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RESEARCH ARTICLE

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Sublethal doses of glyphosate modulates mitochondria and oxidative stress in honeybees by direct feeding

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Abstract

Honeybees are essential for the ecosystem maintenance and for plant production in agriculture. Glyphosate is a broad-spectrum systemic herbicide widely used in crops to control weeds and could affect honeybees' health in sublethal doses. Our aim was to study how sublethal doses of glyphosate affects to oxidative stress and mitochondrial homeostasis in honeybees. We exposed honeybees to glyphosate at 5 and 10 $\text{mg}\cdot\text{l}^{-1}$ for 2 and 10 h for the gene expression analysis by reverse transcription polymerase chain reaction and for 48 and 72 h for the antioxidant enzymes activity and lipid peroxidation determination. We observed a general increase in antioxidant- and mitochondrial-related genes expression in honeybees after 2 h of exposition to glyphosate, as well as a rise in catalase and superoxide dismutase enzymatic activity after 48 h and an increment in lipid peroxidation adducts generation after 72 h. These results suggest a direct effect of glyphosate on honeybees' health, with an insufficient response of the antioxidant system to the generated oxidative stress, resulting in an increase in lipid peroxidation and, therefore, oxidative damage. Altogether, results obtained in this work demonstrate that sublethal treatments of glyphosate could directly affect honeybee individuals under laboratory conditions. Therefore, it is necessary to investigate alternatives to glyphosate to determine if they are less harmful to non-target organisms.

KEYWORDS

antioxidant enzymes, glyphosate, honeybees' health, oxidative stress, redox status

Research Highlights

- Glyphosate toxicity was evaluated on *Apis mellifera*, as non-target living organism.
- Glyphosate modulated mitochondria and antioxidant enzymes, rising oxidative damage, and producing lipid peroxidation.
- Sublethal doses of glyphosate affected honeybees' health in a short period of time by direct feeding.

1 | INTRODUCTION

Populations of honeybees have declined worldwide in recent years (Gray et al., 2020). In some countries, the overwintering losses of honeybees colonies have reached 30% (Gray et al., 2020), and this is a concern due to honeybees providing pollinator service to natural and agricultural ecosystems (Goulson, 2019). A variety of stressors have been implicated as potential causes, such as pathogens (Aufauvre et al., 2014), parasites (Zaobidna et al., 2015), predators (Leza et al., 2019), land use changes (Ricigliano et al., 2019), poor nutrition (Di Pasquale et al., 2013), and agricultural pesticides (Chakrabarti et al., 2020). The widespread indiscriminate use of pesticides in crop management is a major cause of reduced bee health (Crall et al., 2018; Johnson, 2015; Johnson et al., 2010; Tsvetkov et al., 2017).

Oxidative stress has been widely used to study the effect of stressors on honeybees. Among them, we find abiotic factors like the season of the year (Orčić et al., 2017), high and low temperatures (Li et al., 2019; Mucci et al., 2021), natural elements like selenium, mercury, or lead (Alburaki et al., 2019; Gizaw et al., 2020) or pesticides (Aufauvre et al., 2014; Carvalho et al., 2013; Chakrabarti et al., 2020; Qi et al., 2020). Likewise, biotic factors like diseases (Aufauvre et al., 2014; Dussaubat et al., 2012; Zaobidna et al., 2015), predators (Leza et al., 2019), factors related to honeybees' lifestyle (James and Xu, 2012; Korayem et al., 2012; Margotta et al., 2018) or supplementary winter feeding (Tawfik et al., 2020).

Foraging worker honeybees collect the pollen, nectar, water, and other products needed for the colony. During this activity, foragers encounter toxic materials (synthetic and natural origin) and can accidentally introduce them into the colony. The most hazardous pesticides to bees are the neonicotinoid, pyrethrin, organochloride, and organophosphate types (Pohorecka et al., 2017). Most studies have focused on lethal doses (Bailey et al., 2018; Dai et al., 2018, 2019; Helmer et al., 2015), nonetheless, sublethal doses are significant to the behavior, physiology, and health of individual bees (Desneux et al., 2007). However, it is more difficult to prove the effects of sublethal amounts on honeybees (Berry et al., 2013). Recently, it has been shown that the exposure to neonicotinoids is able to induce changes in brain gene expression in bumble bee queens, reflecting the importance of using molecular approaches to explore sublethal consequences of stressors (Costa et al., 2022).

Other toxic materials are herbicides that may have indirect (loss of flowers) and direct effects. Their acute toxicity to adult honeybees is generally low (Johnson, 2015). For example, paraquat has demonstrated effects on honeybees under laboratory studies and it is a model of oxidative stressor in mammals (Cousin et al., 2013). Glyphosate is the most used pesticide worldwide, which is used in the control of several wild weeds, as crop desiccant, spot treatment, field clearance or re-seeding (Duke, 2018). Glyphosate has the molecular formula $C_3H_8NO_5P$ and acts by inhibiting the growth of plants, interfering in the protein synthesis process (Kafarski et al., 1988). Moreover, glyphosate is able to induce oxidative stress in some algae (*Chlorella kessleri*) (Romero et al., 2011), plant (*Arabidopsis thaliana*) (de Freitas-Silva et al., 2017), and animal species such *Salmo trutta* (Webster and Santos, 2015) and *Rattus norvegicus* (El-Shenawy, 2009). However, the effect of glyphosate on molecular and biochemical parameters is still poorly understood, making further research necessary to assess the effects on gene expression, enzymatic action, and oxidative stress on honeybees (Battisti et al., 2021).

The main objective of this study was to establish a molecular approach to define the impact of the herbicide glyphosate on honeybees' oxidative stress. Following this objective, parameters such as the expression of antioxidant genes, mitochondrial-related genes, enzymatic activity of antioxidant enzymes, and lipid peroxidation were performed.

2 | MATERIAL AND METHODS

2.1 | Honeybees (*Apis mellifera*) and glyphosate treatments

Honeybees used in this study were obtained from three different colonies placed at the University of the Balearic Islands (Spain). Honeybee workers of each colony were transported to the laboratory where, once there, a sample of each colony was introduced in three different entomological boxes (25 × 25 × 25 cm). In this way, we obtained a pool of bees of at least 50 individuals coming from different colonies, avoiding pseudoreplication.

Entomological boxes were maintained in a temperature-controlled room (darkness, 25 ± 1°C with 55 ± 5% relative humidity). Honeybees were fed with a small plastic feeder ad libitum of unscented 684.6 $g \cdot l^{-1}$ (2 $mol \cdot l^{-1}$) sucrose solution, either with or without glyphosate. Control honeybees were fed the solution without herbicide. On the other hand, two different concentrations of glyphosate (N-(Phosphonomethyl) glycine; ≥98.0% purity; Sigma-Aldrich) were used: 5 and 10 $mg \cdot l^{-1}$. Both concentrations of glyphosate have proven to be sublethal for honeybees (Balbuena et al., 2015; Motta et al., 2018) and the honeybees began to be fed with both concentrations in less than 1 h after their preparation.

Six random honeybees were sampled from each entomological box after 2, 10, 48, and 72 h and directly introduced in liquid nitrogen and preserved at −80°C until being processed to evaluate different oxidative stress parameters. The different exposure times of bees to glyphosate correspond to immediate exposure to the pesticide (2 h) and another nonimmediate exposure, but still short-term (10 h), to evaluate its effects on gene expression. At 48 and 72 h, the aim was to observe the more medium-term effect of glyphosate on the physiology of worker bees.

2.2 | Reagents

Routine chemicals were supplied by Panreac, Sigma-Aldrich, Takara Bio Inc. and Bio-Rad Laboratories. Primers were purchased from Metabion.

2.3 | Reverse transcription polymerase chain reaction (RT-PCR)

To evaluate the effects of glyphosate on gene expression, whole honeybees were homogenized in 1 mL of Tri-Reagent® (Sigma-Aldrich) after glyphosate exposure at 2 or 10 h. After that, total RNA was isolated from using following the manufacturer's protocol and quantified with a BioSpec-nano spectrophotometer (Shimadzu Biotech) set at 260 nm. One microgram of total RNA was reverse-transcribed to cDNA at 42°C for 60 min with 25 U of MuLV reverse transcriptase in a 10 µL volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 mM random hexamers, 10U RNase inhibitor, and 500 mM of each dNTP. Each resulting cDNA was diluted 1/10.

PCR was done for the target genes shown in Table 1 using SYBR green technology (Takara Bio Inc.) on a Light-Cycler 480 rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 µL, containing 7.5 µL Lightcycler® 480 Master SYBR Green I (with 0.5 µM sense and antisense specific primers) and 2.5 µL of the cDNA template. The amplification program consisted of a preincubation step for

TABLE 1 Primers and conditions used for reverse transcription polymerase chain reaction.

Gene	Forward Primer (5′-3′)Reverse Primer (5′-3′)	T. An. (°C)	Accession number
RPL8	TGGATGTTCAACAGGGTTCATA CTGGTGGTGGACGTATTGATAA	60	XM_393671.7
Catalase	TAGCCTTCGATCCGGCTCAT TGTTTGGGCCAAGACGATGT	60	NM_001178069.1
Sod1	GTGTGCGTTCTTCAGGGTGA GGGTTGAAATGTGCACCAGC	61	NM_001178027.1
Sod2	ATCTTTCCCCAAATGGCGGT TATCCAAGCCAACCCCAACC	60	NM_001178048.2
Gtpx1	GCTCCTGGAGTGTAGTTCGT GCCGCATTTTGACGCTACAT	60	NM_001178021.1
Tpx-3	CACCGAGAAAACAGGGTGGA TGAAGAGGCCTCGTAAAGCA	60	NM_001178024.1
Tpx-4	TGCTGATCATGACCGCACTT ACGTAATCGATGATCGGGGC	60	NM_001170973.1
TrxR	GCAAGTACTGTTGCCCAGGA TGAGCGGTTTGCGACCAATA	60	NM_001178026.1
GstD1	TCCTTGGAACGAGGAGAGGT CGCAAATGGTCGTGTGGATG	60	NM_001178028.1
GstS1	ATTCCTGGTCTTGCCGAACC GCGATCAAGCGAGAAATAGCC	60	NM_001160270.1
CoxI	CATGAACAGTTCCATCCTTAGGT TACCACAAATTTCTGAACATTGACC	60	OM203348.1
Cox6b	TGTGTTCTTTCAGTCACCAACG TGGTGCTGTGTTTGGTTTCAA	60	NM_001172403.1
CytC	CGTCTGGTACTCGAGCGTTT TCGCGAGAGAGCACCAAAAT	60	NM_001177490.1
IF-2mt	GGGCATGTTGATCATGGCAAA TGAAAGCAGCATGTCCAGGT	60	NM_001011586.1

denaturation (10 min 95°C) followed by 40 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depends on each pair of specific primers), and an extension step (12 s, 72°C for all). Table 1 shows the primers and conditions used for RT-PCR. A negative control without cDNA template was run in each assay.

The C_t values of the real-time PCR were analyzed, considering the efficiency of the reaction and referring these results to the messenger RNA (mRNA) expression levels of RPL8, used as a housekeeping gene, using the GenEx Standard Software (MultiDAnalises).

2.4 | Measurement of lipid peroxidation

Each whole honeybee was homogenized in 1 mL of homogenization buffer (40 mM sodium phosphate pH 7.4, 10 mM NaCl) and the protein content was determined with the Bradford's method (Bradford, 1976) and samples were kept at −20°C until 4-hydroxy-2-nonenal levels determination. Bees exposed to glyphosate for 48 and 72 h were analyzed.

For 4-hydroxy-2-nonenal (4-HNE) analysis, 20 µg of protein from honeybee homogenates were separated in an SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S as a loading control, without differences between samples (data not shown), and then blocked with 5% nonfat powdered milk with TBS-Tween. Antiserum against 4-HNE (HNE11-S) was used as a primary antibody (Alpha Diagnostic International). Bands were visualized by Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad Laboratories). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and the density of bands was analyzed using Quantity One software (Bio-Rad Laboratories).

2.5 | Superoxide dismutase and catalase activities

Bees exposed to glyphosate for 48 h were analyzed to evaluate the effect of this pesticide on enzymatic activities. Each whole honeybee was homogenized in 1 mL of homogenization buffer and protein content was determined with the Bradford's method (Bradford, 1976). The enzymatic activities assays were run immediately.

Superoxide dismutase activity was determined by following the cytochrome c reduction by measuring the absorbance at 550 nm on a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) at 37°C, as described previously by Quick et al. (2000).

Catalase activity was determined using the spectrophotometric method based on the Purpald® oxidation described by Johansson and Borg (1988) and measured with a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.).

2.6 | Statistical analysis

The statistical analyses were performed with the Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc.). Data are presented as mean ± standard error of the mean. In the statistical analysis, the colony of origin was treated as a fixed effect due to the experimental design, assumed to be consistent across all observations. The statistical differences between control bees and 5 and 10 mg·l^{-1} glyphosate concentration exposed bees in gene expression, enzymatic activities, and 4-HNE levels were analyzed using Student's *t*-test or *U* Mann–Whitney test depending on normality of data. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Glyphosate exposure produced changes on honeybee workers' antioxidant genes expression

The effects of two concentrations of glyphosate (5 and 10 mg·l^{-1}) at two different exposure times (2 and 10 h) in honeybee workers are shown in Figure 1. In Figure 1a, it is shown how glyphosate produced a rise in the gene expression of Catalase and *SOD2* after 2 h of exposure and without an increase of expression after 10 h. Moreover, Figure 1b shows that glyphosate exposure generates different effects on oxidative stress-related genes expression, with an increment in the expression of *Tpx3* and *Tpx4*, and a drop in the expression of *Gtpx1* and *TrxR* at 2 h of glyphosate treatment, with no significant differences at 10 h of exposure. Finally, Figure 1c indicates that glyphosate induced an increase in *GstD1* gene expression at both concentrations, but a decrease in *GstS1* gene expression after 2 h of glyphosate exposure and, in this case, a rise in *GstD1* and a decrease in *GstS1* after 5 mg·l^{-1} concentration treatment of glyphosate at 10 h of exposure.

3.2 | Glyphosate exposure produced changes on honeybee workers' mitochondrial-related genes expression

Figure 2 indicates the effects of glyphosate at two concentrations (5 and 10 mg·l^{-1}) in honeybee workers after 2 and 10 h of exposure. The results show that glyphosate exposure at 5 mg·l^{-1} increased *CoxII* and *CytC* gene expression, while glyphosate exposure at 10 mg·l^{-1} significantly decreased *CoxII* and increased *Cox6b* gene expression at 2 h. On the other hand, exposure to glyphosate at 10 h showed only a significant increment in *CytC* (at 5 mg·l^{-1}) and a decrease in *If2mt* (at 10 mg·l^{-1}) gene expression.

3.3 | High concentration of glyphosate (10 mg·l^{-1}) induced an increase in superoxide dismutase and catalase enzymatic activities

As shown in Figure 3, the exposure of honeybee workers to 10 mg·l^{-1} concentration of glyphosate induced a more than double increase in *SOD* activity, as well as a significant increase (+28%) in catalase activity.

3.4 | Glyphosate exposure increased lipid peroxidation in honeybee workers

We studied the effects of glyphosate exposure on lipid oxidative damage through the analysis of 4-HNE adducts levels. As it can be observed in Figure 4, the exposure of honeybee workers to both concentrations of glyphosate for 48 h did not produce any statistically significant change in lipid peroxidation compared to control bees. However, as shown in the same Figure 4, the effects of glyphosate exposure for 72 h are significantly marked, showing an increase in 4-HNE adducts formation in both concentrations of glyphosate.

4 | DISCUSSION

In a recent meta-analytical review, Battisti et al. (2021) underlined that the effects of glyphosate on the molecular and biochemical parameters are poorly understood. These authors highlighted the need for further research to assess the effects of glyphosate on gene expression, enzymatic action, and oxidative stress of bees (Battisti

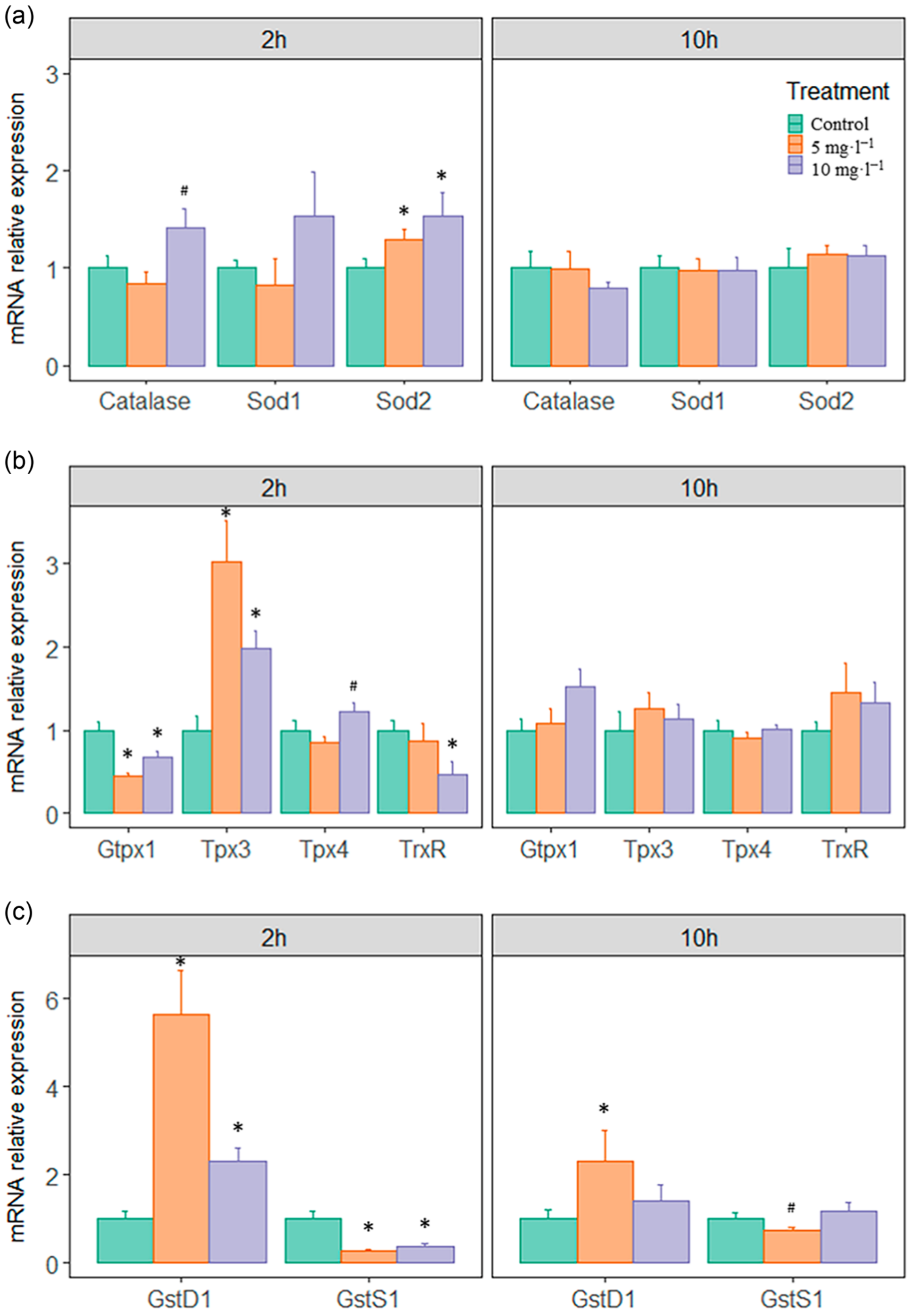

FIGURE 1 Effects of 5 and 10 mg·l^{-1} concentrations of Glyphosate on antioxidant enzymes gene expression at 2 and 10 h of exposure. Data represent means ± SEM mRNA relative expression to RPL8 mRNA expression levels ($n = 6$). (a) Effects of Glyphosate on Catalase, *SOD1* and *SOD2* gene expression. (b) Effects of Glyphosate on *Gtpx1*, *Tpx3*, *Tpx4* and *TrxR* gene expression. (c) Effects of Glyphosate on *GstD1* and *GstS1* gene expression. Statistically significant difference between experimental groups (Student's *t*-test, $^*p < 0.05$ and $^\#p < 0.1$). mRNA, messenger RNA; SEM, standard error of the mean.

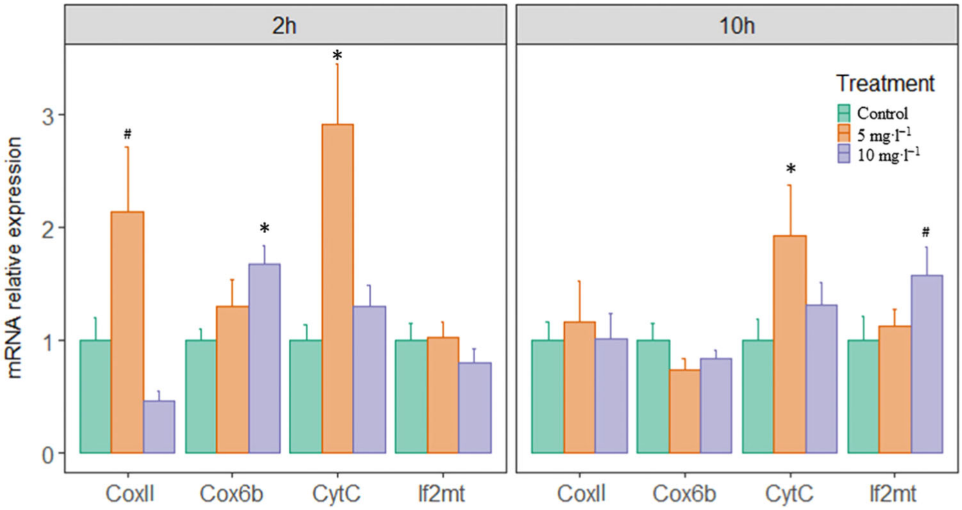

FIGURE 2 Effects of 5 and 10 mg·l^{-1} concentrations of Glyphosate on mitochondrial-related gene expression at 2 and 10 h of exposure. Data represent means ± SEM mRNA relative expression to RPL8 mRNA expression levels ($n = 6$). Statistically significant difference between experimental groups (Student's t-test, $^*p < 0.05$ and $^\#p < 0.1$). mRNA, messenger RNA; SEM, standard error of the mean.

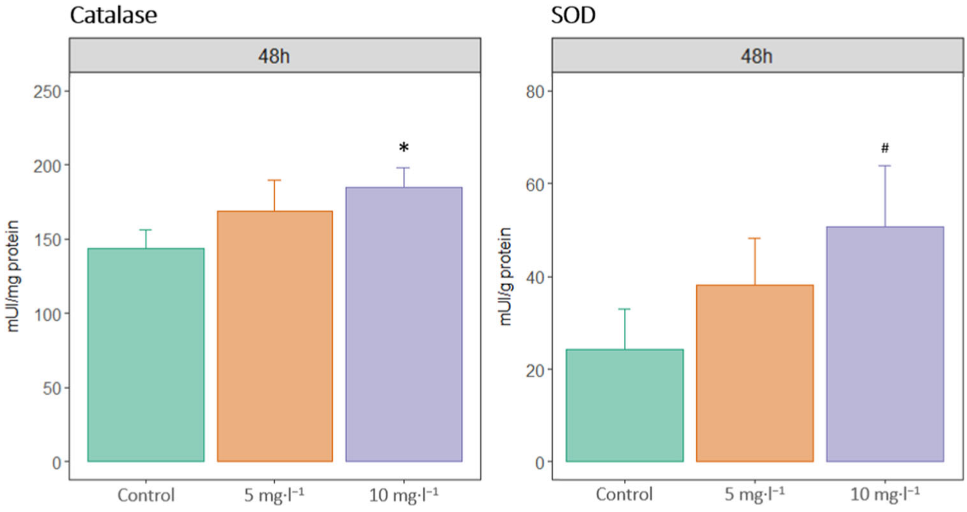

FIGURE 3 Effects of 5 and 10 mg·l^{-1} concentrations of Glyphosate on Catalase and *SOD* activity at 48 h of exposure. Data represent means ± SEM ($n = 6$). Statistically significant difference between experimental groups (Student's t-test, $^*p < 0.05$ and $^\#p < 0.1$). SEM, standard error of the mean.

et al., 2021). Precisely, in this study we present an analysis of the effects of glyphosate on oxidative stress- and mitochondrial-related genes expression, antioxidant enzymatic activities, and oxidative damage in honeybees. To our knowledge, it is the first time describing short-term sublethal exposure to glyphosate on honeybees' oxidative stress status.

The results we have observed showed an early response in antioxidant- and mitochondrial-related genes after 2 h of glyphosate exposure. Moreover, the antioxidant enzymes activity increased after 48 h, while the lipid peroxidation levels raised after 72 h of treatment.

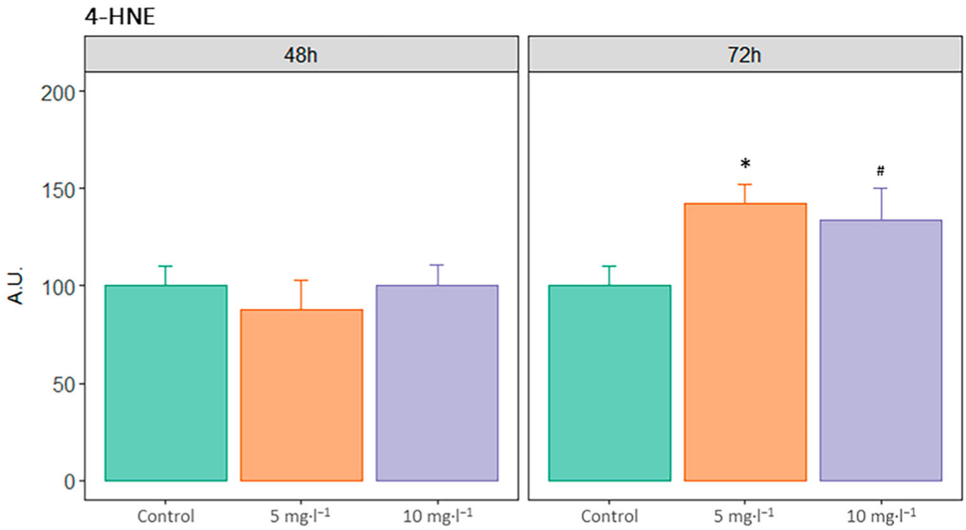

FIGURE 4 Effects of 5 and 10 mg·l^{-1} concentrations of Glyphosate on 4-HNE quantification at 48 and 72 h of exposure. Data represent means ± SEM ($n = 6$). Statistically significant difference between experimental groups (Student's t-test, $^{*}p < 0.05$ and $^{\#}p < 0.1$). SEM, standard error of the mean.

In recent studies, it has been shown that chronic exposure to glyphosate modifies gene expression of genes implicated in the immunity, plant-herbivore interaction, epigenetic mechanisms of disrupted microbiota and detoxification, like oxidative stress-responsive genes (Vázquez et al., 2020). Moreover, the same authors suggest that glyphosate exposure in larval stages causes an increment of catabolism and oxidative metabolism which, in the long run, can produce adverse effects in honeybee populations (Vázquez et al., 2018). Our results suggest that sublethal treatment with glyphosate modifies antioxidant- and mitochondrial-related genes expression patterns of honeybees in the very short term, after 2 h, while after 10 h this effect is attenuated. These results are in agreement with those obtained by Balbuena et al. (2015), who observed that treatment for 1 h with similar concentrations of glyphosate to those used in this study caused alterations in honeybees' navigation, which can be related to an increase in oxidative stress (Margotta et al., 2018). Concretely, another study carried out in honeybees evaluated the response in antioxidant-related genes expression to pesticides Coumaphos and Imidacloprid, showing, among others, Catalase and *SOD2* antioxidant genes expression modulation (Gregorc et al., 2018). Our results demonstrate that external products applied to protect plants like herbicides (such as glyphosate) can modulate the antioxidant response in honeybees. Precisely, in this same study from Gregorc et al. (2018), the authors showed a decrease in *TrxR1* gene expression after 20 days of Imidacloprid treatment. However, in our results we observed a decrease in this gene expression after sublethal treatment of glyphosate only for 2 h, suggesting that, at these concentrations, glyphosate could induce a rapid response in the antioxidant system expression. It could be some compensatory mechanisms which could explain the difference between the effects of glyphosate after 2 and 10 h of exposure. In 2018, Vazquez et al. described compensatory mechanisms for the effects of glyphosate on honeybee larvae, although these mechanisms may have some effects such as increased energy consumption by increasing intake, which could cause the death of less tolerant individuals (Vázquez et al., 2018). This could be an explanation for why the effects of 10 h of glyphosate exposure appear to be more attenuated.

Insects do not have genes encoding glutathione reductase and glutathione peroxidase, two important genes for the antioxidant defense in vertebrates (Chaitanya et al., 2016). Their functions are performed by homologous genes that encode thioredoxin reductase (*TrxR*) and thioredoxin peroxidase (*Tpx*), in addition to other genes which encode enzymes of the antioxidant system that act as peroxidases like *GPx* homologues with *Tpx* activity (*Gtpx*) and glutathione S-transferase (*GST*) (Chaitanya et al., 2016). In this sense, the downregulation of *TrxR* and *Gtpx1* expression after the higher concentration of glyphosate, followed by an increase in *Tpx3* and *Tpx4* expression could be an indicator of an altered antioxidant system in honeybees. In fact, it is known that *Gtpx1* acts eliminating hydrogen peroxide but in a different pathway than catalase (Missirlis et al., 2003), which in our study increases its expression and activity in response to glyphosate at 10 mg·l^{-1}.

In the case of *GSTs*, the major detoxifying enzymes, catalyze the conjugation of glutathione with electrophilic substrates resulting in lower reactivity and higher solubility in water products (Nikolić et al., 2019). In their study, Nikolić et al. (2019) demonstrated that *GstD1* presented a greater response to contaminants like copper than *GstS1*. Interestingly, delta class of *Gst* (*GstD1*) is specific to insects and it is involved in resistance to pesticides (Udomsinprasert et al., 2005), while sigma class of *Gst* (*GstS1*) has a protective effect against oxidative stress and participates in the elimination of lipid peroxides (Singh et al., 2001). Our results are in accordance with these observations, since *GstD1* increased its expression in response to the herbicide treatment, and *GstS1* expression was decreased, a fact that could explain the results obtained in lipid peroxidation.

In a previous publication, we demonstrated that some biotic stressors, like an invasive species predator, could affect mitochondrial-related genes expression (Leza et al., 2019). In the same way, it is known that glyphosate can affect mitochondrial function in nontarget living organisms (Strilbyska et al., 2022), so we planned to study the effects of glyphosate on honeybees' mitochondria. In other animals, like zebrafish, glyphosate at 10 mg·l^{-1} induced the inhibition of the cytochrome c oxidase (*Cox*) expression levels (Pereira et al., 2018), agreeing with our results in *CoxII* expression levels after 2 h of glyphosate treatment. However, at the same concentration and time of exposure to glyphosate, the expression of another subunit of cox, *Cox6b*, was increased, in a possible compensatory effect due to the decrease in the expression of subunit 2. Interestingly, exposure to 5 mg·l^{-1} of glyphosate produced an increase of *CoxII* expression, suggesting that at these two different concentrations the response of honeybees is different. Another interesting data is that cytochrome c (*CytC*) gene expression was increased after 2 and 10 h of glyphosate exposure at 5 mg·l^{-1}, but not at 10 mg·l^{-1}. These two data, the raises in the expression of *CoxII* and *CytC*, could suggest that bees, at a concentration of 5 mg·l^{-1}, can act against the stress generated by glyphosate by increasing the expression levels of mitochondrial genes in response to the herbicide, but not at 10 mg·l^{-1} glyphosate concentration. Moreover, the increase in the expression of the *IFmt2* gene, a fundamental product for the translation of mitochondrial-encoded mRNAs, after 10 mg·l^{-1} glyphosate concentration, suggests that the higher mitochondrial activity in bees exposed to glyphosate requires an increase in the whole set of mitochondrial products, as occurs with the antioxidant genes.

A recent study has observed the effects of glyphosate on antioxidant enzymes activity, showing contradictory results, reflecting that 16 days of glyphosate treatment decreased catalase activity and increased *SOD* activity in honeybees' midgut, but at very low concentrations (Pal et al., 2022). Another recent study has demonstrated that physical changes like exposure of honeybees to an electromagnetic field frequency increased catalase and *SOD* activities (Migdał et al., 2020). Even biological stressors are able to modify antioxidant enzymatic activities to respond to the generated oxidative stress (Leza et al., 2019). This increment in this antioxidant enzymes activity is a symptom of response to general free radical production increase (Ighodaro & Akinloye, 2018), suggesting a rise in the oxidative stress of honeybees exposed to glyphosate.

It is important to note that Thompson et al. (2014) estimated the total maximum daily intake of glyphosate residues in honeybee broods in field to be 66 mg. In our experiment, both concentrations of glyphosate used have proven to be sublethal for honeybees (Balbuena et al., 2015; Motta et al., 2018). Helmer et al. (2015) did not detected lipid peroxidation on honeybees at maximum dose of 5 ng gliphosate per bee, meanwhile Jumarie et al. (2017) observed lipid peroxidation a dose 5 ng/bee. In this study, after 72 h of glyphosate treatment lipid peroxidation of honeybees at both concentrations (5 and 10 mg·l^{-1}) was increased, thus promoting oxidative damage. This results agree with Jumarie et al. (2017), likewise, other authors have shown in other species like fishes and rats, even in plants, that glyphosate induced an increase in lipid peroxidation (El-Shenawy, 2009; Nwani et al., 2013; Puértolas et al., 2010). Moreover, the effects of glyphosate on lipid peroxidation in honeybees have been demonstrated to be an indicator of oxidative damage produced by this herbicide (Pal et al., 2022). At this point, it is important to remember the results obtained in the GstS1 gene expression. This gene is involved in the protector effect against oxidative stress and one of its functions is to eliminate the lipid peroxides (Singh et al., 2001), so the decrease of this gene's expression after glyphosate treatment could explain the increase of 4-HNE adducts, a product related to lipid peroxidation (Esterbauer et al., 1991).

5 | CONCLUSION

In this study we demonstrated that sublethal treatments of glyphosate modifies the expression of mitochondrial-related genes and induces oxidative stress in honeybees in a very short period of time by direct feeding. Honeybees' mitochondria and antioxidant defenses are modified to reduce the negative effects of glyphosate; however, the oxidative damage markers are still increased. Although more studies are necessary, this research provides evidence that the use of glyphosate could directly affect honeybee colonies. It is therefore necessary to invest in other types of plant protection products that do not affect nontargeted living organisms, especially honeybees because of their importance in ecosystem maintenance and in plant production.

AUTHOR CONTRIBUTIONS

Conceptualization: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. *Methodology*: Cayetano Herrera and Margalida Torrens-Mas. *Validation*: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. *Formal Analysis*: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. *Investigation*: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. *Data Curation*, Daniel Gabriel Pons, Cayetano Herrera, Margalida Torrens-Mas, Mar Leza, and Jorge Sastre-Serra. *Writing—Original Draft Preparation*: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. *Writing—Review and Editing*: Daniel Gabriel Pons, Cayetano Herrera, Margalida Torrens-Mas, Mar Leza, and Jorge Sastre-Serra. *Supervision*: Daniel Gabriel Pons, Mar Leza, and Jorge Sastre-Serra. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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