanding of the internal state of larvae exposed to this herbicide, even in the absence of external symptoms.

The aim of the present work has been to measure how GLY affects honey bee larva when there are no observable signs of toxicity. For this purpose, we carried out a

longitudinal randomized experiment in brood fed with food containing or not GLY, and assessed a transcriptomic analysis to compare gene expression between groups.

2. Materials and Methods

2.1. Study site and animals

The rearing experiment was performed in January 2018 (summer season in the southern hemisphere). Female honey bee larvae were sampled from brood frames from three healthy colonies (henceforth: A, B and C) and reared *in vitro* (see section 2.2). The colonies were housed in Langstroth hives at the experimental apiary of the Universidad de Buenos Aires, Argentina (34° 32' S, 58° 26' W). The queens from the three colonies are not genetically related and they had been naturally inseminated by multiple mates during free flights in the field (i.e., inter and intra-colony genetic diversity).

2.2. *In vitro* rearing

An empty frame was introduced into each colony (A-C) and monitored for 8 hours until the queen laid enough eggs. Three days later the brood frames were withdraw and carried to a room with suitable environmental conditions for grafting. Around 60 first stadium larvae were grafted (0-8 hour old post-hatching) from the brood frame to plastic cups and placed in Petri dishes (Crailsheim *et al.* 2013). This number of larvae represents around 5 % of a cohort (eggs laid in one day by the queen) and up to 0.5 % of colony in an average hive. The same person carried out this procedure to avoid variability in grafting effect. Larvae were reared inside an incubator with constant temperature and relative humidity (34.5 °C and 95%, respectively) during five days. To standardize larval food administration and GLY exposure, 110 µL of food spread in five aliquots of increasing volume were provided to each larva during the 5 days of the feeding period: 10 µL during grafting, 10 µL at 24 h, 20 µL at 48 h, 30 µL at 72 h and 40 µL at 96 h (Aupinel *et al.* 2005). A previously established diet was used: 6% D-glucose, 6% D-fructose, 1% yeast extract (Sigma-Aldrich) and 50% commercial royal jelly (Kaftanoglu *et al.* 2011, Vandenberg and Shimanuki 1987). In order to prevent bacterial or fungal contamination dead larvae were removed every day.

135

136 **2.3. Exposure to GLY**

137 The accurate concentration of GLY in brood food (bee bread or royal/worker jelly)
138 offered to larvae inside the hive during field exposure have not yet been determined, but the
139 herbicide is actually ingested by larvae (Farina *et al.* 2019, Thompson *et al.* 2014, USEPA
140 2012, Raina-Fulton 2014). Therefore, the worst-case exposure scenario was assumed, and a
141 chronic exposure (0-120 h post-hatching) was chosen, considering the highest
142 concentration of GLY reported in agricultural landscapes and its median expected
143 environmental concentration (Farina *et al.* 2019). Two treatments for larva reared *in vitro*
144 were defined: control group (food without herbicide) and exposed group, food with 2.5 mg
145 a.e. (acid equivalent) of GLY (analytical standard provided by Sigma-Aldrich, purity of
146 99.2 %) per litre of food. For this, a GLY stock solution of 100 mg a.e. L^{-1} (bi-distilled
147 water as solvent) was diluted in food solution. Food was renewed once a week due to the
148 slight photodegradation of GLY (Duke and Powles 2008).

149

150 **2.4. Endpoints**

151 The status of each larva during the 120 h of exposure was daily checked. Each
152 larval stadium can be identified by its morphological traits (Human *et al.* 2013). Whenever
153 a larva had a smaller size or different characteristics from the stadium in that is expected to
154 be, it was classified as "in delay" and separated from the remainders with an optimal
155 growth (Vázquez *et al.* 2018; Wu *et al.* 2011). Besides, larvae were classified as dead when
156 their colour changed to brownish, they developed oedema or remained immobile
157 (Crailsheim *et al.* 2012). The "relative risk ratio" and its confidence interval (Katz *et al.*
158 1978) were calculated when significant differences in either survival or successful moulting
159 proportions were observed.

160

161 **2.5. Biological samples and RNA isolation**

162 After the chronic *in vitro* exposure, larvae with optimal growth (i.e., with a success
163 moult in each moulting event and similar size) represented the asymptomatic
164 subpopulation, regardless if they belonged to GLY exposed or control groups. The

biological replicates for the gene expression experiments were pools of 6 asymptomatic larvae (2 larvae from each colony A-C) per treatment. Larvae were sampled in TRIzol® reagent (Sigma-Aldrich) and temporally stored in liquid nitrogen. We sampled only 5-day old larvae with complete intake to homogenize nutritional state. Each larva was weighed with a precision balance (Mettler Toledo AG285, ±0.1 mg). Therefore, the 110 µL of ingested food was equivalent to a dose of 0 in the control group or 275 ng a.e. of GLY in exposed group. Pooled larvae were homogenized using sterilized pestles in cold and total RNA was extracted using TRIzol® according to the supplier's protocol and resuspended in 90 µL of DEPC-treated water. RNA integrity and quality were assessed by means of a 1% agarose electrophoresis gel and in Agilent 2100 Bioanalyzer (Agilent Technologies).

2.6. RNA sequencing

The gene expression profiling of pooled larvae (section 2.5) was carried out with 3 biological replicates per treatment (control vs. GLY exposed). Library construction and high-throughput sequencing services were hired at Novogene Corporation Inc. (Sacramento, USA). A total of 6 cDNA libraries were constructed using the NEB Next® Ultra™ RNA Library Prep Kit (New England Biolabs) with an insert length of 250-300 base pairs (bp). The libraries were sequenced using Illumina HiSeq2000 equipment (paired-end 150 bp) with a sequencing depth of at least 21 million paired-end reads per library (Conesa *et al.* 2016, Rajkumar *et al.* 2015, Fang and Cui 2011). The raw sequence dataset is available at the NCBI BioProject database with the accession number PRJNA587756 (Table S1 and S2).

2.7. Bioinformatic analysis

Differential expression analysis was performed with an empirical Bayes approach for small samples based on a negative binomial distribution using the edgeR package (Robinson *et al.* 2010) (for details in the bioinformatic procedure and cites see Supplementary information). Those genes with a false discovery rate (FDR) < 0.1 were considered as differentially expressed between control and exposed groups and genes with logarithm2-fold-change more than ± 0.5 were considered with relevant biological signal.

Enrichment analysis of all genes of the transcriptomic result was carried out with the ermineR package using the Gene Score Resampling method (Gillis *et al.* 2010). The functional categories were determined using Gene Ontology (GO) terms from BeeBase (Gene Ontology Consortium 2018, Elsik *et al.* 2016, Honey Bee Genome Sequencing Consortium 2014).

2.8. qRT-PCR

The differential expression results from RNA-Seq were complemented with qRT-PCR (Everaert *et al.* 2017). For this purpose, we selected 5 genes from the set of differentially expressed genes (DEGs). Their expression level was analysed using 4 biological replicates per treatment (control and exposed group) independent from those used for RNA-Seq and prepared as described above (section 2.5). A total of 1.5 µg of total RNA were treated with DNaseI (Promega). cDNA was synthesized with 15.5 µL of treated RNA per sample by means of the M-MLV reverse transcriptase system (Promega). qRT-PCRs were performed in an AriaMx Real-Time PCR System (Agilent Technologies) using 6 µL of FastStart Universal Master Mix (Hoffmann-La Roche), 0.5 µL of a 10 µM forward and reverse primer solution and 1.5 µL of 4-fold diluted cDNA in final volume of 12 µL. Primers were designed using Primer3 v4.0.0 (http://primer3.wi.mit.edu) and efficiency was calculated for each primer pair (Table S2). Reactions were performed in technical triplicate under the following conditions: 10 min at 95 °C; 40 cycles of 20 s at 95 °C; 20 s at 56-58 °C and 30 s at 72 °C. In all qRT-PCR experiments, no-template controls were included. The efficiency and amplification of a single fragment was evaluated for each primer pair (Table S3) (Taylor *et al.* 2010). The expression of *GAPDH, Rp18S* and *Rp49S* was used to normalize target gene expression (Table S4 and Fig S1A). These genes were previously described as stable reference genes in *A. mellifera* (Lourenço *et al.* 2008, Scharlaken *et al.* 2008). Gene expression ratio was calculated by means of the Pfaffl formula (Pfaffl 2001).

2.9. Statistics

We performed data analysis and graphics in R (for details and cites see Supplementary Information). Survival and developmental data were analyzed with Cox

Proportional Hazard models (CPH). Weighing data were transformed with Box-Cox method to meet the statistical assumptions and analyzed with generalized linear models (GLM). Gene expression data from qRT-PCR were analyzed with principal components (PCA) and Mann-Whitney *U* test for comparison between treatments for each gene. The alpha level was set at 0.1 and p-value corrected for multiple post-hoc comparisons with Benjamini-Hochberg procedure.

3. Results

3.1. Signs of toxicity in larvae fed with GLY

In order to detect changes in the physiology of honey bee larvae without observable signs of disturbed development after ingesting GLY (Table S5), we tracked brood during the exposure period (0-120 h) searching for the occurrence of the endpoints (death or delay, Figure 1).

Thirty percent of the brood in the control group died at the age of 84 ± 27 h, around the last larval moult. Those larvae exposed to GLY dead at a similar age (80.9 ± 24 h) and did not differ with the control group in survival proportion (CPH model: survival ~ [GLY] + strata(colony), $\chi^2(1) = 1.74$, P = 0.187, N = 364). However, a significant increase in the proportion of larvae with delayed development during the exposure to GLY was observed (CPH model: successful moulting ~ [GLY] + strata(colony). $\chi^2(1) = 10.57$, P = 0.001, N = 364). Forty-four percent of the brood in the control group displayed a delay in at least one moult with delay of 55.0 ± 26 h. Nevertheless, sixty-two percent of larvae in the group exposed to GLY displayed a moulting process with delay of 60.7 ± 33 h. Hence, the relative risk ratio associated with the delayed development and the exposure to GLY was 1.43 (confidence interval of 95%: 1.20-1.69). Therefore, GLY affected the larval development of a subpopulation of brood in the longitudinal experiment, increasing the incidence of delayed moulting.

At the end of the experiment (120 h), 53% of brood in the control group and 29% in the exposed group did not show disruptions in the larval development (delay, death or both). Thereupon, we sampled from these asymptomatic subpopulations those larvae with full intake and similar size (89. 89 ± 8.8 mg in control group and 89.95 ± 12.44 mg in

exposed group. GLM model: Box-Cox(weight) ~ [GLY]. F(1,94) = 0.64, P = 0.426, N = 96).

3.2. Gene expression profiling of asymptomatic larvae

The transcriptomes derived from the samples of asymptomatic larvae with or without the intake of GLY allowed us to explore differences in gene expression using RNA-Seq. Less than 10% of the raw read pairs were eliminated after quality control. Between 20 and 24 million of clean reads were obtained per library (biological replicate). More than 88% of reads mapped to the honey bee genome (Table S1 and S2).

Nineteen coding transcripts (0.22% of protein-coding genes expressed in samples) were found to be differentially expressed (7 up and 12 down-regulated in exposed larvae relative to control group) (Figure 2 and Table S6). The magnitude of the fold change ranged from 0.6 to 0.1 for the genes under-expressed and from 1.7 to 2.7 for the genes over-expressed in the exposed group. The DEGs were characterized by information about their function and dominant expression in tissues provided by different genome databases (Table S7). Moreover, all genes in the transcriptomes were classified according to functional categories based on the Gene Ontology classification (Table 1). The most enriched GO terms of biological processes and molecular functions in the exposed larvae were lipid metabolism (with a high percentage of genes with low FDR) and oxidoreductase activity (with a high percentage of genes with large fold-change).

Finally, we choose 5 transcripts from the DEG set (*CYP6AS3*, *GB46620*, *SLC1*, *EST* and *UGT1-3*) to quantify their expression levels by qRT-PCR in independent biological replicates from the same cohort under study (Figure 3). *UGT1-3* showed a significant up-regulation between treatments while *SLC1* and *EST* showed a meaningful biological modulation on their average expression ratio, 50% of down-regulation and 83% of up-regulation respectively (Mann Whitney *U* test: *CYP6AS3*: W = 4, *P* = 0.248. GB46620: W = 8, *P* = 1. *SLC1*: W = 14, *P* = 0.08. *EST*: W = 2, *P* = 0.08. *UGT1-3*: W = 1, *P* = 0.04). Furthermore, two principal components (PC1 and PC2) achieving 68% of the cumulative proportion of deviation were obtained from a PCA with the 5 genes (Table S8). Although there was variation in the gene expression ratio among samples in qRT-PCR (Table S9), the

PCA showed more similarity among samples from the same treatment in the internal state based on those genes (Figure S1B). Lastly, the effect of GLY exposure in the transcript´s abundance of *UGT1-3* had similar response in direction (over-expression) in both qRT-PCR and transcriptomic procedures, with high read counts and moderate variability in the latter (Table S6).

4. Discussion

4.1. Transcriptional changes in asymptomatic larvae

In agreement with previous results (Vázquez et al 2018), the chronic self-dosing of GLY in the honey bee brood has sub-lethal effects in the larval development under *in vitro* rearing conditions. GLY acts as a risk factor increasing the incidence of delayed moults. Moreover, the gene expression profiling in the asymptomatic subpopulation suggests alterations in their physiology after the chronic intake of GLY. A set of 19 coding transcripts was found to be differentially expressed in their whole body. This modulation was mainly restricted to genes related to the defensive response against environmental stressors (37%) and the intermediary metabolism (26%) (Table S7). Furthermore, the most enriched functional categories in the whole transcriptome (Table 1) were those associated to enzymes with catalytic and redox activities. Most of the DEGs have been reported in *Drosophila melanogaster* with predominant transcription in the gut epithelium, integument and Malpighian tubules (Table S7) (Thurmond *et al.* 2019). These organs are directly exposed to the herbicide which would indicate an inner adjustment of the larval physiology as a consequence of the oral and epidermal exposures.

A complete correlation between RNA-Seq and RT-qPCR results should be expectable for those experiments dealing with an acute treatment with a relative simple mode of action and a great modulatory effect on gene expression. In the current study, we identified transcriptional changes triggered by chronic exposure to a chemical whose action mechanism is unknown in insects and seems to act at different levels, e.g. in gut microbiota (Blot *et al.* 2019, Dai *et al.* 2018, Motta *et al.* 2018). Besides, Vázquez and co-workers (2018) observed that the impact of GLY among colonies was not homogeneous. Even individuals of the same colony could present different response profiles to GLY due to the

different self-dosing of each larva and the variation in susceptibility of its microbiota (Motta *et al.* 2018, Vázquez *et al.* 2018). Hence, the results presented here are a snapshot of a dynamic process and several individuals within the same group could have been caught in different points along their toxicological response. We consider both RNA-Seq and RT-qPCR as complementary procedures to identify changes, that in our case we expect to be subtle. Therefore, the current detection of signs of transcriptional modulation constitutes a meaningful piece of information revealing a physiological reaction of the larva against the herbicide in an allostatic process (Juster *et al.* 2010), even in the absence of evident symptoms of toxicity.

4.2. Signs of detoxification and dysbiosis

Phytochemicals and pesticides present in food or nest were shown to modulate similar genes during the defensive response in honey bees (Poquet *et al.* 2016, Mao *et al.* 2013, James and Xu 2012, Johnson *et al.* 2012). In the current study, genes related to immunity (*pacifastin* and *MME*), plant-herbivore interaction (*G12-like protein*), epigenetic mechanisms of disrupted microbiota (GB46620) and detoxification were modulated by GLY. In the latter process, three genes belong to phase I (*CYP6AS3*) and phase II (a sulfotransferase and *UGT1-3*) in the biotransformation of xenobiotics (Timbrell 2008, Claudianos *et al.* 2006). Previous works showed that *CYP6AS3* detoxified xenobiotics present in honey and beebread, such as quercetin (Mao *et al.* 2013, Johnson *et al.* 2012). Meanwhile, the UDP-glycosyltransferase gene (*UGT1-3*) was related to the glycosylation (UDP-glucose as sugar donor) of small hydrophobic molecules (Ahn *et al.* 2012). Many endogenous compounds are glycosylated, such as ecdysteroid hormones and cuticle tanning precursors (Hu *et al.* 2019, Ahn *et al.* 2012). The xenobiotic metabolism and immunity have been consistently modulated by the intake of GLY in honey bee brood in different experiments (Vázquez *et al.* 2018, Gregorc *et al.* 2012). Nevertheless, it is currently unknown if these enzymes metabolize GLY or other chemical that could be generated secondarily, e.g. toxins from the dysbiosis in gut microbiota (Blot *et al.* 2019, Dai *et al.* 2018, Motta *et al.* 2018). Therefore, the long-term trend in gene modulation does not necessarily reflect a specific gene activation or repression capacity of GLY (Samsel and

Seneff 2013). Besides that, expression of an apoptotic signalling gene (*SMPD1*) was significantly modulated in our experiment. The induction of apoptosis suggested by this result is in agreement to a previous study (Gregorc and Ellis 2011). Furthermore, the most up-regulated transcript was a metalloprotein related to inflammatory response (*MME*). These are toxicity signs frequently associated with dysbiosis (Samsel and Seneff 2013).

4.3. Signs of metabolic stress

Triggering of stress compensatory mechanisms induces energy consumption (Wang *et al.* 2019, Li *et al.* 2017, Avigliano *et al.* 2014), which could disrupt the moulting process in honey bees due to a trade-off between growth and defensive response. The herbicide showed adverse effects in growth of various invertebrates including honey bees (Vázquez *et al.* 2018, Dutra *et al.* 2011, Marc *et al.* 2004, Tate *et al.* 1997). Although more physiological experiments should be performed, the functional analysis presented here provides evidence of alterations in the energetic metabolism. On one hand, the most down-regulated transcripts were a branched-chain-amino-acid transaminase (GB49819) and a fatty acid hydroxylase (GB40899) related to protein and lipid catalytic metabolism respectively. Lipids and amino acids contribute to energy metabolism by providing carbon source into the Krebs cycle, especially when the primary sources of energy (trehalose and glycogen) are scarce (Nation 2015) (GO:0006629 and GO:0008610). On the other hand, one of the most enriched functional categories was iron-binding proteins (GO:0005506). These are important metalloproteins that contribute in solute transport (*SLC1*) and defense response, such as *MME* and CYP450s (Dlouhy and Outten 2013, Claudianos *et al.* 2006, De Sousa *et al.* 1988). The other most enriched category was alcohol oxidoreductase enzymes (GO:0016614 and EC 1.1). These enzymes are mainly dehydrogenases and also take part in the energetic metabolism and phase I in xenobiotic metabolism (IUBMB 1992). Isocitrate dehydrogenase is the major control point in the Krebs cycle modulated by the concentration of ATP and other metabolites (Nation 2015). In addition, cells use the coenzyme FAD (Flavin Adenine Dinucleotide) associated with flavoproteins (GO:0050662 and GO:0050660) in many energetically difficult oxidation reactions such as dehydrogenation, because it is a very strong oxidizing agent. Flavoproteins take part in a

large variety of energetic metabolic pathways including beta-oxidation of fatty acids and amino acid catabolism (Nation 2015, Iida *et al.* 2007, Patterson and Bates 1989). All the mentioned enzymes and biological processes have a crucial role in the redox homeostasis. Disturbances in the normal redox state of cell can cause toxic effects through the production of reactive oxygen species. Oxidative stress is associated with increased production of oxidizing compounds during catabolism and severe oxidation can trigger apoptosis and energy depletion (Lelli *et al.* 1998). These toxic oxidants are removed by antioxidant metabolites and different enzymes. In this sense, in a previous laboratory assessment with adult honey bees exposed chronically to GLY, the authors reported a decrease in antioxidants (Helmer *et al.* 2015).

4.4. Implications in field assessments

The present results suggest that open or semi-field assessments need to consider measurements of internal state regarding conspicuous endpoints, such as death or delayed development (Thompson *et al.* 2014). The hazard analysis of stressors, one at a time, in laboratory gives the advantage to identify useful biomarkers of effect or exposure for biomonitoring (Gerhardt 2002). Although samples of whole body insects makes more difficult the detection of organ-specific changes, this kind of sampling allowed us to describe in a holistic way changes in the transcriptional state of brood and establish a reference due to the unfeasible dissections of larvae *in situ*. Finally, in open field assays it is important not to lose sight of the exposome of each honey bee colony that is a result of different kind of acute or chronic disturbances in brood or adults honey bees (Traynor *et al.* 2016, Miller and Jones 2013). These exposures could affect the biological fitness to cope with concomitant stressors, as previously demonstrated for GLY and mosquitoes (Riaz *et al.* 2009) even if there are not observable signs of toxicity.

Conclusion

Our results suggest an increase of the catabolism and oxidative metabolism in honey bee asymptomatic larvae chronically exposed to GLY. A maladaptive physiological response in early stages in life cycle could lead to long-term negative effects on bee populations.

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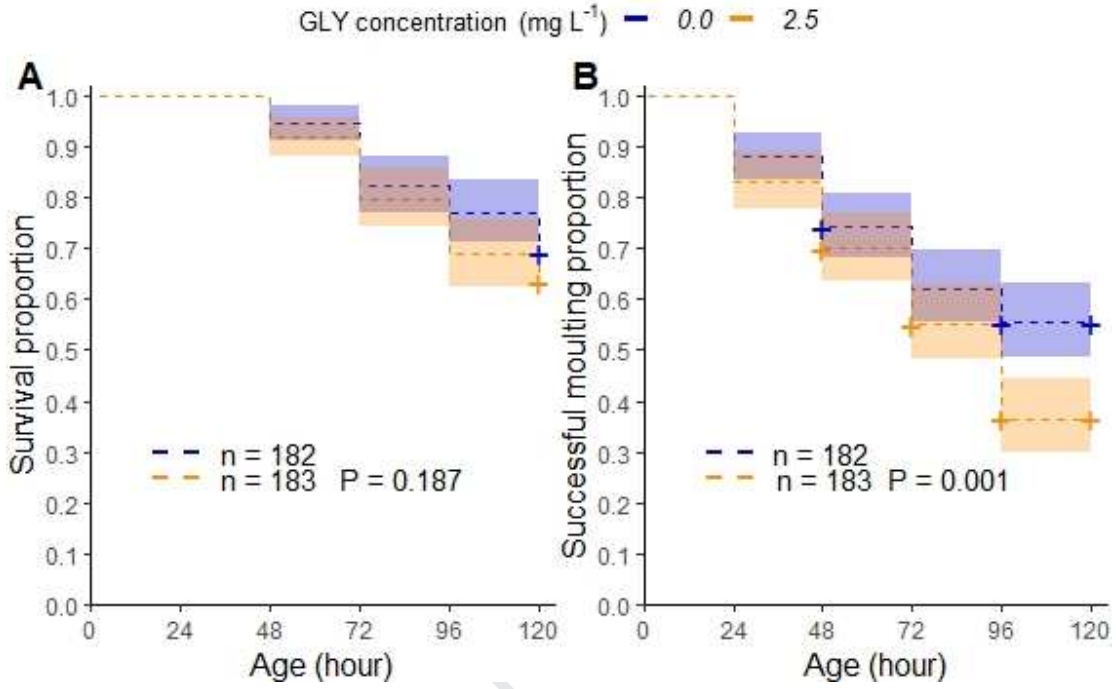
709 **Figures**

710 Figure 1.



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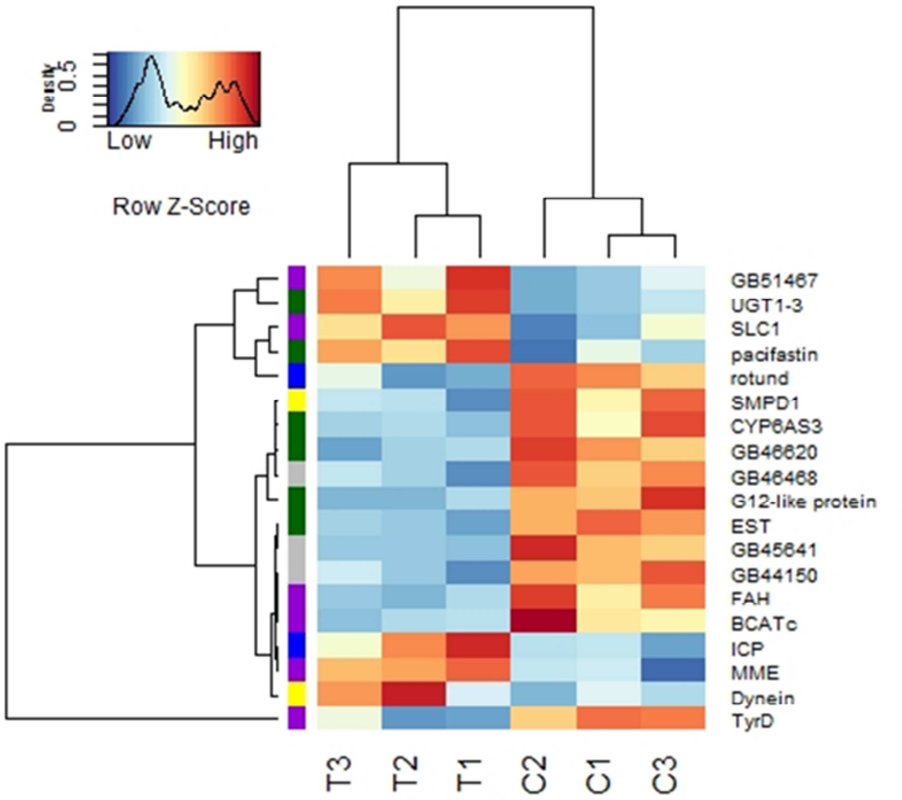
713 Figure 2.



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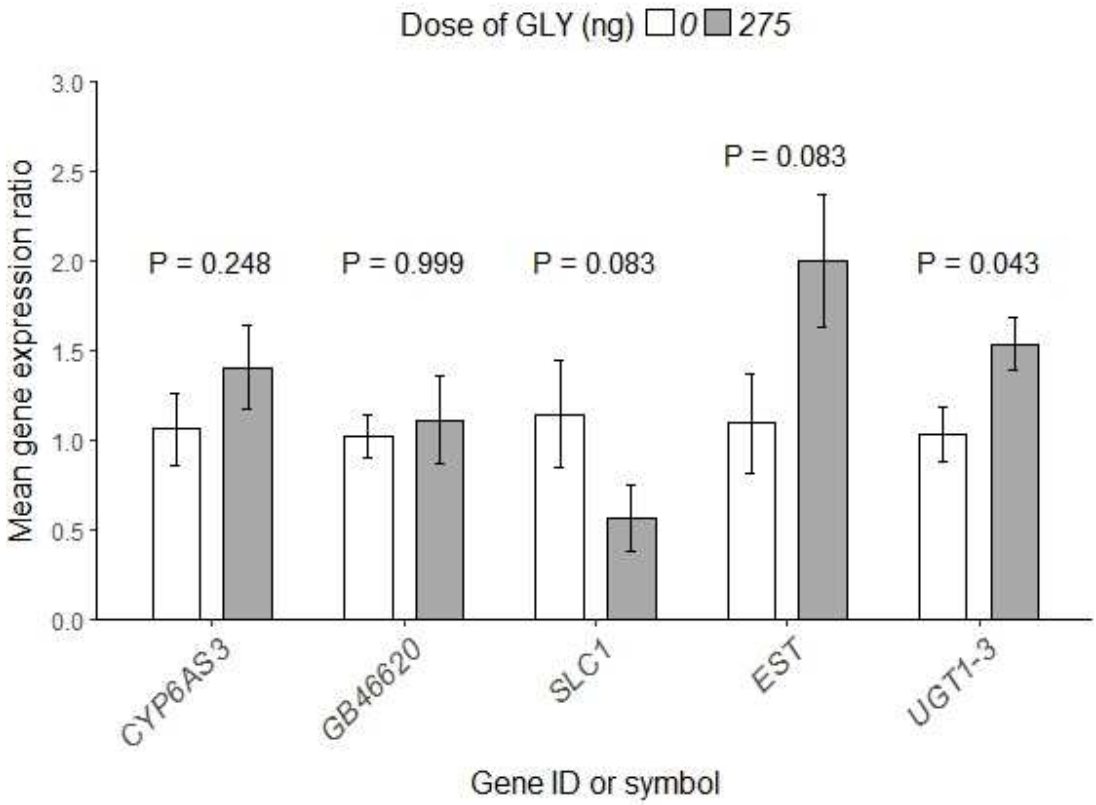
716 Figure 3.



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719 **Tables**

720 Table 1.

ID	Description	N° genes	P-value	FDR
GO:0016614 §	oxidoreductase activity, acting on CH-OH group of donors	46	1.00E-12	2,48E-10
GO:0005506 *	iron ion binding	66	2.03E-05	2,48E-10
GO:0050660 §	flavin adenine dinucleotide binding	45	1.00E-12	1,24E-10
GO:0050662 §	coenzyme binding	134	7.61E-07	1.22E-06
GO:0008610 *	lipid biosynthetic process	73	0.011	2.94E-06
GO:0006629 *	lipid metabolic process	152	0.022	0.015

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Figures Captions

Fig 1. Endpoint assessment in honey bee larvae exposed *in vitro* to GLY. A) The proportion of survival and **B)** the proportion of larvae with successful moulting (without delay events) during the chronic exposure (0-120 h post-hatching) to contaminated food with GLY (0 or 2.5 mg a.e. of herbicide per litre). The curves are plotted with their confidence interval (95%) and with different colours per treatment: control group in blue and exposed larvae in orange. The + indicates time points with censoring data. Both survival and developmental data were fitted to CPH models (survival or successful moulting ~ [GLY] + strata(colony)). The number of assessed larvae and p-values for each test are shown in the graph.

Figure 2. Effect of GLY on gene transcription in asymptomatic larvae. Heatplot of differentially expressed genes (FDR < 0.1, Table S6) comparing transcription levels among samples of pooled bees (6 asymptomatic larvae, i.e. without signs of toxicity after chronic exposure of 120 h to GLY) in both treatments (control: C or exposed: T, total dose of 0 or 275 a.e. ng of GLY respectively). Transcription levels per gene (fragments per kilobase per million, a.k.a. FPKM) were standardized with z-score and represented by means of a color scale, in which blue/red represent lowest/highest expression respect to average FPKM among all samples per gene. The density subplot allows to identify the trend in transcription level. Genes are identified by their symbol or Beebase code (GB-number). A bar color code identifies the functional category of genes: violet for intermediary metabolism, green for defensive response, yellow for cellular processes, blue for development and grey for genes without functional information (Table S7). Dendrograms were plotted with hierarchical clustering among samples and genes based on Euclidean distances and Ward method for clustering.

Figure 3. Gene expression measured with qRT-PCR. Mean gene expression ratio (Pfaffl formula) of 5 genes (differently expressed in RNA-Seq, Table S6) has been performed with 4 samples of pooled larvae (6) per treatment (control or exposed) using qRT-PCR. The samples were different from those in sequencing data. *GAPDH, Rp18S* and *Rp49S* expression levels (Table S3) has been used to normalize the expression level of every gene. Bars indicate means $\pm$ s.e.m. The *p*-values for each test to compare between treatments (Mann-Whitney *U* test) are shown in the graph for each gene.

Tables Captions

Table 1. GO enrichment analysis of the RNA-Seq data. Gene Set Enrichment Analysis was performed with the list of filtered genes (8567) from the RNA-Seq result (see Supplementary). Functional gene sets were defined using the Gene Ontology (GO) annotations (2554) of the *Apis mellifera* genome in BeeBase (93% of the filtered genes). Gene Score Resampling method (GSR) applied to identify significantly enriched functional categories with high-scoring genes (§ mean absolute fold-change or * mean $-\log_{10}$(FDR) from the RNASeq result). The reported IDs correspond to the significantly enriched GO terms (FDR with multifunctionality correction < 0.1).

Highlights

1. Honey bee larvae were chronically fed *in vitro* with food containing glyphosate.
2. At the end of the cohort study, larvae without signs of toxicity were sampled.
3. Exposed asymptomatic larvae showed differentially expressed genes in RNA-Seq.
4. Enriched functional categories suggested high catalytic and oxidative metabolism.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: