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The effects of short-term glyphosate-based herbicide exposure on insect gene expression profiles

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ABSTRACT

Glyphosate-based herbicides (GBHs) are the most frequently used herbicides worldwide. The use of GBHs is intended to tackle weeds, but GBHs have been shown to affect the life-history traits and antioxidant defense system of invertebrates found in agroecosystems. Thus far, the effects of GBHs on detoxification pathways among invertebrates have not been sufficiently investigated. We performed two different experiments—**1)** the direct pure glyphosate and GBH treatment, and **2)** the indirect GBH experiment via food—to examine the possible effects of environmentally relevant GBH levels on the survival of the Colorado potato beetle (*Leptinotarsa decemlineata*) and the expression profiles of their detoxification genes. As candidate genes, we selected four cytochrome P450 (CYP), three glutathione-S-transferase (GST), and two acetylcholinesterase (AChE) genes that are known to be related to metabolic or target-site resistances in insects. We showed that environmentally relevant levels of pure glyphosate and GBH increased the probability for higher mortality in the Colorado potato beetle larvae in the direct experiment, but not in the indirect experiment. The GBHs or glyphosate did not affect the expression profiles of the studied CYP, GST, or AChE genes; however, we found a large family-level variation in expression profiles in both the direct and indirect treatment experiments. These results suggest that the genes selected for this study may not be the ones expressed in response to glyphosate or GBHs. It is also possible that the relatively short exposure time did not affect gene expression profiles, or the response may have already occurred at a shorter exposure time. Our results show that glyphosate products may affect the survival of the herbivorous insect already at lower levels, depending on their sensitivity to pesticides.

1. Introduction

Glyphosate-based herbicides (GBHs) are the most frequently used herbicides worldwide (Woodburn, 2000; Benbrook, 2016; Myers et al., 2016). The function of glyphosate (N-(phosphonomethyl)glycine) is based on the inactivation of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Steinrücken and Amrhein, 1980; Duke and Powles, 2008) by blocking phosphoenolpyruvate (PEP) binding sites, thereby inhibiting the reaction between shikimate 3-phosphate (S3P) and PEP (Funke et al., 2006) in the shikimate pathway. The shikimate pathway is responsible for the synthesis of aromatic amino acid (e.g., phenylalanine, tyrosine, and tryptophan) in numerous plants, fungi, and bacteria (Bentley, 1990; Haslam, 1993; Leino et al., 2021), but it does not appear in animals. Glyphosate use is intended to tackle weeds, but evidence for the potential negative effects of glyphosate products on the cell function, tissues, physiology, microbial function, and survival rate of animals has also been shown (Mesnage et al., 2015; Claus et al., 2016; Margus et al., 2019b; Gomez-Gallego et al., 2020; Motta et al., 2018; Rainio et al., 2020; Ruuskanen et al., 2020). GBHs are known to affect non-target organisms, which may be directly exposed to glyphosate products in several ways. GBHs exposure in non-target organisms may occur during chemical transportation, handling and storage, by wind action during field application (Torretta et al., 2018), or when synchronizing and accelerating the ripening of forage cereals (Helander et al., 2012). Glyphosate is also used to control invasive plant species in nature conservation (Weidenhamer and Callaway, 2010). There is a growing concern regarding using GBHs as the primary weed management strategy due to increasing evidence of its toxicity for non-target

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organisms (Helander et al., 2012; Torretta et al., 2018; Van Bruggen et al., 2018). However, agrochemicals are required to secure crop productivity in agricultural fields in order to feed the increasing population worldwide. Thus, more ecosystem-level studies are required to identify the effects of pesticides used on non-target organisms and to find sustainable methods for plant protection in the future.

GBHs have been shown to affect the survival, development, and reproduction of invertebrates found in agroecosystems (Castilla et al., 2008; Schneider et al., 2009; Benamu et al., 2010; Castilla et al., 2010; Evans et al., 2010; Saska et al., 2016; Gill et al., 2018), although there are also studies that report little or no effects of the use of GBHs (Thompson et al., 2014; Salvio et al., 2016; Margus et al., 2019b; Rainio et al., 2020). GBHs are also known to affect the antioxidant defense system of invertebrates, such as the antioxidant enzyme activities of glutathione-S-transferases, catalase, and superoxide dismutase (Che-Mendoza et al., 2009; Contardo-Jara et al., 2009; Rainio et al., 2020). However, the effects of GBHs on detoxification pathways among invertebrates are not well investigated. Earlier studies have revealed changes in gene expression and alteration in transcript expression in relation to pesticide exposure in bees, leaf-beetles, and several vertebrate species (Gregorc et al., 2012; Uren Webster and Santos, 2015; Margus et al., 2019b; Vazquez et al., 2020). For example, studies on Colorado potato beetles (*Leptinotarsa decemlineata*) have revealed that several genes involved in antioxidant defense and detoxification processes are linked to insecticide resistance (Wan et al., 2013; Kumar et al., 2014). However, whether these genes are affected when individuals are exposed to herbicides has not been well studied.

Glyphosate is an organophosphorus compound (more specifically organophosphonate), which is similar to a few commonly used insecticides (Larsen et al., 2016; Sidhu et al., 2019); however, it lacks certain features of such insecticides, such as the inhibition of cholinesterase (Sandrini et al., 2013), which is a common effect of most organophosphates (OP) (Marrs, 1993). Nevertheless, several GBHs have been shown to inhibit acetylcholinesterase (AChE) activity, which is the target enzyme for anticholinesterase insecticides such as OPs and carbamates (Revuelta et al., 2011), in vertebrates (Glusczak et al., 2006; Glusczak et al., 2007; Modesto and Martinez, 2010: Menéndez-Helman et al., 2012) and invertebrates (Margus et al., 2019b; Margus et al., 2021), thereby being an important group that must be studied with regard to the effects of GBHs.

If herbicides function as xenobiotics, they may modify different detoxification pathways of insects (Larsen et al., 2012; David et al., 2013; Zhu et al., 2016; Gaines et al., 2020), including phase I cytochrome P450 enzymes (CYPs) and phase II conjugation enzymes (e.g., glutathione-S-transferases, GSTs) (David et al., 2013; Hodges and Minich, 2015; Gaines et al., 2020). The phase I CYP450 enzymes are generally the first defense employed by the body to metabolize xenobiotics and are responsible for oxidation, peroxidation, and reduction of several endo- and exogenous substrates (Paine, 1981; Danielson, 2002). Insect CYP genes belong to four major clades: CYP2, CYP3, CYP4, and mitochondrial clades (Claudianos et al., 2006), all of which reveal successful physiological adaptations of insects to their food sources and environments (Feyereisen, 2006). In phase II, the active sites of hydrophilized xenobiotics can be conjugated by enzymes, like GSTs, whose main function is to attach a glutathione group to a bio-transformed metabolite (Hodges and Minich, 2015). GSTs are a diverse family of dimeric proteins, which are important in detoxification processes, function as transport proteins, and protect against oxidative stress (Hayes and Pulford, 1995; Ding et al., 2003; Enayati et al., 2005; Hayes et al., 2005). Insects are known to have at least six different classes of GSTs that are encoded by multigene families (Ding et al., 2003). Both CYPs and GSTs are involved in the detoxification processes related to metabolizing hormones and external substances, such as medication, environmental pollutants, pesticides, and plant secondary compounds (Feyereisen, 2006; Guengerich et al., 2008); thus, they are important but poorly studied groups in relation to herbicides as well. The measurement of AChE inhibition has been often used as a biomarker for OP pesticides (Lionetto et al., 2013). AChE is a key enzyme in the nervous system (Lionetto et al., 2013) and its function in animals is to terminate neurotransmission by rapidly hydrolyzing the neurotransmitter acetylcholine at cholinergic synapses (Soreq and Seidman, 2001; Lu et al., 2012). Moreover, it forms a common mechanism of insecticide resistance through its reduced sensitivity to OP insecticides (Fournier and Mutero, 1994; Kono and Tomita, 2006).

In this study, we examined the effects of pure glyphosate and GBHs on beetle survival and the changes in the expression of the genes that participate in the detoxification processes of pesticides by using a non-target herbivore, the Colorado potato beetle (*Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae), as a model species. The Colorado potato beetle is an economically important invasive potato pest found worldwide (Casagrande, 1987; Grapputo et al., 2005; Walsh, 1865). In Finland, it is classified as a quarantine pest species (Vänninen et al., 2011). The beetles may be exposed to GBH residues or their metabolites directly during field application (Torretta et al., 2018) or indirectly via diet (potato plant) or soil (in their pupal phase or during diapause). Therefore, we performed two different experiments—**1)** an experiment with direct pure glyphosate and GBH exposure and **2)** an experiment involving indirect GBH exposure via food—to examine the possible effects of environmentally relevant levels of GBHs on beetle survival and the expression levels of the detoxification genes. In the direct exposure manipulation, we studied the effects of GBHs and pure glyphosate because glyphosate is applied as part of formulated products (Mesnage et al., 2015). The impacts of GBHs on non-target organisms may vary substantially depending on the use of commercial formulations, as these differ in terms of their surfactants and salts, which are added to enhance the effectiveness of glyphosate on weed (e.g., cellular uptake) (Mesnage et al., 2015). In addition, a few adjuvants used in the GBHs may be even more toxic than pure glyphosate (Mesnage et al., 2014). In the indirect experiment, we planted potatoes in the soil treated with GBH; thus, the larvae were likely exposed to GBHs indirectly via food.

We evaluated survival and quantitative changes in the expression of genes belonging to CYP superfamilies, GSTs, and AChEs in beetle larvae, which are more vulnerable to environmental chemicals than adult beetles. As candidate genes, we selected four CYP, three GST, and two AChE genes, which are known to be related to metabolic resistance (including OP resistance) in the Colorado potato beetle or relative species (Shi et al., 2013; Wan et al., 2013; Margus et al., 2021, Lindström et al., unpublished). The CYP genes used in this study belonged to CYP3 (*CYP6BH1v1, CYP6BJ1, CYP9z14v2*) and CYP4 clades (*CYP4G29*) and were designed based on the thesis of Sovelius (2014). The GST genes used in this study were *GST1, GST3*, and *GST_c10663*. The GST1 and GST3 were designed based on the GSTe2 gene in *Anopheles gambiae* (Ding et al., 2003) and the GST_c10663 based on GST_c in *Tribolium castaneum* (GenBank, Benson et al., 2013). From the AChE group, we used *Ldace1* and *Ldace2* genes, from which *Ldace1* has been shown to be affected by pesticides, including GBHs, in the Colorado potato beetle (Margus et al., 2019b).

We hypothesize that **1)** both pure glyphosate and GBHs increase the mortality of the beetle larvae when exposed either directly or indirectly to GBHs; **2)** pure glyphosate and GBHs affect the expression of detoxifying genes belonging to CYP, GST, and AChE gene families; and **3)** GBH shows stronger effects on gene expression and mortality than pure glyphosate due to the adjuvants used in GBH.

2. Materials and methods

2.1. Direct exposure to GBH

GBH (Roundup® Bio containing 360 g/l glyphosate, Monsanto, USA) treatment was conducted during summer 2017 in a licensed quarantine laboratory in the University of Jyväskylä, Finland (62°1348“N 25°44‘34“E). In this experiment, we used the Colorado potato beetles

that originated from Vermont in the United States (US). The Vermont beetle population was field-collected (44°43́N, 73°20'W) in 2010, after which it was grown in laboratory conditions in Jyväskylä (a more detailed description of laboratory conditions is provided in Margus et al., 2019a).

In our experiment, we used 222 beetle larvae from 18 families. The newly hatched larvae were reared in the potato plants (*Solanum tuberosum* var. Challenger) covered by light-permeable fabric bags for 11–14 days until the larvae were at their fourth (and last) instar. These larvae were randomly divided into three groups: 1) pure glyphosate (5 µl of 13.68 g/l technical glyphosate PESTANAL, Sigma-Aldrich, USA), 2) Roundup (5 µl of 3.8% Roundup Bio, 13.7 g/l of glyphosate isopropylamine salt), and 3) control group (5 µl of distilled water). We selected the 3.8% Roundup concentration to represent the commonly used concentration in fields after harvesting in Finland. The recommended volume of formulated glyphosate solution (Roundup Bio) for field application is 1.5 l/ha–3.0 l/ha (spring, before sowing) and 3.0 l/ha–8.0 l/ha (autumn, after harvesting) for weed (Finnish Food and Safety Authority). The larvae were treated with pure glyphosate and Roundup by topical application to simulate direct glyphosate spraying in crop fields; 199 larvae were treated (pure glyphosate: n = 67; Roundup: n = 65; control: n = 67). The treatment was performed by placing five larvae at a time in a petri dish (Ø 92 mm) covered with a filter paper (Ø 70 mm, grade 1002), and pipetting 5 µl of herbicide on top of each larva. After the exposure, the larvae were kept without food for 2 h to ensure that they were not exposed via food. The control group was treated similarly, but using distilled water instead. The larvae were exposed for 24 h, after which their mortality was checked; the viable larvae were collected and immediately frozen with liquid nitrogen and stored at −80 °C for subsequent genetic analyses.

2.2. Indirect exposure to GBH

The indirect GBH exposure experiment was conducted in the summer of 2014 in Jyväskylä (62° 13′ N, 25° 44′ E). We mixed 100 L of soil (W HS R8017, Kekkilä Oy, Finland) with 20 L of water and divided it equally among 40 plastic pots (Ø 16.5 cm). The soil consisted of fine white sphagnum peat and sand. For the indirect GBH exposure, the soil in half of the pots was sprayed (4 L/ha) with Roundup Bio (360 g/l glyphosate isopropylamine salt, Monsanto, United States) according to the manufacturer's instructions (see (Margus et al., 2019b). We used 1.5% Roundup Bio solution (5.4 g/l of glyphosate isopropylamine salt) to imitate sprayings in natural fields in spring. The other half of the pots were not sprayed and used as controls. After 11 days, we mixed the soil and planted the potatoes (*Solanum tuberosum* variety Van Gogh) in the pots; the potatoes were grown outside, under ambient climatic conditions in July (day length ca. 18 h in Central Finland, under a mean temperature of July 2014, ca. 18 °C –19 °C in Jyväskylä). We checked the potatoes regularly and watered the pots when needed.

We transferred the potatoes into a greenhouse in late August, after they were grown for 6–7 weeks. The temperature was set to 23 °C–25 °C and the day length was the ambient late August day length in Central Finland (ca. 15 h). We divided 406 two-day-old larvae from 9 different families into the GBH and control treatment groups and grew them either on the GBH or control potato plants in family groups for four days. The plants were covered with clear plastic bags to separate the families and prevent the larvae from escaping from the plants. At the age of six days, we checked larval mortality and collected the viable larvae from the plants. We transferred the larvae to Eppendorf tubes, froze them with liquid nitrogen, and stored them at − 80 °C for further analyses.

2.3. Real-time qPCR analyses

For gene expression analyses, we selected 27 larvae, with 9 larvae per treatment group (pure glyphosate, GBH, and control) from the direct experiment; in addition, we selected 9 families, with 3 larvae pooled for each family per treatment group (GBH and control) for the indirect experiment. Furthermore, we extracted the RNA from whole larvae or pooled larvae using the TriReagent (Sigma-Aldrich, USA) and RNeasy Mini kit (Qiagen, Germany). The extraction was followed by a DNase treatment (RNAse-Free DNase Set, Qiagen), which is suggested by the manufacturer for applications that are sensitive to small amounts of DNA. We measured RNA concentration and purity with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA), and the integrity and quality of the samples was assessed using Agilent RNA ScreenTape System, analyzed with 2200 TapeStation (Agilent Technologies, USA). Thereafter, all RNA samples were diluted to 100 ng/µl concentration. Then, the cDNA was synthesized from 100 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, USA) following the manufacturer's instructions.

In order to investigate the gene expressions of *CYP6BH1v1* (direct experiment only), *CYP6BJ1* and *CYP9z14v2* (direct experiment only), *CYP4G29* and *GST1* (direct experiment only), and *GST3, GST_c10663, Ldace1*, and *Ldace2* genes, we performed RT-qPCR using a SYBR Green Supermix (Bio-Rad, USA) kit. The qPCR reaction mix contained 5 µl of diluted cDNA (1:8 dilution), 10 µl of 2 × SYBR green supermix, 1 µl of each primer (forward and reverse; Supplementary Table 1), and 3 µl of nuclease-free water. Primers used for RT-qPCR were obtained from the Master's thesis of Sovelius (2014) (Table S1). Then, the RT-qPCR reactions were run with a Bio-Rad CFX96 instrument. The following were the cycling conditions in qPCR: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s, and extension at 72 °C for 30 s. The melt curve was measured from 65 °C to 95 °C at the end of each amplification reaction to ensure a single amplification product. The efficiency of qPCR amplification was calculated for each gene using 2-fold serial dilutions of pooled cDNA containing 7 points (three technical repeats per dilution). Based on the serial dilution curves, 1:8 dilution was chosen for further analyses. The amplification efficiencies were between 93.3% and 109.5%, which are commonly accepted (Taylor et al., 2010, see more details in Table S1). We used nine biological replicates for each group, with three technical replicates; the standard deviations among the technical replicates were less than 0.3 Ct (CV less than 10%). In addition, we used a pooled sample as a positive control in each run for inter-run calibration. We also included *L13e* (ribosomal protein L13e, Yocum et al., 2009) and *βTub* (β- tubulin, Revuelta et al., 2011) as reference genes.

2.4. Statistical analyses

Statistical analyses were performed using R (R Core Team, 2022) and the brms package (Bürkner, 2017). The survival rate of the beetles among the treatment groups (direct experiment: GBH-treated, pure glyphosate and control; indirect experiment: GBH, control) was analyzed with a generalized linear mixed model (GLMM with binary distribution and logit link function). Family was used as a random factor to control for the non-independence of larvae used from the same family. A Bayesian approach was chosen for the survival analysis. Bayesian inference assumes that the model parameters are random variables, and their posterior distribution is to be estimated given prior information such as expert knowledge or evidence from previous studies (Gelman et al., 2013). Flat priors were chosen for the regression coefficients of the model and a truncated student t-distribution prior was chosen for the standard deviation of the family effects.

Principal component analysis (PCA, SAS statistical software 9.4, 2013) was performed for the studied genes to illustrate the large family level variation and to show which genes are more closely connected. The first principal component (PC1: CYP6BJ1, CYP9Z14v2, and GST1) explained 38.6% of the variation (eigenvalue: 2.70), the second component (PC2:CYP6BH1v1, GST_c10663, GST3) explained 28% of the variation (eigenvalue 1.95), and the third component (PC3: CYP4G29) explained 16.5% of the variation (eigenvalue: 1.15) in the direct

experiment (Fig. S1). Correspondingly, in the indirect experiment, the first principal component (PC1:CYP4G29, GST_c10663) explained 41% of the variation (eigenvalue: 1.64) and the second component (PC2: CYP6BJ1, GST3) explained 37.9% of variation (eigenvalue: 1.52) (Fig. S2).

Further, inter-run calibration was performed with CFX manager software (v 4.1, Bio-Rad CFX Maestro 1.1, Bio-Rad Laboratories, United States). Thereafter, the calibrated expression values for each biological replicate were normalized using the NORMA-Gene method (Heckmann et al., 2011) prior to REST analyses. The statistical significance of pairwise comparisons of the cycle threshold (Ct) values among the treatments was tested with REST (Relative Expression Software Tool) 2009 software (https://www.gene-quantification.de/rest-index.html), with a Pair Wise Fixed Reallocation Randomization Test, the iterations set at 10 000 (Pfaffl et al., 2002). REST is based on an efficiency corrected mathematical model for data analysis, calculating the relative expression ration in the basis of the PCR efficiency and crossing point deviation of the investigated expressions. This program calculates the

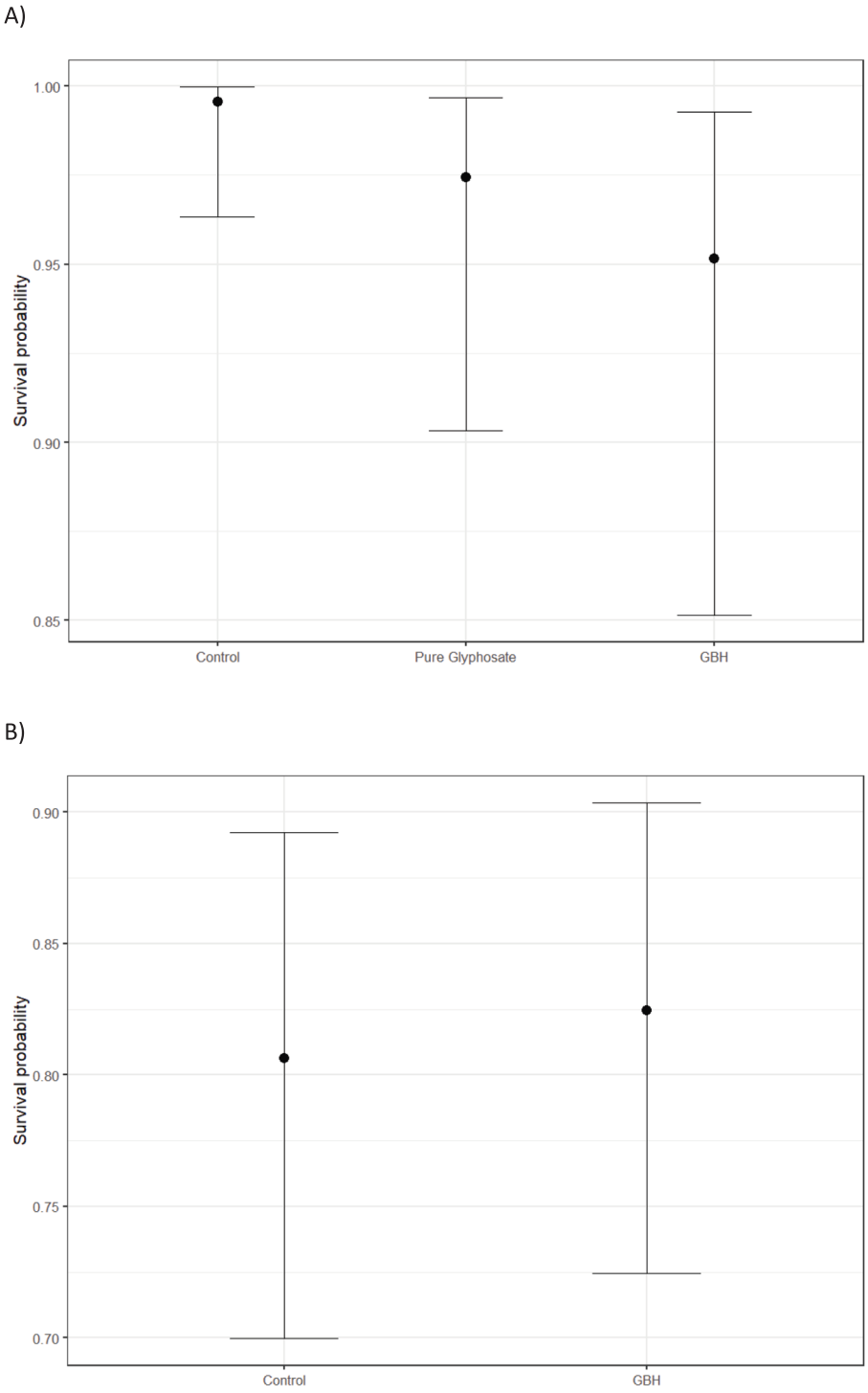

Fig. 1. (A-B). Predicted survival probabilities of the Colorado potato beetle larvae and their 95% credible intervals for each treatment group in the A) direct (GBH, pure glyphosate, control) and B) indirect (GBH, control) experiment. Circles denote the median survival probability.

statistical significance of expression ratios of the target genes using bootstrapping and randomization (iteration) techniques to provide 95% confidence intervals for expression ratios, without normality or symmetrical distribution assumptions (Pfaffl et al., 2002), while still remaining as powerful as more standard tests (Horgan and Rouault, 2000). The randomization test repeatedly and randomly reallocates the observed values to the two treatment groups, and notes the apparent effect (expression ratio) each time. The proportion of these effects, being as great as that actually observed in the experiment, gives the p value of the test (Pfaffl et al., 2002). We analyzed the data by testing the differences among the treatment groups and also by testing the differences separately for each family.

3. Results

Based on the posterior distribution of the model parameters, there was evidence that both direct exposure to GBH and pure glyphosate increased the probability for mortality in the Colorado potato beetle larvae. There was a 99% probability that direct exposure to GBH results in a lower survival rate compared to the control group. Similarly, there is a 93% probability that direct pure glyphosate exposure results in a lower survival rate compared to the control group. Also, there was 81% probability that the decrease in survival is greater resulting from the direct GBH exposure than from the direct pure glyphosate exposure (Fig. 1). The median survival posterior probabilities and their 95% posterior intervals for each treatment group are shown in Fig. 1 (see also Table S2 and Fig. S1). Larval survival in the control, pure glyphosate-treated and GBH-treated groups were 98.7%, 94.8% and 91.3%, respectively in the data. We note that family effects can be especially large and comparable in size to the effects of the treatments in the survival analysis (Table S2, Fig S1).

There were no significant differences in the gene expression levels in the studied genes of the larvae in the pure glyphosate, GBH, and control groups. This suggests that pure glyphosate or GBH did not increase or decrease the gene expression levels of the detoxification genes compared to the controls, at least in the environmentally relevant concentrations used in this study (Fig. 2 and S2). Moreover, larvae exposed to pure glyphosate did not differ significantly from those exposed to GBHs (Table 1a and Table 1b). On the other hand, we observed large family level variation in the expression profiles of the studied genes (Fig. 2).

Further, there was no strong evidence that indirect exposure to GBH would result in lower survival rate compared to the control group, as the posterior probability is only 32%. The survival rates in the control and GBH groups were 78.9% and 80.7%, respectively in the data (Fig. 1b). In addition, there were no significant differences in the gene expressions of the studied genes between the GBH and control groups in the indirect treatment (Fig. 1).

4. Discussion

We found evidence that direct exposure to pure glyphosate and GBH affect negatively to the survival of the Colorado potato beetle larvae, whereas the indirect exposure to GBH did not show any evidence of lower survival rates in GBH compared to control group. There was also 81% probability that the decrease in survival was higher in the direct GBH exposure compared to direct pure glyphosate exposure, suggesting that GBH is more harmful to the larvae than pure glyphosate. This may be due to adjuvants used in the GBHs, which can be even more toxic than pure glyphosate (Mesnage et al., 2014). For example, POEA (polyethoxylated tallowamine), which has been commonly used as adjuvant in GBHs showed higher toxicity compared to pure glyphosate in several studies (Contardo-Jara et al., 2009, Mesnage et al., 2014, Chłopecka et al., 2017, Bednářová et al., 2020). The indirect exposure to GBH exposure via food did not significantly affect the survival of the Colorado potato beetle. This may be due to different transportation route of glyphosate to the beetle system (via skin vs. via food). Studies on other invertebrate species have shown negative effects of GBHs on survival rate (Castilla et al., 2008; Benamu et al., 2010; Evans et al., 2010; Janssens and Stoks, 2017), although the opposite results have also been found (Haughton et al., 2001; Michalkova and Pekar, 2009; Baker et al., 2014; Thompson et al., 2014; Salvio et al., 2016) depending on exposure time and concentration. In both our current experiments, we used environmentally relevant concentrations that are commonly used in the agricultural field in Finland, depending on weed type and the time of year. The exposure time is essential in herbicide studies; it is possible that the relatively short exposure time to GBH (24 h in indirect experiment and 4 days in the indirect experiment) may not be long enough to show clear effects on larval survival thus underestimating the negative effects of GBH (see also Relyea, 2005). On the other hand, the expression of detoxification genes may happen shortly after pesticide exposure, which is why we focused only on the short-term effects of GBH in this study.

Here we studied the environmentally relevant GBH concentrations to simulate the conditions in the agricultural fields. GBH or the pure glyphosate did not affect the expression profiles of the studied genes; this may be related to the sublethal concentrations or the exposure time used in our experiments (direct and indirect exposure). Moreover, there were no differences between the pure glyphosate and GBH groups in the studied genes. Here, we studied genes CYP6BH1v1, CYP6BJ1, and CYP9Z14v2, belonging to clade CYP3, which is the most common of insect P450 genes. A few members of this clade, such as CYP6 and CYP9 gene clusters, have been shown to be affected by several insecticides, such as pyrethroid resistance (Kasai and Scott, 2001; Nikou et al., 2003; Claudianos et al., 2006; Feyereisen, 2012) as well as dichlorodiphenyltrichloroethane (DDT) and neonicotinoid resistances (Daborn et al., 2002) in insects. In Sovelius' (2014) study, genes CYP6BJ1 and CYP4G29 showed decreased expression in the Colorado potato beetle larvae treated with OP insecticide compared to control larvae. However, there is no knowledge of the effects of GBHs on the expression of the studied genes thus far in the Colorado potato beetles. In addition, we studied CYP4G29, a gene belonging to the CYP4 gene cluster, which is known to be substantially expressed in insect genomes and reflect a diversity of functions (Feyereisen, 2006). However, its expression was not affected by pure glyphosate or GBH either. The results suggest that the studied genes may not be activated in the Colorado potato beetle system in relation to GBH exposure. In comparison, the mammalian CYP genes—such as the enzyme activities of CYP1A1/2, CYP2B, and CYP3A—that participate similarly to the detoxification processes have been shown to be affected by glyphosate products (Larsen et al., 2014). In general, the genes in the CYP6 and CYP4 clusters have been shown to have increased expression in relation to pesticide exposure and the increased expression is also shown to be associated with pesticide resistance in several species (Kasai and Scott, 2000; Hu et al., 2021; Daborn et al., 2002; Pridgeon et al., 2003; Wan et al., 2013). It is important to note that 89 CYP genes have been identified in the Colorado potato beetle (Schoville et al., 1931), and there are a number of CYP6s, CYP9s, and CYP4s that deal with several potential substrates. Therefore, is it likely that certain genes other than the studied ones in the same clusters may be involved in GBH exposure in this species. In general, the Colorado potato beetle tolerates pesticides relatively well and has developed resistance to several synthetic insecticides, including OPs (Alyokhin et al., 2008; Piiroinen et al., 2013; Kostic et al., 2016; Brevik et al., 2018a), which can potentially make them less sensitive to other OP pesticides as well.

Further, increased GST enzyme activity has been associated with resistance to major classes of insecticides (Prapanthadara et al., 1993; Huang et al., 1998; Vontas et al., 2002), even in studies on Colorado potato beetles (Clements et al., 2016; Han et al., 2016). In Rainio et al.'s (2020) study, the larvae of the Colorado potato beetle that were indirectly exposed to GBH showed higher GST enzyme activity than the control larvae. Thus, we expected to find changes in the studied GST gene expression profiles as well. However, there were no differences in

Table 1a
Expression profiles of target genes in the Colorado potato beetle larvae that were directly exposed to pure glyphosate and glyphosate-based herbicides (GBHs). Upward and downward arrows indicate the significant differences (positive or negative) between the treatment groups.

Target gene	Family ID	Control vs. Pure glyphosate		Control vs. GBHs		Pure glyphosate vs. GBHs	
		Log2Fold change		Log2Fold change		Log2Fold change	
CYP4G29	1	−0.059		0.773	↑	0.833	
	2	1.669	↑	0.873	↑	−0.796	
	3	−2.102	↓	−1.370	↓	0.729	
	4	0.797	↑	−0.117		−0.913	↓
	5	−0.567		−1.127		−0.556	↓
	6	1.218		1.009		−0.209	
	7	3.101	↑	1.470	↑	−1.630	↓
	8	0.816	↑	−1.806	↓	−2.617	↓
	9	1.025		1.075	↑	0.050	
CYP6BH1v1	1	−0.496	↓	−0.657	↓	−0.162	↓
	2	0.905		0.114	↑	−0.791	↓
	3	−0.183	↓	1.034	↑	1.218	↑
	4	−1.062	↓	−1.077	↓	−0.014	↓
	5	0.282	↓	−0.921		−1.204	↓
	6	1.908	↑	1.397		−0.513	
	7	0.395		0.814	↑	0.419	
	8	0.960	↑	0.891		−0.069	↓
	9	1.357	↑	1.516	↑	0.158	
CYP6BJ1	1	−0.527		−0.811		−0.283	↓
	2	−1.204	↓	−2.139	↓	−0.935	
	3	2.034	↑	0.996		−1.038	↓
	4	1.679	↑	0.128	↑	−1.552	
	5	−3.427	↓	−0.786	↓	2.641	↑
	6	−1.272	↓	−3.184	↓	−1.916	
	7	−1.535	↓	−0.573	↓	0.961	↑
	8	−0.791	↓	−0.819	↓	−0.026	↓
	9	−3.680	↓	−2.388	↓	1.302	↑
CYP9Z14v2	1	0.768	↑	1.381		0.614	↑
	2	−0.718	↓	−0.460	↓	0.258	↑
	3	−0.023	↓	−0.214	↓	−0.189	↓
	4	−0.226		0.205	↑	0.433	
	5	2.001	↑	0.241	↑	−1.761	↓
	6	−1.130	↓	1.218	↑	2.349	↑
	7	−1.868	↓	−0.211		1.657	↑
	8	0.315	↑	0.647		0.332	↑
	9	−0.619	↓	−1.044	↓	−0.427	
GST_c10663	1	−0.283	↓	−1.136	↓	−0.852	↓
	2	0.027	↓	0.240		0.213	
	3	0.341	↑	−0.385	↓	−0.725	↓
	4	−0.188	↓	0.047	↑	0.234	
	5	0.352		0.537	↑	0.187	↑
	6	−0.016		−0.392		−0.375	↓
	7	−0.056		−0.619		−0.565	↓
	8	0.614		0.908		0.295	
	9	2.364		1.865		−0.500	
GST1	1	0.325	↑	0.192	↑	−0.134	
	2	0.364	↑	0.588	↑	0.223	↑
	3	1.052		0.742	↑	−0.309	
	4	−0.580	↓	−0.396	↓	0.185	
	5	−1.123		0.654		1.777	↑
	6	0.994		−1.136	↓	−2.133	↓
	7	−1.023	↓	−1.221	↓	−0.199	↓
	8	1.379	↑	1.401		0.021	
	9	−1.168	↓	−1.221	↓	−0.053	
GST3	1	0.662		−0.578		−1.241	↓
	2	0.301		0.507	↑	0.205	↑
	3	0.760	↑	−0.718		−1.478	↓
	4	1.893		1.559	↑	−0.335	↓
	5	−1.431	↓	0.169	↑	1.601	↑
	6	0.151		−1.026	↓	−1.178	
	7	0.034		0.293		0.258	↑
	8	−1.713	↓	−1.029	↓	0.683	↑
	9	−2.023	↓	−2.308	↓	−0.279	
Ldace1	1	0.463	↑	2.470	↑	2.008	
	2	−1.262		−0.092		1.171	↑
	3	−1.053		0.139	↑	1.191	↑
	4	−2.059	↓	−0.246		1.810	↑
	5	1.867	↑	1.402	↑	−0.466	↓
	6	−0.842	↓	−0.580	↓	0.262	
	7	−0.351	↓	−0.602	↓	−0.252	↓
	8	−0.208	↓	−0.066		0.141	↑
	9	1.318	↑	1.188	↑	−0.130	↓

(*continued on next page*)

Table 1a (*continued*)

Target gene	Family ID	Control vs. Pure glyphosate		Control vs. GBHs		Pure glyphosate vs. GBHs	
		Log2Fold change		Log2Fold change		Log2Fold change	
Ldace2	1	−0.265		−1.490	↓	−1.224	↓
	2	0.532		−0.271	↓	−0.803	↓
	3	0.825		−0.209		−1.035	↓
	4	−0.531	↓	−0.317	↓	0.214	↑
	5	2.431		0.162	↑	−2.265	↓
	6	−0.400	↓	2.260		2.660	↑
	7	2.141	↑	0.992		−1.149	↓
	8	−1.194	↓	−0.669		0.525	
	9	2.352		0.879	↑	−1.474	↓

Table 1b
Expression profiles of the target genes in the Colorado potato beetle larvae that were indirectly exposed to glyphosate-based herbicides (GBHs). Upward and downward arrows indicate the significant differences (positive or negative) between the treatment groups.

Target gene	Family ID	Control vs. GBHs	
		Log2Fold change	
CYP4G29	1	−0,396	
	2	−0,407	
	3	0,559	↑
	4	−0,405	↓
	5	−0,366	↓
	6	−0,082	
	7	−0,204	
	8	−0,492	↓
	9	0,639	
CYP6BJ1	1	0,1	
	2	0,734	↑
	3	−0,837	
	4	−0,338	↓
	5	−1,02	↓
	6	−0,158	↓
	7	−0,974	↓
	8	−0,411	↓
	9	−0,697	↓
GST_c10663	1	0,124	
	2	−0,196	↓
	3	−0,889	↓
	4	0,171	↑
	5	−0,271	↓
	6	0,346	↑
	7	0,180	
	8	0,754	
	9	−0,353	
GST3	1	−0,233	
	2	0,057	
	3	−0,54	↓
	4	−0,32	↓
	5	0,502	↑
	6	−0,206	
	7	0,537	
	8	−0,302	↓
	9	−0,37	
Ldace1	1	−0,127	↓
	2	−0,264	↓
	3	−1,315	↓
	4	0,635	
	5	−1,685	↓
	6	0,055	↑
	7	0,419	↑
	8	−0,173	
	9	0,057	
Ldace2	1	−0,798	↓
	2	−0,091	
	3	−0,155	↓
	4	−0,014	↓
	5	−1,989	↓
	6	−0,554	↓
	7	−0,105	↓
	8	−2,45	↓
	9	−0,146	↓

the expressions of the GST1, GST3, and GST_c10663 genes among the treatment groups, exposed either directly or indirectly to pure glyphosate or GBH. Contrary to our study, a recent study by Vazquez et al., (2020) revealed transcriptional changes in several genes related to catabolism and oxidative metabolism in honey bees (*Apis mellifera*) chronically exposed to pure glyphosate in sub-lethal concentrations. It is possible that the sublethal concentrations used in the current experiments in our study are not sufficiently high to cause changes in the gene expression level; however, they can be still evident at the enzyme activity level (e.g. Rainio et al., 2020), which is often the first response in chemical exposure (Nikinmaa, 2014). Since the Colorado potato beetle has 27 GSTs (Schoville et al., 1931), it may be that the genes selected for this study are not the ones that are expressed by glyphosate or GBH or, alternatively, the selected genes are not active in the Colorado potato beetle larvae. Moreover, it is also possible that the relatively short exposure time (24 h in the direct experiment and 4 days in the indirect experiment) does not show any effect on gene expression profiles. Alternatively, if the response in the gene expression level is extremely fast (e.g. only few hours), the 24 h exposure in the direct experiment may be too long to show an effect. Thus, the use of several time points would be necessary to study this effect.

Further, we studied two acetylcholinesterase genes, *Ldace1 and Ldace2*. *Ldace1* has been previously shown to be inhibited by GBHs (Margus et al., 2019b) in the Colorado potato beetle larvae. However, in the present study, both genes were unaffected by direct and indirect GBH exposure or exposure to pure glyphosate. AChE has shown to be inhibited by GBHs in insects (Margus et al., 2019b), aquatic organisms (Modesto and Martinez, 2010; Ruamthum et al., 2011; Menéndez-Helman et al., 2012; Sandrini et al., 2013; de Melo Tarouco et al., 2017; Pala, 2019), and mammals (Cattani et al., 2017; Gallegos et al., 2018; Bali et al., 2019); however, only weak inhibition (Kwiatkowska et al., 2014; Larsen et al., 2016) or no inhibition has also been reported (de Aguiar et al., 2016; Jin et al., 2018; Ruuskanen et al., 2020) in different animal groups. Most of the studies have concentrated on AChE enzyme activities, but only a few studies have concentrated on the effects of GBHs on AChE gene expression or transcription profiles (Lopes et al., 2017; Margus et al., 2019b; Margus et al., 2021), thereby revealing time-dependent decrease or increase in expression levels.

We found surprisingly large family-level variation within all studied genes in both experiments (direct and indirect), thereby suggesting that certain families may be more sensitive to GBHs than others. The variation was visible in both survival- and the gene expression analyses, supporting each other. The Colorado potato beetles are known to adapt relatively fast to novel pesticides. Also, their heterogeneity is high and can be highly localized (Chen et al., 2022). Temporal and spatial sampling of the beetles from the same field population or nearby have shown that beetles can have different sensitivities even to the same insecticide (Clements et al., 2017, Crossley et al., 2017, Crossley et al., 2018), which could partly explain the high family-level variation observed in our study as well. It is also possible that the families react differently when exposed to glyphosate products due to their different pesticide history (i.e. exposure to pesticides). The variability in sensitivity to novel pesticides among or within populations appears to be due to differences in

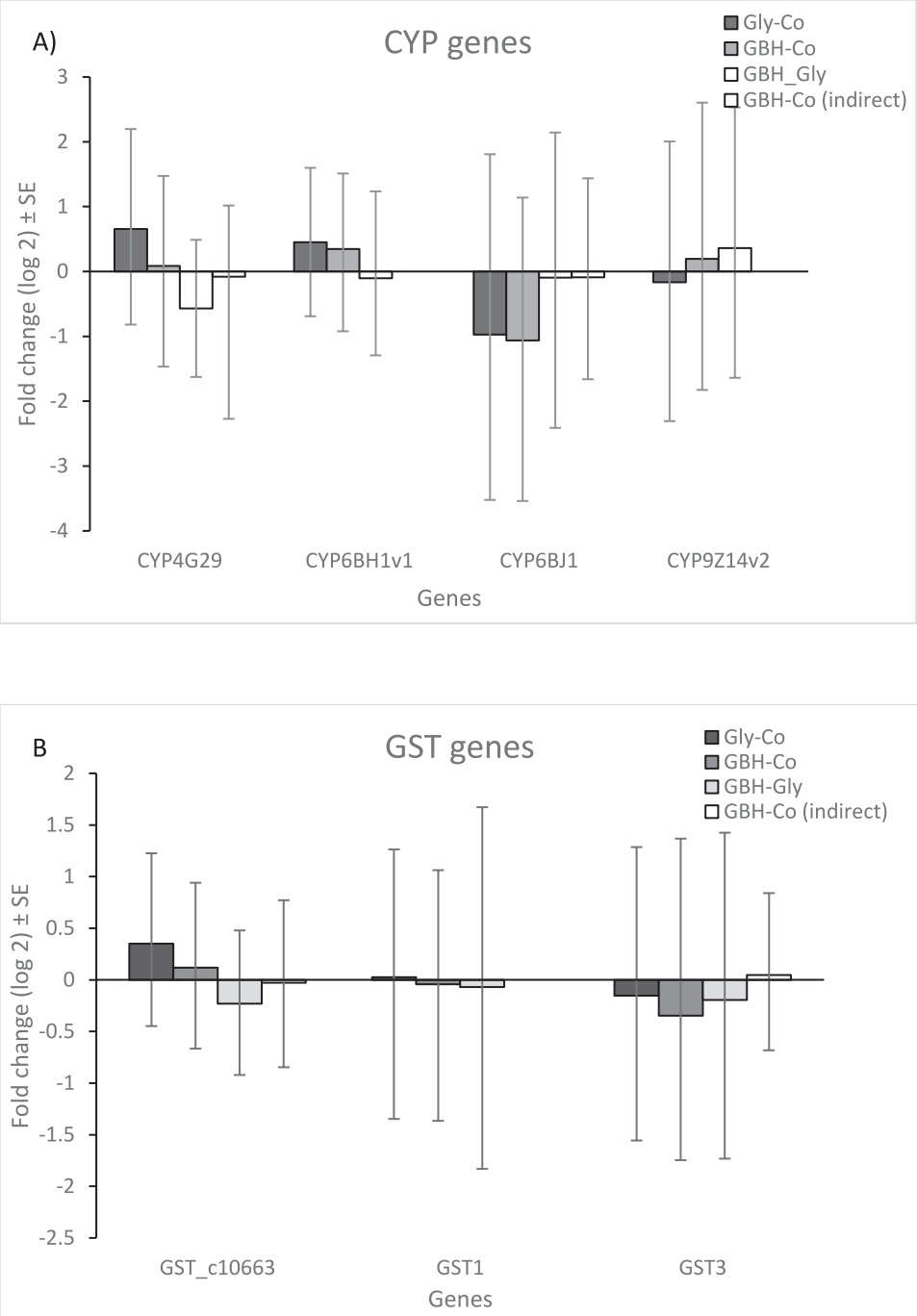

Fig. 2. (A-C). Gene expression fold change (log2 transformed ± SE) of the target A) CYP genes (CYP4G29, CYP6BH1v1, CYP6BJ1, CYP9Z14v2) B) GST genes (GST_c10663, GST1 and GST3), and C) Ldace genes (Ldace1, Ldace2) in the Colorado potato beetle larvae under either direct (Gly = pure glyphosate, GBH = glyphosate-based herbicide, Co = control) or indirect (GBH, control) exposure to herbicides (24 h). The expression levels of the study genes were normalized using the NORMA-Gene method.

the expression of detoxification mechanisms and cuticular proteins (Dively et al., 2020, Chen et al., 2022). Based on recent population genomics studies, genetic variation underlying resistance of the Colorado potato beetle is likely polygenic (Chen et al., 2022), involving a range of target genes, such as CYPs, GSTs, insecticide target sites and cuticle proteins (Crossley et al., 2017) and is repeatedly evolving. It has been suggested that environmental stressors (e.g. temperature and drought) interact with pesticides improving the stress tolerance (Gutiérrez, 2020), and this has been shown also in the Colorado potato beetle (Clements et al., 2018a, Clements et al., 2018b). Moreover, exposure to sublethal levels of stress has been shown to cause epigenetic changes that may be related to the rapid adaptation of many insect pest species (Brevik et al., 2018b). There is evidence that exposure to insecticides alters epigenetic modifications also in the Colorado potato beetles (Chen et al., 2022).

To conclude, the probability for higher mortality increased in pure glyphosate and GBH treated Colorado potato beetle larvae compared to control larvae in the direct exposure experiment, but had no effect in the indirect experiment. Neither the direct or the indirect experiment

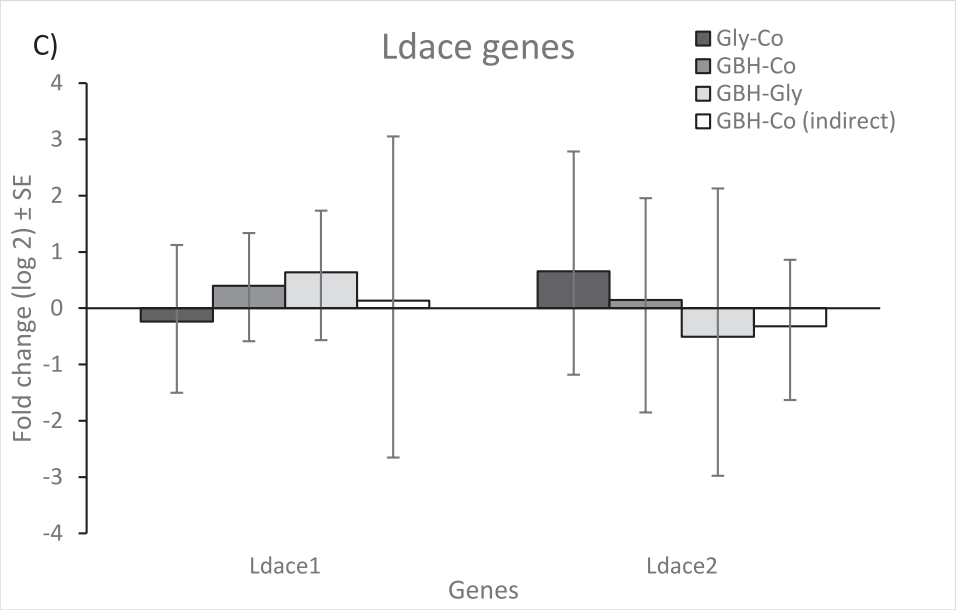

Fig. 2. (*continued*).

showed any effect on the expression of the studied CYP, GST or AChE genes; however, a large family-level variation in expression profiles was found in both the direct and the indirect experiments. The large observed variation in expression profiles of different genes along with the relatively small sample size may affect the interpretation of the results and thus the results need to be interpreted with caution and verified with larger sample size. This issue may become more obvious particularly when employing relatively short exposure time and using sublethal concentrations of herbicides, which generally may show only marginal effects on animal physiology. The use of a larger number of families and several larvae from each family could help in reducing variation, thereby enabling a more reliable interpretation of the expression profiles of the studied genes. Overall, it appears that the detoxification system of the Colorado potato beetle can handle the glyphosate compounds relatively well, which could be related to its high resistance against several insecticides (including OPs). Pesticide resistance is a growing risk—with multiple resistance to herbicides, insecticides, and antibiotics—that could potentially lead to health risks for animals and humans from exposure to the increased use of pesticides.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104503.

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