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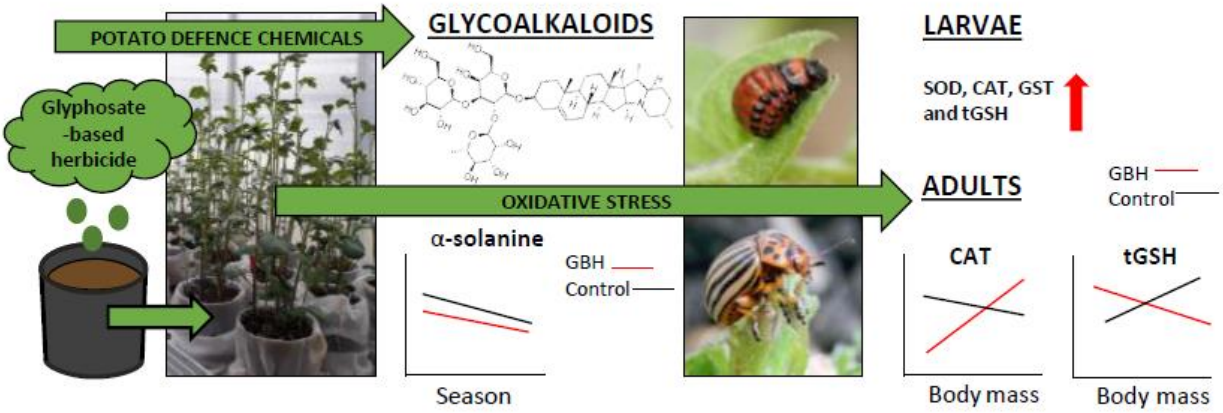

Glyphosate-based herbicide has soil-mediated effects on potato glycoalkaloids and oxidative status of a potato pest

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Abbreviations

CAT = catalase, GBH = glyphosate-based herbicide, GP = glutathione peroxidase, GR = glutathione reductase, GSH = glutathione, GSH:GSSG = reduced vs. oxidized form of glutathione, GST = glutathione-S-transferase, LHP = lipid hydroperoxides, ROS = reactive oxygen species, SOD = superoxide dismutase, tGSH = total glutathione

Highlights

The α-solanine levels were reduced in potato plants grown in GBH-treated soil.

The survival of the beetles was not affected by the soil-mediated GBH treatment.

Indirect GBH treatment modify the antioxidant defense of the Colorado potato beetle larvae.

Soil-mediated GBH treatment at larval stage may have long-term effects on the adult beetles.

Abstract

Glyphosate is the most used herbicide worldwide, targeting physiological pathways in plants. Recent studies have shown that glyphosate can also cause toxic effects in animals. We investigated the glyphosate-based herbicide (GBH)-induced changes in potato (*Solanum tuberosum*) plant chemistry and the effects of a GBH on the survival rate and oxidative status of the Colorado potato beetle (*Leptinotarsa decemlineata*). The beetles were reared on potato plants grown in pots containing soil treated with a GBH (Roundup Gold, 450 g/l) or untreated soil (water control). The 2nd instar larvae were introduced to the potato plants and then collected in 2 phases: as 4th instar larvae and as adults. The main glycoalkaloids of the potato plants, α-solanine and α-chaconine, were measured twice during the experiment. The α-solanine was reduced in potato plants grown in GBH-treated soil, which can be detrimental to plant defenses against herbivores. GBH treatment had no effect on the survival rate or body mass of the larvae or the adult beetles. In the larvae, total glutathione (tGSH) concentration and the enzyme activity of catalase (CAT), superoxide dismutase, and glutathione-S-transferase were increased in the GBH treatment group. In the adult beetles, CAT activity and tGSH levels were affected by the interactive effect of GBH treatment and the body mass. To conclude, environmentally relevant concentrations of a GBH can affect the potato plant's glycoalkaloid concentrations, but are not likely to directly affect the survival rate of the Colorado potato beetle, but instead, modify the antioxidant defense of the beetles via diet.

Keywords: Antioxidant defense, Herbivores, Insects, Potato defense chemicals, Roundup, α-solanine

1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) is the most commonly used herbicide worldwide, given its effectiveness and broad spectrum ability to kill weeds (Myers et al., 2016; Woodburn, 2000). It has been proclaimed to be safe for the environment due to its low accumulation rate and rapid inactivation in soils (Giesy et al., 2000, Vereecken, 2005). However, accumulating evidence has demonstrated that glyphosate and its degradation metabolites (e.g., aminomethylphosphonic acid, AMPA) can remain in the soil for years and affect non-target organisms (Helander et al., 2018; Larsen et al., 2012). Furthermore, non-target organisms may be directly exposed to glyphosate products by the unwanted loss of substance during transportation, handling, and storage, and by wind action during field application (Torretta et al., 2018). Glyphosate exposure may also occur when it is used to synchronize and accelerate the ripening of forage cereals (Helander et al., 2012). Glyphosate use is intended to tackle weeds, but recent toxicological studies have shown harmful effects of glyphosate products in animals, such as changes in cell function, tissues, physiology, and survival rate of the animals (Claus et al., 2016; Margus et al., 2019; Mesnage et al., 2015).

Glyphosate is also the most important herbicide directly affecting the synthesis of secondary compounds in plants (Duke and Powles, 2008). The glyphosate-based reduction of secondary compounds in plants (i.e., defense chemicals) may expose plants to herbivore attacks; influence the flavor-producing chemicals important in herbivore behavior or food quality (El-keltawi and Croteau, 1987); and reduce plant resistance to pathogens and fungal infections (Lydon and Duke, 1989). On the other hand, glyphosate may also increase the production of plant secondary metabolites (Ossipov et al. 2003). Overall, the sub-lethal effects of herbicides on non-target plants may affect agricultural ecosystems by altering the synthesis of compounds that are important in inter- and intraspecific interactions (Lydon and Duke, 1989). Plant-herbivore interactions are

central to both food production and biological diversity, affecting the dynamics of various ecosystems (Blumenthal and Augustine, 2009).

Glyphosate is the only herbicide affecting the inactivation of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Duke and Powles, 2008; Steinrücken and Amrhein, 1980). This enzyme belongs to the shikimate metabolic pathway, which appears in plants and in some bacteria and fungi (Bentley, 1990; Haslam, 1993; Helander et al., 2018). Glyphosate blocks phosphoenolpyruvate (PEP) binding sites, thus inhibiting the reaction between shikimate 3-phosphate (S3P) and PEP (Funke et al., 2006). An inactivation of EPSPS leads to the accumulation of high levels of shikimate in plant tissues (Amrhein et al., 1980; Lydon and Duke, 1989), preventing the biosynthesis of essential aromatic amino acids (e.g., phenylalanine, tyrosine, and tryptophan) necessary in protein synthesis (Duke and Powles, 2008) and as precursors for several secondary metabolites important in plant growth (Tzin and Galili, 2010). This can result in shortages of carbon for other essential pathways (Siehl, 1997) and reduce (Kishore and Shah, 1988; Martinez et al., 2018; Shilo et al., 2016; Sihtmäe et al., 2013) or increase (Ossipov et al., 2003) secondary metabolites in some species of plants and microbes. For example, while blocking the production of arogenic acid, glyphosate may direct the conversion of secondary metabolites into hydrolysable tannins via 3-dehydroshikimic acid, which have been shown to accumulate under glyphosate treatment (Ossipov et al., 2003). Glyphosate is also a strong chelating agent that creates the complexes that immobilize the mineral micronutrients of soil, making them unavailable to plants (Glass, 1984).

Both glyphosate and plant defense chemicals are known to impair the antioxidant defense system and increase the production of reactive oxygen species (ROS) in plants (Adamski et al., 2014; Chowański et al., 2016; Gomes et al., 2016; Liu et al., 2010, Radman and Fayez, 2016) and animals

(Annett et al., 2014; Hultberg, 2007; Modesto and Martinez, 2010; Uren Webster and Santos, 2015), which can, in turn, cause cellular biochemical stress, called oxidative stress, and consequent oxidative damage to biomolecules (George and Gatehouse, 2013; Halliwell and Gutteridge, 2007). Previous studies in animals have shown increased oxidative stress or alteration in antioxidant defense systems in relation to various glyphosate-based herbicides (thereafter GBHs; Contardo-Jara et al., 2009; El-Shenawy, 2009; Glusczak et al., 2007; Modesto and Martinez, 2010; Rainio et al., 2019; Uren Webster and Santos, 2015). Also, the breakdown products of glutathione (e.g. γ-glutamylglutamine and cysteinylglycine), involved in the regulation of redox balance, have been shown to increase in rats exposed to GBH (Mesnage et al. 2019). Moreover, GBHs have been shown to affect the survival rate, development, and reproduction of invertebrates found in agroecosystems (Benamú et al., 2010; Castilla et al., 2010; Evans et al., 2010; Saska et al., 2016; Schneider et al., 2009), though there are also studies reporting little or no effects (Margus et al., 2019; Salvio et al., 2016; Thompson et al., 2014). The impacts of GBHs on plants and non-target organisms may differ substantially depending on the use of commercial formulations that differ in their surfactant and salts, which are added to enhance the effectiveness of glyphosate. Some adjuvants used in GBHs may be even more toxic than the glyphosate itself (Mesnage et al., 2014). Previous studies have shown that the consequences of GBH use in target ecosystems and their surrounding areas are relatively poorly known and require further studies from a multidisciplinary approach.

The increasing evidence of glyphosate toxicity on non-target organisms has caused growing concern about the use of glyphosate as the primary weed management strategy (Helander et al., 2012; Torretta et al., 2018; Van Bruggen et al., 2018). The environmental risks of glyphosate are likely to be pronounced in northern ecosystems, which are characterized by long biologically inactive winters and short growing seasons, limiting the time period of peak glyphosate degradation

activity to the summer months (Laitinen, 2009; Helander et al., 2012; Helander et al., 2018; Silva et al., 2018). On the other hand, plant-protective agents are required for effective crop production, thus it is important to find safe and sustainable ways to protect plants in the future.

In this study, we investigated the soil-mediated effects of a GBH on the glyphosate-induced changes in plant chemistry, and the survival rate and oxidative status of a non-target herbivore, by using potato plant (*Solanum tuberosum*) and the Colorado potato beetle (*Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae) as a model system. The Colorado potato beetle is an economically important potato pest worldwide (Casagrande, 1987; Grapputo et al., 2005; Walsh, 1865;), including in Finland, where it is classified as a quarantine pest species (Vänninen et al., 2011). Potato plants and the Colorado potato beetle form an excellent study system, since glyphosate is known to affect herbivores not only directly, but also via potato plant defense chemicals. At the larval stage, the beetles can be exposed to glyphosate residues or glyphosate metabolites via diet or due to possible changes in potato plant quality; whereas, at the pupal stage, the beetles may be exposed to GBH residues also via the soil.

Potato plants are characterized by the presence of steroidal glycoalkaloids, such as α-solanine and α-chaconine (Lachman et al., 2001; Matthews et al., 2005), which are biosynthetically derived from cholesterol (Chowański et al., 2016). These glycoalkaloids are produced in all parts of the plant, having the highest concentrations in the leaves, flowers, and unripe fruits (Adamski et al., 2014; Friedman, 2006). Glycoalkaloids have insecticidal and fungicidal properties, and are often synthesized when plants are under stress, such as when they have been injured by herbivores (Chowański et al., 2016). They disrupt the cellular functions of herbivores, increase the generation of ROS (Chowański et al., 2016), act as acetylcholinesterase inhibitors (Friedman et al., 1997), and also elicit behavioral responses by insects (Lyytinen et al., 2007; Nylin and Janz, 1993). Potato

plant glycoalkaloids have been previously shown to reduce the growth rate and food consumption rate in the khapra beetle (*Trogoderma granarium*; Nenaah, 2011), decrease reproduction rates in the potato aphid (*Macrosiphum euphorbiae*; Güntner et al., 1997); decrease fertility, survival rate, and hatchability in the greater wax moth (*Galleria mellonella*; Adamski et al., 2014); and increase mortality in peach potato aphids (*Myzus persicae*; Fragoyiannis et al., 1998). On the other hand, it is possible that under a certain threshold level of foliage glycoalkaloids, the herbivores may still feed and reproduce (Khan et al., 2013). Colorado potato beetle larvae have shown either negative (Hare, 1987) or no response (Kowalski et al., 1999) in relation to glycoalkaloids, suggesting that the effects of glycoalkaloids may vary with the life-stage of the beetle or the length of exposure (Lyytinen et al., 2007).

To examine the soil-mediated effects of the GBH on the oxidative status of the beetles, we measured antioxidant glutathione (total glutathione, tGSH) and the ratio of its reduced and oxidized form (GSH:GSSG). Glutathione (GSH) is one of the most important small antioxidant molecules in almost all organisms (Andrews, 2000) and the GSH:GSSG ratio, which indicates the overall redox status of cells, is commonly used as an indicator of oxidative stress (Halliwell and Gutteridge, 2007; Isaksson et al., 2005; Rainio et al., 2013). In addition, we measured the activity of insect homologs' antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR), as well as glutathione-S-transferases (GSTs) related to GSH metabolism (Halliwell and Gutteridge, 2007). GSTs are a ubiquitous and important family of enzymes (isozymes) participating in detoxification processes by catalyzing the conjugation of GSHs on xenobiotics (Alghamdi and Frey, 2017; Halliwell and Gutteridge, 2007) and showing the peroxidative activity function in insects (Corona and Robinson, 2006; Farjan et al., 2012). ROS regulation enzymes, superoxide dismutase (SOD) and catalase (CAT), were measured to study first-line antioxidant defense (Fridovich, 1974), where superoxides are transformed to hydrogen peroxide (H_2O_2) by SOD and further catalyzed to water

(H_2O) and molecular oxygen by CAT (Finkel and Holbrook, 2000; Pinto et al., 2003). To determine oxidative damage, we measured lipid hydroperoxides (LHP), which have been suggested to increase with ROS production. Lipid peroxidation can be harmful in insects, because, in addition to being essential components in cell membranes, they also have unique physiological functions (e.g., in developmental and reproductive physiology; Downer, 1985).

We hypothesize the following: **1)** Environmentally relevant levels of a GBH in the soil may cause quantitative effects in the production of glycoalkaloids, since GBHs affect the aromatic amino acid L-tryptophan (Santos-Sánchez et al., 2019), which is a precursor of alkaloids in secondary metabolism (Dewick, 2009). If the GBH affects plant defense chemicals, it may change the plant quality and resource allocation for growth and defense and change plant-herbivore interactions by making the potato plants more (or less) sensitive to herbivore attacks. **2)** The GBH may reduce the survival rate and body mass of the beetle larvae and adult beetles, and increase the developmental time of the adult beetles in cases where the GBH is absorbed into the potato plant via the soil**. 3)** The GBH may further show negative soil-mediated effects during the pupal stage of the beetles, which may reflect the adult's survival rate as well. **4)** The GBH may affect the antioxidant defense system of the beetles by changing the antioxidant enzyme activity or GSH concentrations, either via diet or soil-mediated effects during the pupal stage of the beetles.

2. Materials and methods

2.1. Study design

The GBH (Roundup Gold, Monsanto, USA) treatment was conducted in summer 2016 in a licensed quarantine greenhouse in the Botanical Garden of the University of Turku (60° 26' N, 22°10' E).

We preferred to use the commercial formulation of glyphosate rather than pure glyphosate, since those are more relevant in the agricultural context. To study the soil-mediated effects of the GBH on the Colorado potato beetles in the greenhouse experiment, we used soil that had been pre-treated with the GBH. The soil was collected from a long-term field experiment established in 2013 at the Botanical Garden (see more details in Hagner et al., 2019). The experimental soil was treated with a permitted dose of Roundup Gold (450 g/l isopropylamine glyphosate salt, CAS: 38641-94-0, application rate: 6.4 l/ha) that was applied twice per year (specifically, May 2014, 2015, and 2016; and October 2014 and 2015). The control soil received the same amount of tap water as the treated soil. The soil type in the field was medium clay with a high organic matter content (>120 g kg^{-1}) and pH 5.9. In June 2016, the soil for the greenhouse experiment was collected from the field experiment 2 weeks after a GBH treatment and divided into 100 pots (Ø 19 cm; 50 controls, 50 GBH-treated). The organic variety 'Ditta' potatoes were planted in the pots with the GBH-treated and control soils, and the pots were then fully randomized in the greenhouse. The position of the pots was further changed during the growing period to prevent the uneven growth of the potato plants. The plants were grown in ambient June-July day-lengths in southwest Finland (about 17-19 h day length) under a 20°C/15°C day/night temperature.

We used the United States (Vermont) Colorado potato beetle population collected from the field (44°43´N, 73°20`W) in 2010, which had been since grown in laboratory conditions (see Lehmann et al., 2015). Altogether, 500 Colorado potato beetle larvae (250 larvae/treatment group, 30 larvae/family) from 16 families (full-sib design) were used in this experiment. After 3.5 weeks of the potato planting, small 2-day-old larvae (2^{st} instar) were randomly introduced to the potato plants (5 larvae to each plant), which were covered by light-permeable fabric bags. After 9 days, when the larvae were at their 4^{th} instar, 184 larvae (94 controls, 90 GBH-treated) were collected, weighed, and stored in a freezer at -80°C for oxidative status analyses. The remaining larvae were grown

until they dropped from the plant and burrowed into the soil to pupate. Once all larvae had burrowed into the soil, the potato plant shoots were cut and removed. Emerged adult beetles (133 controls, 134 GBH-treated) were collected every day, weighed, sexed, and used for oxidative status analyses to study the possible soil-mediated or carry-over effects of the GBH. To analyze potato plant glycoalkaloids, α-solanine and α-chaconine, we took ca 5 leaves per potato plant a) before placing the larvae on the plants (1st measurement) and b) when the larvae had pupated and the shoots had been cut down (2nd measurement). Leaves were freeze-dried, ground (TissueLyser, Qiagen, Austin, TX, USA), and stored in a freezer at -20°C until the chemical analyses. The licenses for rearing quarantine pest species in laboratory conditions were given by the Finnish Food Authority, Finland (Ruokavirasto, permission 4057/0614/2016). Licenses for conducting experiments with insects are not necessary in Finland.

2.2. Determination of potato plant defense chemicals

For the quantitation of potato plant glycoalkaloids, α-chaconine and α-solanine, 5 mg of ground potato plant leaf material was weighed in a 2 ml Eppendorf tube. Samples were extracted with 2 ml of 5% aqueous acetic acid (5:95, v/v) utilizing overnight maceration in a cold room (4°C) and were shaken with a planar shaker (280 min^{-1}) for 3 hours at room temperature. Extracts were centrifuged (14,000 min^{-1}) for 10 min and decanted into new 2 ml Eppendorf tubes. 100 × dilutions were made with the extraction solvent and samples were filtered via polytetrafluoroethylene filters (13 mm i.d.; 0.2 µm) and analyzed with a UHPLC-DAD-ESI-Orbitrap-MS instrument. One of the potato plant leaf extracts was chosen as the quality control sample. It was analyzed before and after every 10 samples to monitor the changes in the performance of the mass spectrometer. The ultrahigh performance liquid chromatograph was coupled to a photodiode array detector (UHPLC-DAD, Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q

Exactive, Thermo Fisher Scientific, Bremen, Germany). ACQUITY UPLC BEH Phenyl (100*2.1 mm i.d., 1.7 µm, Waters Technologies Ireland, Wexford, Ireland) columns were utilized. The mobile phase consisted of acetonitrile (A) and 0.1% aqueous formic acid (99.9:0.1, v/v) (B): 0-0.5 min, 0.1% A in B; 0.5-6 min, 0.1-30% A in B; and 6-10.5 min, column wash and stabilization. The heated electrospray ionization (ESI) source (H-ESI, Thermo Fisher Scientific, Bremen, Germany) was operated in the positive ion mode. Source parameters were as follows: spray voltage, +3.8 kV; sheath gas (N_2) flow rate, 60 (arbitrary units); auxiliary gas (N_2) flow rate, 20 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); capillary temperature, 380°C. The Orbitrap spectrometer was operated with a resolution of 35,000 and a mass range of *m/z* 150-2250. Data processing was done using Thermo Xcalibur Quan Browser software (Version 4.1.31.9, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations of α-chaconine and α-solanine in samples were quantified using external calibration curves made from the commercial standards of both α-chaconine and α-solanine (Carbosynth, Compton, UK).

2.3. Oxidative status analyses

Beetle homogenates (larvae and adults) were used to measure oxidative status biomarkers (GST, GPx, GR, CAT, SOD, tGSH, and GSH:GSSG) and oxidative damage (LHP) of the beetles. All antioxidant and enzyme activities was measured in triplicate (intra-assay coefficient of variability [CV] < 15% in all cases) using 96- (CAT and LHP) or 384-well (GPx, GR, GST, SOD, tGSH, and GSH:GSSG) microplates, which in most cases required reducing the reagent volumes as per the kit instructions. All analyses were measured with an EnVision® microplate reader (PerkinElmer Finland, Turku, Finland). There were 3 control samples used with each plate to be able to correct inter-assay precision with the ratio specific to the particular plate (range 0.8-1.2).

Samples were homogenized individually (TissueLyser, Qiagen, Austin, TX, USA) with 180 µl (larvae) or 150 µl (adults) KF buffer (0.1 M K_2HPO_4 + 0.15 M KCl, pH 7.4). The protein concentration (mg/ml) was measured with bicinchoninic acid (BCA) protein assay (Smith et al., 1985) using bovine serum albumin (BSA) as a standard (Sigma-Aldrich Finland, Espoo, Finland) with an EnVision® microplate reader at an absorbance of 570 nm.

GST assay (Sigma-Aldrich CS0410) was adjusted from a 96- to 384-well plate. We used 2 µl of each sample in triplicate and our own reagents: Dulbecco's phosphate-buffered saline (DPBS), 200 mM GSH (Sigma G4251), and 100 mM 1-Chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich C6396) in ethanol. The change in absorbance was measured at 340 nm. GPx assay (Sigma-Aldrich CGP1) was adjusted from a cuvette to a 384-well plate and the activity was measured according to kit instructions, using 2 mM H_2O_2 instead of t-Bu-OOH as a substrate (see details in Rainio et al., 2019). The change in absorbance was measured at 340 nm. GR-assay (Sigma-Aldrich GR-SA) was adjusted from a cuvette to a 384-well plate and modified from the kit instructions by using our own reagents: assay buffer (100 mM potassiumphosphate buffer + 1 mM EDTA, pH 7.5), 2 mM GSSG (Sigma-Aldrich GG4626), 3 mM DTNB (Sigma-Aldrich D8130), and 2 mM NADPH (Sigma-Aldrich N1630). The change in absorbance was measured at 412 nm. SOD assay (Sigma-Aldrich 19160) was adjusted from 96- to 384-well plate and measured according to kit instructions. We used 0.3 mg/ml sample dilution and the activity was expressed as inhibition % at an absorbance of 450 nm. CAT-assay (Sigma-Aldrich CAT100) was adjusted from a cuvette to a 96-well plate. We used 0.6 mg/ml sample dilution and tested each sample in triplicate. We made our own reagents: 10 × CAT assay buffer (500 mM KF, pH 7.0), CAT dilution buffer (50 mM KF + 0.1% TritonX, pH 7.0), chromogen reagent (0.25 mM 4-aminoantipyrene + 2 mM 3,5-dicloro-2-hydroxybenzenesulfonic acid in 150 mM potassium phosphate buffer, pH 7.0), peroxidase solutions (from horseradish), stop solution (15 mM NaN_3, Sigma-Aldrich), and 200 mM and 10 mM H_2O_2

according to information provided in the technical bulletin (see also Deisseroth and Dounce, 1970; Fossati et al., 1980). The change in absorbance was measured at 520 nm. Total GSH and the ratio of GSH:GSSG were measured with a ThioStar® Glutathione Fluorescent Detection Kit (K005-FI, Arbor Assays, Ann Arbor, MI, USA) according to kit instructions, and the fluorescence was measured at an excitation/emission wavelength of 405/510 nm. Prior to analyses, the sample homogenate was deproteinized with 5% sulfosalicylic acid (SSA), incubated on ice for 10 min, and centrifuged for 10 min at 10,000 g in 4°C.

For the LHP measurement, the larvae were first weighed and then homogenized with 125 µl methanol. LHP were measured using the FOX-II method, modified from Nourooz-Zadeh et al. (1995) and Bou et al. (2008). We used 45 µl of the sample, 5 µl 10 mM thiamine pyrophosphate (TPP) or methanol, and 950 µl of FOX reagent (see also Vuori et al., 2015). Cumene hydroperoxide (0/8/16/32/64/96/128/160 mM, Sigma-Aldrich, USA) was used as a standard (see more details in Rainio et al. 2019). The absorbance was measured at 570 nm. The results were set against the weight of the body mass of the beetles.

2.4. Statistics

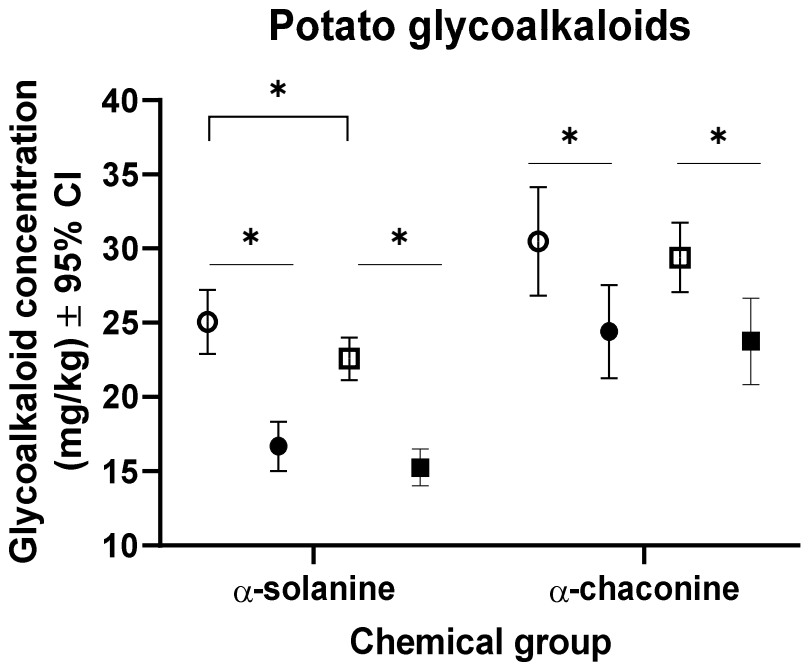
All statistical analyses were performed with SAS statistical software 9.4 (SAS, 2013) and the figures were prepared with GraphPad Prism 8.4.2. software (GraphPad Prism, 2020). Differences in potato plant glycoalkaloids (α-solanine and α-chaconine) between the treatment groups (GBH-treated and control) were analyzed with repeated generalized linear models (GLMs; Gaussian distribution and identity link function, Glimmix procedure in SAS). Degrees of freedom were calculated with the Kenward-Roger method. The Pearson correlation coefficient was used to test the correlations between potato plant defense chemicals.

The survival rate of the beetles between the developmental stage (larvae, adults) and treatment groups (GBH-treated, control) and their interaction was analyzed with a generalized linear mixed model (GLMM; with binary distribution and logit link function, events/trials syntax in GLIMMIX procedure, SAS). Family was used as a random factor to control for the non-independence of larvae used from the same family. Degrees of freedom were calculated with the Kenward-Roger method.

The developmental time of the adult beetles was calculated from hatching of the larvae to newly emerged adult beetles, and the differences in developmental time between the treatment groups was analyzed with a GLMM (Gaussian distribution and identity link function), using treatment (GBH-treated, control), sex (female, male), and treatment × sex interaction as explanatory variables. Family was used as a random factor. The effect of GBH treatment on body mass (larvae and adults, female and males) was analyzed with a GLMM (Gaussian distribution and identity link function) using family as a random factor.

To examine the effects of GBH treatment on the oxidative status of the beetles, we performed a GLMM (with lognormal distribution and identity link function, except for CAT and tGSH [for larvae only], in which we used Gaussian distribution and identity link function) for each parameter, separately for larvae and the adult beetles, using treatment (GBH treatment, control), body mass, treatment × body mass, sex (female, male, adults only), and treatment × sex (adults only) as explanatory variables. Family was used as a random factor in the models when applicable (larvae: GST, GR, SOD, tGSH, LHP; adults: GP, CAT, tGSH). Non-significant terms were dropped sequentially from the final model, but the main effect of treatment was always kept in the model, as this was our main study question. Degrees of freedom were calculated as mentioned above. Prior to GLMMs, the normality of each parameter was checked. If the parameter was not normally

376 distributed, lognormal distribution was used in the models. The Spearman correlation coefficient
377 was used to test the correlations between oxidative status parameters, body mass, and potato plant
378 glycoalkaloids for larvae and adult beetles, separately in both treatment groups.
379
380 **3. Results**
381
382 *3.1. Potato plant defense chemicals*
383
384 The α-solanine levels were significantly reduced in the potato plants grown in the GBH-treated soil
385 ($F_{df}=6.05_{1,\ 98}$, p=0.016), and the concentrations differed between the measurement times
386 ($F_{df}=98.08_{1,\ 98}$, p= <0.001, Fig. 1), being clearly lower at the second measurement. The treatment ×
387 measurement time interaction was not significant ($F_{df}=0.44_{1,\ 97}$, p=0.509). The α-chaconine levels
388 did not differ between the treatment groups ($F_{df}=0.36_{1,\ 98}$, p=0.552, Fig. 1), but the concentrations
389 differed between the measurement time ($F_{df}=16.17_{1,\ 98}$, p=0.0001, Fig. 1), being likewise lower at
390 the second measurement. There was no significant treatment × measurement time interaction
391 ($F_{df}=0.02_{1,\ 97}$, p=0.880). The defense chemicals also correlated with each other. The first
392 measurement of α-solanine correlated positively with the second measurement of α-solanine
393 (r_P^2=0.64, p = <0.001) and with the first measurement of α-chaconine (r_P^2=0.30, p=0.036); whereas,
394 the second measurement of α-solanine correlated positively with the first (r_P^2=0.42, p=0.002) and
395 second measurement (r_P^2=0.74, p= <0.001) of α-chaconine. The first measurement of α-chaconine
396 further correlated positively with the second measurement of α-chaconine (r_P^2=0.61, p= <0.001).
397



398

399 **Figure 1.** Potato glycoalkaloid (α-solanine and α-chaconine) concentrations (mean ± 95% CI)
400 between the treatment groups (GBH treatment, control) at two measurement points (measurement 1,
401 measurement 2). The color of the symbols indicates measurement time (white=measurement 1,
402 black=measurement 2) and different symbols the treatment groups (circle=control, square=GBH).
403 The star above the bars indicate the significant difference between the treatment groups
404 (generalized linear mixed model, $p<0.05$).

405

406 *3.2. Survival rate and changes in developmental time*

407

408 GBH treatment had no effect on the survival rate of the Colorado potato beetle larvae or the adult
409 beetles (Fig. 2). The survival rate of the larvae and the adult beetles differed significantly from each
410 other, but there was no treatment × age interaction (Table 1). Larval survival rate in the GBH and
411 control groups was 98.9% and 97.9%, respectively; whereas, adult survival was 83.9% and 83.4%,
412 respectively (Table 1). The body mass of the larvae or the adult beetles was not affected by GBH
413 treatment (larvae: $F_{df}=0.58_{1,\ 166.2}$, $p=0.447$; adults: $F_{df}=0.01_{1,\ 254.5}$, $p=0.929$). In the adult beetles,

414 neither the body mass of the females ($F_{df}=0.61_{1, 129.6}$, p=0.434) nor males ($F_{df}=0.27_{1, 111.4}$, p=0.606)
415 differed between the treatment groups. However, the developmental time of the adult beetles was
416 significantly increased in the GBH-treated group compared to the control group (Table 1). Yet, the
417 estimated difference was only 0.56 days (marginal means: GBH-treated: 30.22, SE: 0.268; control:
418 29.66, SE: 0.268). Developmental time was not affected by sex or sex × treatment interaction
419 (Table 1).

420

421 **Figure 2.** Survival of the Colorado potato beetle (*L. decemlineata*) larvae (2^{nd} instar to 4^{th} instar)
422 and adults (2^{nd} instar to adult) between the treatment groups (control=black circle, GBH
423 treatment=black square). The bars represent mean survival (± 95% Cl) between the treatment
424 groups.

Table 1. The relationship between glyphosate-based herbicide (GBH) treatment and age (larvae and adults) on survival rate of the Colorado potato beetle (*L. decemlineata*). Significant results are indicated in bold.

	Survival	
Model*	**F_{df}**	**p**
Treatment	$0.07_{1, 502}$	0.797
Age	**$16.93_{1, 502}$**	**<0.001**
Treatment × age	$0.24_{1, 501}$	0.623
	Developmental time	
Model**	**F_{df}**	**p**
Treatment	**$6.26_{1, 253.2}$**	**0.013**
Sex	$1.77_{1, 255.1}$	0.185
Treatment × sex	$0.19_{1, 252.9}$	0.667

* Generalized linear mixed model (GLMM) with binary distribution and logit link function, family used as a random factor in the model.
** GLMM with Gaussian distribution and identity link function, family used as a random factor in the model.

3.3. Oxidative status

Oxidative status parameters (GR and GPx homologs, GST, tGSH, GSH:GSSG, CAT, SOD and LHP) were analyzed separately between the developmental stages (larvae, adults, Table A1). Oxidative status parameters of the larvae were associated with GBH treatment and body mass, but the body mass × treatment interaction was not associated with any of the oxidative status parameters (Table 2). In the larvae, tGSH concentration and the activity of GST, CAT, and SOD were up-regulated in the GBH treatment group compared to the control group (Table 2, Fig 3.). The other oxidative status parameters (GPx, GR, GSH:GSSG, and LHP) were not associated with GBH treatment. In addition, GST activity was negatively associated with larval body mass, while tGSH concentrations had a positive association with body mass (Table 2). No association between body mass and other oxidative status parameters were found.

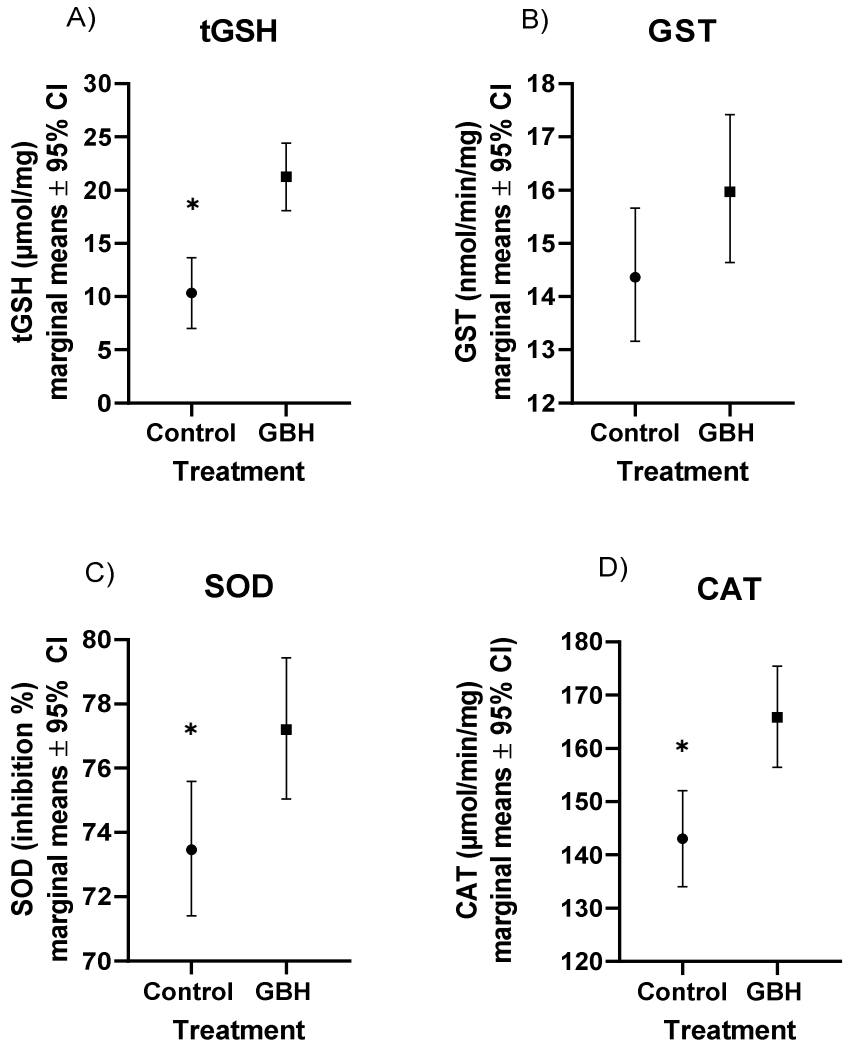
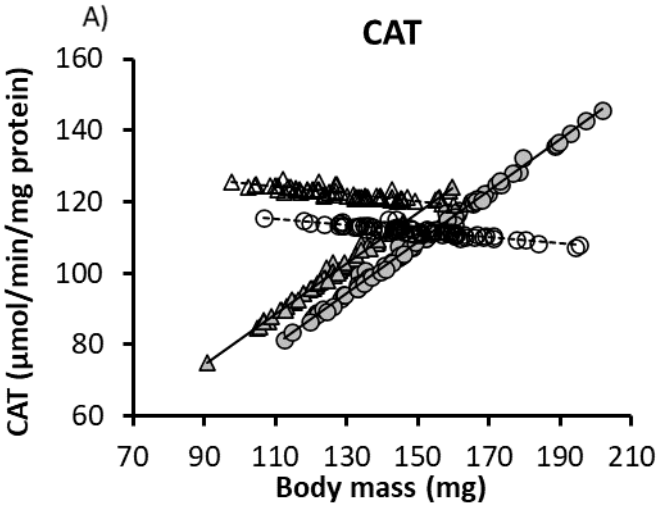


Figure 3. Variation in A) total glutathione (tGSH) concentration, B) glutathione-S-transferase (GST), C) superoxide dismutase (SOD), and D) catalase (CAT) activity in larvae of the Colorado potato beetle (*L. decemlineata*) between treatment groups (control=black circle, GBH treatment=black square). The bars represent the marginal means from the models (± 95% CI). The star above the bars indicate significant difference between the treatment groups (generalized linear mixed model, $p<0.05$).

Table 2. The effects of glyphosate treatment (GBH, control), body mass (bm), sex (female, male), body mass × treatment, and sex × treatment interactions on oxidative status parameters glutathione-S-transferase (GST), glutathione peroxidase (GPx). glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG) and lipid hydroperoxides (LHP) in larvae and adult Colorado potato beetles (*L. decemlineata*). Non-significant terms were dropped sequentially from each model, starting from interactions (generalized linear mixed model with lognormal distribution and identity link function). Significant results are shown in bold.

Parameters	**Model**	**Larvae**			**Adults**		
		F_{df}	**p**	**n**	**F_{df}**	**p**	**n**
GST	treatment	***3.88*** $_{1,\ 49.97}$	***0.054***	68	0.31 $_{1,\ 60}$	0.578	64
	bm	**33.99** $_{1,\ 46.49}$ **est. -0.007 SE 0.001**	**<0.001**		**4.59** $_{1,\ 60}$ **est. -0.005, SE 0.002**	**0.036**	
	bm*treatment	0.72 $_{1,\ 61.41}$	0.399		1.60 $_{1,\ 59}$	0.211	
	sex	-	-		1.08 $_{1,\ 60}$	0.303	
	sex*treatment	-	-		0.00 $_{1,\ 58}$	0.979	
GPx	treatment	0.44 $_{1,\ 65}$	0.511	68	0.39 $_{1,\ 43.98}$	0.536	61
	bm	0.75 $_{1,\ 65}$	0.389		***3.48*** $_{1,\ 47.6}$	***0.068***	
	bm*treatment	1.02 $_{1,\ 64}$	0.316		0.19 $_{1,\ 48.8}$	0.669	
	sex	-	-		1.14 $_{1,\ 55.17}$	0.289	
	sex*treatment	-	-		0.30 $_{1,\ 43.35}$	0.588	
GR	treatment	0.05 $_{1,\ 47.76}$	0.823	66	***3.39*** $_{1,\ 59}$	***0.071***	64
	bm	0.55 $_{1,\ 58.7}$	0.460		**6.77** $_{1,\ 59}$ **est. 0.003, SE 0.004**	**0.012**	
	bm*treatment	0.47 $_{1,\ 55.41}$	0.495		**3.33** $_{1,\ 59}$	**0.073**	
	sex	-	-		1.76 $_{1,\ 59}$	0.189	
	sex*treatment	-	-		0.04 $_{1,\ 58}$	0.842	
CAT	treatment	**11.48** $_{1,\ 63}$	**0.001**	65	**5.57** $_{1,\ 50.62}$	**0.022**	64
	bm	2.21 $_{1,\ 62}$	0.142		1.65 $_{1,\ 48.13}$	0.206	
	bm*treatment	1.92 $_{1,\ 61}$	0.171		**4.61** $_{1,\ 50.81}$	**0.037**	
	sex	-	-		0.81 $_{1,\ 56.95}$	0.373	
	sex*treatment	-	-		1.11 $_{1,\ 47.53}$	0.297	
SOD	treatment	**7.79** $_{1,\ 50}$	**0.007**	68	3.16 $_{1,\ 62}$	0.080	64
	bm	0.03 $_{1,\ 46.77}$	0.862		1.57 $_{1,\ 61}$	0.215	
	bm*treatment	1.80 $_{1,\ 60.44}$	0.184		0.28 $_{1,\ 58}$	0.599	
	sex	-	-		0.00 $_{1,\ 60}$	0.999	
	sex*treatment	-	-		0.43 $_{1,\ 59}$	0.512	
tGSH	treatment	**42.10** $_{1,\ 32.51}$	**<.001**	43	**9.22** $_{1,\ 44.43}$	**0.004**	56
	bm	**5.10** $_{1,\ 37.36}$ **est. 0.089, SE 0.039**	**0.030**		0.11 $_{1,\ 42.31}$	0.736	
	bm*treatment	1.65 $_{1,\ 38.53}$	0.206		**10.04** $_{1,\ 44.9}$	**0.003**	
	sex	-	-		0.85 $_{1,\ 48.42}$	0.362	
	sex*treatment	-	-		2.68 $_{1,\ 39.84}$	0.110	
GSH:GSSG	treatment	1.14 $_{1,\ 41}$	0.291	43	0.11 $_{1,\ 51}$	0.743	54
	bm	0.15 $_{1,\ 40}$	0.704		0.10 $_{1,\ 50}$	0.756	
	bm*treatment	0.22 $_{1,\ 39}$	0.642		0.38 $_{1,\ 49}$	0.543	
	sex	-	-		0.71 $_{1,\ 51}$	0.402	
	sex*treatment	-	-		0.00 $_{1,\ 48}$	0.991	
LHP	treatment	1.40 $_{1,\ 15.11}$	0.255	33	0.01 $_{1,\ 53}$	0.908	57
	bm	0.26 $_{1,\ 27.94}$	0.613		2.48 $_{1,\ 53}$	0.122	
	bm*treatment	0.78 $_{1,\ 18.12}$	0.390		0.15 $_{1,\ 52}$	0.700	
	sex	-	-		0.57 $_{1,\ 53}$	0.452	
	sex*treatment	-	-		0.50 $_{1,\ 51}$	0.484	

In adult beetles, tGSH concentration and CAT activity had a significant association with treatment × body mass interaction (Table 2), and a similar tendency was also found for GR activity (see Table 2). The GR and CAT activity increased with body mass in the adult beetles in GBH treatment; whereas, in the control adult beetles, the enzyme activity decreased with increased body mass (Fig. 4). The tGSH had the opposite trend; the adult beetles in the GBH treatment showed decreased tGSH concentrations with increased body mass; while in the control, adult beetle tGSH concentrations increased with body mass (Table 2, Fig. 4). Further, GST activity was negatively associated with body mass; whereas, GPx had a tendency to be positively associated with body mass (Table 2). No associations were found for the other measured parameters (SOD, GSH:GSSG, and LHP) of the oxidative status.



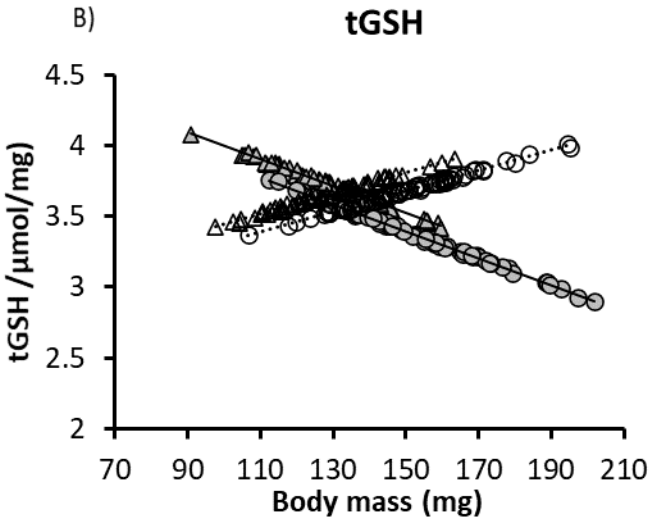


Figure 4. The relationship between oxidative status parameters (CAT and tGSH) and body mass in adult Colorado potato beetles (*L. decemlineata*) indirectly exposed to glyphosate (predicted values from the model; tGSH log transformed values). Legend: white triangle = control male, white circle = control female, grey triangle = GBH male, grey circle = GBH female.

We further examined the correlations between the oxidative status parameters and potato plant glycoalkaloids in the larvae and the adult beetles separately in both treatment groups. We found that in the GBH treatment group, the CAT activity of the larvae correlated negatively with both measurements of α-chaconine (r_S^2=-0.606, p=0.028 and r_S^2=-0.628, p=0.022, respectively) and with the second measurement of α-solanine (r_S^2=-0.694, p=0.009, Table A2 A). Also, the GST levels of the larvae in the GBH treatment group correlated negatively with the second measurement of α-solanine and α-chaconine (r_S^2=-0.558, p=0.038 and r_S^2=-0.593, p=0.025, respectively, Table A2 A). There was also a tendency for a negative correlation between GST and the first measurement of α-chaconine (r_S^2=-0.513, p=0.061, Table A2 A). The GSH:GSSG ratio had a nearly significant negative correlation with the first measurement of α-solanine (r_S^2=-0.592, =0.055, Table A2 A). The larvae in the control group had a negative correlation between CAT and the second measurement of α-solanine (r_S^2=-0.824, p=0.006), and a nearly significant negative correlation between CAT and the second measurement of α-chaconine (r_S^2=-0.656, p=0.055, Table A2 B). There were no significant correlations between the other parameters (p>0.05). In the adult beetles, no correlations between the potato plant glycoalkaloids and oxidative status parameters were shown in the GBH treatment group (p>.05, Table A2 C), but in the control group, LHP correlated negatively with the first measurement of α-solanine (r_S^2=-0.558, p=0.031, Table A2 D). There were no significant correlations between the body mass of the larvae and the adult beetles and the potato plant glycoalkaloids (p>0.05) in either of the treatment groups.

4. Discussion

4.1. Potato plant defense chemicals

Soil-mediated exposure to a GBH affected potato plant glycoalkaloid levels. The amount of α-solanine, one of the main defense chemicals of potato plants, was reduced in the potato plants grown in GBH-treated soil compared to the controls; whereas, the α-chaconine levels did not differ between the treatment groups. Correspondingly, Mesnage et al. (2019, preprint) showed in their studies a notable decrease in solanidine (a steroidal alkaloid likewise found in plants of the Solanaceae family) levels in the cecal content of rats exposed to GBH, suggesting that GBH may have a role in the microbial metabolism of alkaloids. GBH has been shown to reduce other secondary compounds in plants as well, such as flavonoid synthesis in barley (*Hordeum vulgare*) seedlings (Laanest, 1987), medicarpin in alfalfa (*Medicago sativa*; Latunde-Dada and Lucas, 1985), and glyceollin in soybeans *(Glycine max*; Ward, 1984). However, opposite results have also been reported, such as the increase of hydrolysable tannins in mountain birch (*Betula pubescens ssp. czerepanovii*; Ossipov et al., 2003). Overall, the effects of GBHs on secondary compounds in plants are surprisingly little studied. The reduction in α-solanine levels may have negative effects on potato plant defense against herbivores, but may benefit the beetles due to lower toxicity of their food items. On the other hand, Colorado potato beetles are specialist herbivores, feeding on *Solanaceae* species with high glycoalkaloid contents, and are well adapted to the defense chemicals of the host plant (Harvey et al., 2005).

Both α-solanine and α-chaconine levels were reduced in the second measurement compared to the first measurement. The observed difference is most likely related to the size of the potato plant leaves, since the leaves were bigger at the time of the second measurement. Thus, the amount of

glycoalkaloids may have become diluted with the leaf growth (personal observations by Rainio and Salminen). However, we cannot entirely rule out the influence of larval feeding or changes caused by potato plant growth on the levels of defense chemicals. For example, Colorado potato beetles have been shown to secrete symbiotic bacteria to suppress plant defenses in tomato plants (*Solanum lycopersicum*; Chung et al., 2013), which may apply to potato plant defense chemicals as well. Moreover, GBHs have been shown to affect the growth (Helander et al., 2019) and quality of plants, such as nutrient accumulation (Zobiole et al., 2012) as well as antioxidant defense (Radwan and Fayez, 2016). For example, glyphosate has been shown to lower photosynthesis and reduce protein- and free amino acid levels as well as induce antioxidant enzyme activities (e.g. CAT, SOD and peroxidases) in peanut (*Arachis hypogaea* L. cv. Giza; Radwan and Fayez, 2016). We did not monitor potato plant growth in this study, but Helander et al. (2019) have shown in their greenhouse experiment that potato plants growing in GBH-treated soil had shorter sprouts soon after planting, but the height of the plants did not differ later during the growing season. However, in the field experiment, the potato plant shoot and tuber biomass was 25% and 14% higher, respectively, from plants grown in GBH-treated soil compared to those grown in control soil (Helander et al., 2019).

4.2. Survival rate and developmental time

Soil-mediated exposure to a GBH had no effect on the survival rate of the Colorado potato beetle larvae or the adult beetles, indicating that the environmentally relevant concentrations used in the soil did not increase mortality during the larval stage or show carry-over or soil-mediated effects in adult beetles. The soil used in our experiment contained some glyphosate residues (glyphosate July: 0.41-0.91 mg/kg, AMPA: 0.24-1.00 mg/kg, certified laboratory, Groen Agro Control, Delfgauw, Netherlands, LC-MS/MS, with a detection limit of 0.01 mg/kg). The glyphosate concentrations of the leaves from the present study were not measured, but potato plant leaves, measured from the

potato plants grown outside in the field, had no detectable residues (<0.01 mg/kg), unlike potato tubers (glyphosate: 0.02-0.07 mg/kg, AMPA: 0.06-0.07 mg/kg). The adult beetles were also tested for GBH residues to see whether the GBH accumulates in beetles via food at the larval stage or via soil during the pupal phase. Low levels of AMPA were indeed detected in the beetles (AMPA: 0.11mg/kg, glyphosate: 0.013mg/kg), but the residue levels were low and did not affect the survival rate of the beetles at any developmental stage. Our results are in accordance with some other invertebrate studies, which show no effects of GBHs on survival rate (Baker et al., 2014; Haughton et al., 2001; Michalková and Pekár, 2009; Salvio et al., 2016; Thompson et al., 2014). On the other hand, several studies of invertebrates (Benamú et al., 2010; Castilla et al., 2008; Evans et al., 2010; Janssens and Stoks, 2017; Schneider et al., 2009) have shown either direct mortality effects or sublethal effects when exposed to various GBHs, indicating temporal and dose-dependent effects, as well as species-specific differences in insect susceptibility to GBHs. In our earlier study (Rainio et al., 2019), where the Colorado potato beetle larvae were directly exposed to different concentrations of the GBH, low (environmentally relevant) concentrations had no effect on larval survival rate, whereas high concentrations increased larval mortality.

In the present study, neither the body mass of the larvae or the newly emerged adult beetles (neither females nor males) was affected by GBH treatment, which was expected since the larvae never come in direct contact with the GBH, supporting the finding that the GBH does not affect the beetles' survival rate. However, the developmental time of the adult beetles increased significantly in the GBH treatment group compared to the control group, but the difference (0.56 days) was rather low in a biological sense and likely does not have notable effects on the overall survival rate of the beetles. In general, the Colorado potato beetle tolerates pesticides relatively well, and has developed resistance to several synthetic insecticides, including organophosphates (Kostic et al., 2016; Piiroinen et al., 2013), used as a control method in potato farms. The metabolic adaptation is

manifested by a complex set of detoxifying enzymes, such as GSTs, P450 monooxygenases, and esterases (Ben-Abdallah et al., 2019). Glyphosate also belongs to the organophosphate chemical group, which may potentially affect the susceptibility of the Colorado potato beetles to GBHs. However, this has not been examined in detail.

4.3. Oxidative status

Soil-mediated early-life exposure to the GBH affected the antioxidant defense system of the beetles, more specifically the enzymes related to ROS regulation and detoxification of xenobiotics. From the measured oxidative status parameters, GST, CAT, and SOD activity and the concentration of tGSH were up-regulated in the larvae of the GBH-treated group compared to the control group, but this was not seen in the adult stage. The up-regulation can be due to an activation of antioxidant enzymes that work efficiently against increased ROS production to prevent oxidative stress. However, since we did not measure ROS levels, we do not know the exact levels caused by the GBH. On the other hand, it is possible that the potato plant quality (e.g. antioxidant defence, nutrient accumulation) or microbial changes in potato plant (Nissinen et al., unpublished) might have changed due to the GBH treatment, which, in turn, might explain the differences we observe in beetles. In earlier studies, GST activity has been shown to increase in blackworm (*Lumbriculus variegatus;* Contardo-Jara et al., 2009) or decrease in teleostean fish (Samanta et al., 2014) in relation to GBHs or other organophosphorus pesticides e.g. in fish and amphibian studies (Diepens et al., 2014; Oruc, 2011). Insecticide exposure has also been reported to induce GST activity in many insect species (Che-Mendoza et al., 2009). The up-regulation of SOD and CAT activity—the enzymes that catalytically remove ROS (Halliwell and Gutteridge, 2007)—was shown in the larvae, but not in the adult beetles. Since these enzymes operate together, it was expected that they would show a similar trend in relation to GBH treatment. Elevated hepatic SOD and CAT activity has also

been found in bullfrog (*Lithobates catesbeiana*) tadpoles exposed to Roundup Original (Costa et al., 2008), increased SOD activity in blackworm exposed to Roundup Ultra (Contardo-Jara et al., 2009), and increased CAT activity in teleost fish exposed to GBHs (Samanta et al., 2014). Our previous direct exposure study of Colorado potato beetles (Rainio et al. 2019) did not show any differences in those same markers of oxidative status, which may be related to the exposure time or the absorption of the GBH by the beetles' bodies (absorption through the cuticle and epidermis vs. via food or soil).

In addition to enzyme activity, tGSH concentrations in the larvae were elevated in the GBH treatment group compared to the control group. GSH protects cells from oxidative stress by scavenging and neutralizing ROS and simultaneously converting them to GSSG (Halliwell and Gutteridge, 2007; Singh, 2002). The detoxification capacity of GSH is related to its reduced thiol group, and thus the reduced form is the most important in resisting oxidative stress (Singh, 2002). Larsen et al. (2012) reported elevated GSH concentrations in rats exposed to GBHs via drinking water, while some other studies have shown the opposite trend (El-Shenawy, 2009). Increased GSH synthesis, as an adaptive response during moderate oxidative stress, has been previously reported in aquatic organisms by Slaninová et al. (2009). Furthermore, GSH has been suggested to be depleted after short periods of oxidative stress, but elevated after long-term exposure to oxidants (Slaninová et al., 2009). The contradictory results highlight the species- (see also Berglund et al., 2014; Rainio et al., 2013;) and tissue-specificity (Yang et al., 2013) of antioxidant defense, but also the use of various GBHs, the dose and the susceptibility of different species to GBH exposure may induce opposite results. In the present study, the GSH:GSSG ratio and the LHP levels of the larvae did not differ between the treatment groups, suggesting that the increased tGSH level, together with up-regulated enzyme activities, has been effective enough in keeping the cellular redox balance (i.e., GSH:GSSG ratio) stable (Lushchak, 2012). However, the long-term up-regulation of antioxidant

614 enzyme activity is energetically costly and may, in the long-term, increase oxidative stress,
615 ultimately trading-off with the overall survival rate and fitness of the beetles.
616
617 The effect of body mass on oxidative status parameters was further studied in the larvae and the
618 adult beetles, since it has been previously shown that the enzyme activity can be linked to body
619 mass, which is often associated with overall animal condition (Koivula et al., 2011; Rainio et al.,
620 2015). In the larvae (as also in the adults), the body mass had a negative association with GSTs,
621 meaning that the lighter larvae had higher GST activity compared to heavier larvae. It is possible
622 that, in general, the lighter larvae that are in poorer condition need to up-regulate GST activity more
623 for detoxification processes, which may be energy demanding, than the heavier ones that are in
624 better condition. A similar results between the antioxidant enzyme activities of GPx, SOD, and
625 CAT and body mass have been found in birds, such as the great tit (*Parus major*), when exposed to
626 metal pollution (Rainio et al., 2015). The larvae further showed a positive association between body
627 mass and tGSH concentrations, meaning that heavier larvae had higher tGSH levels, which is
628 opposite to what we found for GST. However, it may be that the heavier larvae can produce more
629 GSH in their system, reflecting better antioxidant capacity, compared to the lighter larvae that are in
630 poorer condition.
631
632 In this study, we were able to follow the individuals from the larvae to the adult stage to examine
633 the long-term effects of early-life GBH exposure. The GBH directly decreased the oxidative status
634 parameters CAT and tGSH in the adult beetles, and there was a significant treatment × body mass
635 interaction. In the adult beetles, CAT activity (and GR activity to some extent) increased with body
636 mass in the GBH treatment group, but decreased in the control group. The opposite was shown for
637 tGSH, where the levels increased with body mass in the control group, but decreased in the GBH
638 treatment group. The higher CAT activity of the heavier adult beetles in the GBH treatment group

639 may be due to being in better condition, allowing them to allocate more resources for their defense
640 in case of increased ROS production compared to lighter ones that are in poorer condition.
641 However, in the controls, the body mass may not be so critical since their activities stay rather
642 constant.
643
644 The increased tGSH levels may reflect the better condition of heavier adults in the control group;
645 whereas, in the GBH treatment group, the decreased tGSH levels may suggest either lesser need of
646 tGSH (e.g. due to up-regulated enzyme activities) or more rapid transformation of GSH to GSSG to
647 cope with the potential increase in ROS production. This is further supported by the higher GR
648 activity in the bigger adults than the smaller ones in the GBH treatment group, since the main
649 function of GR is to transform oxidized GSH (i.e. GSSG) back to its reduced form (GSH; Halliwell
650 and Gutteridge, 2007). The results suggest that the early-life indirect GBH exposure via diet may
651 show some long-term effects on the adult beetles. On the other hand, the pupa may also be directly
652 exposed to GBH residues during their 2-week pupal stage in the soil, which can partly explain the
653 observed effects on the adults' physiology and developmental time between the treatment groups. In
654 future, it would be important to concentrate more on the plant-mediated effects and separate them
655 from the soil-mediated effects at the pupal stage, and, moreover, extend the studies to observe the
656 following breeding season to see whether the GBH affects the overwintering and reproduction
657 success of the adult beetles later in life.
658
659 We also examined the relationships between oxidative status parameters and potato plant
660 glycoalkaloids separately in larvae and the adult beetles to see whether these chemicals affect the
661 beetle's oxidative status. We found that for the larvae in the GBH treatment group (as also in the
662 control group), the activity of CAT and GST correlated negatively with α-solanine and α-chaconine
663 levels, either with both of the measurements (before and after larval feeding) or with only one of the

664 measurements. Interestingly, these are the same parameters that were affected by GBH treatment in
665 larvae, but in the opposite direction. The GST and CAT activity decreased with increased
666 α-solanine and α-chaconine levels, but increased with GBH treatment. The results are logical, since
667 the lower α-solanine levels were shown in the GBH treatment group with higher antioxidant
668 enzyme activity. The observed changes in antioxidant defense of the beetles can be derived from the
669 GBH itself or from the GBH-mediated effects on potato glycoalkaloid levels, in case the
670 glycoalkaloids affect the potato quality as food items. The α-solanine has been previously shown to
671 increase lipid peroxidation (measured as malondialdehyde [MDA] concentration) and GST activity
672 in the mid-gut, but decrease the GST activity in body fat in Lepidoptera, such as *G. mellonella*,
673 indicating the oxidative activity of glycoalkaloids (Adamski et al., 2014). Furthermore, GSH:GSSG
674 ratio had a similar tendency for a negative correlation with only the first measurement of α-solanine
675 (see table S2), reflecting the increased oxidation of GSH to GSSG in the higher concentrations of
676 glycoalkaloids. In the adult beetles, on the other hand, none of the oxidative status parameters
677 correlated with potato plant glycoalkaloids. Even though both potato plant defense chemicals and
678 GBH treatment seemed to affect the same oxidative status parameters of the beetle larvae (e.g.,
679 GST, CAT), we cannot say for sure whether they show additive or synergistic effects on the beetles.
680 More experimental studies with different concentrations of glycoalkaloids and GBHs would be
681 needed to understand the complex combined effects of glycoalkaloids and GBHs on the oxidative
682 status parameters of the beetles.
683
684 *4.4. Conclusions*
685
686 The reduction of α-solanine levels in potato plants grown in GBH-treated soil suggests the potential
687 reduction of potato plant defense against the Colorado potato beetle, but more dose-dependent
688 studies would be needed to examine the significance of the reduction of defense chemicals on

potato plants, since the herbicides may significantly affect the inter- and intraspecies interactions of agricultural ecosystems. The survival rate of the beetles was not affected by the soil-mediated early-life GBH treatment, but the oxidative status parameters, GST, SOD, CAT, and tGSH, were increased in the larvae in the GBH treatment group compared to the control group. The long-term up-regulation of antioxidant enzyme activity is energetically costly and may increase oxidative stress in the larvae, which could in turn delay the developmental time. In the adult beetles, CAT activity and tGSH levels were affected by the interactive effect of GBH treatment and body mass of the adult beetles, suggesting that the early-life glyphosate treatment or soil-mediated effects at the pupal stage may have long-term effects on the adult beetles. Our results highlight the importance of measuring the physiological parameters, such as oxidative status, along with life-history traits in sublethal herbicide studies, since they may be important factors in affecting the health and survival of animals. In future, it would be important to extend the monitoring of the adult beetles to the following breeding season, to study the effects of GBHs on fertility, reproductive success, and overwinter survival rate of the adult beetles.

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Conflicts of Interest

The authors declare no conflict of interest.

Credit Author Statement

Miia J. Rainio: Study design, conducting experiment, biochemical analyses, statistical analyses, manuscript writing. **Aigi Margus:** Study design, experiment preparation, manuscript editing. **Valtteri Virtanen:** Glycoalkaloid analyses, manuscript editing. **Leena Lindström:** Study design, experiment preparation, manuscript editing. **J-P Salminen:** Glycoalkaloid analyses, manuscript editing. **Kari Saikkonen:** manuscript editing. **Marjo Helander:** Study design, manuscript editing.

References

Adamski, Z., Marciniak, P., Ziemnicki, K., Büyükgüzel, E., Erdem, M., Büyükgüzel, K., Ventrella, E., Falabella, P., Cristallo, M., Salvia, R., Bufo, S.A., Scrano, L., 2014. Potato leaf extract and its component, α-solanine, exert similar impacts on development and oxidative stress in *Galleria mellonella* L. *Archives of Insect Biochemistry and Physiology* 87(1), 26-39.

Alghamdi, A.A., Frey, K.M., 2017. Predicting The Toxic Effect of Organophosphates on GST Enzyme Isoforms. *The FASEB Journal* 31, lb623-lb623.

Amrhein, N., Deus, B., Gehrke, P., Steinrucken, H.C., 1980. The site of the inhibition of the shikimate pathway by glyphosate: II. Interference of glyphosate with chorismate formation in vivo and in vitro. *Plant Physiology* 66(5), 830-834.

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochemical Pharmacology* 59(1), 95-104.

Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate□based herbicides on the freshwater environment. *Journal of Applied Toxicology* 34(5), 458-479.

Baker, L.F., Mudge, J.F., Houlahan, J.E., Thompson, D.G., Kidd, K.A., 2014. The direct and indirect effects of a glyphosate-based herbicide and nutrients on *Chironomidae* (Diptera) emerging from small wetlands. *Environmental Toxicology and Chemistry* 33, 2076-2085.

Ben-Abdallah, S., Cáceres, L.A., Wang, Z.L., Renaud, B.J., Lachâal, M., Karray-Bouraoui, N., Hannoufa, A., Scott, I.M., 2019. Host plant defenses of black (*Solanum nigrum L.*) and red nightshade (*Solanum villosum Mill.*) against specialist Solanaceae herbivore *Leptinotarsa decemlineata* (Say). *Archives of Insect Biochemistry and Physiology* 101(2).

Benamú, M.A., Schneider, M.I., Sánchez, N.E., 2010. Effects of the herbicide glyphosate on biological attributes of *Alpaida veniliae* (Araneae, Araneidae), in laboratory. *Chemosphere* 78(7), 871-876.

754 Bentley, R., 1990. The shikimate pathway - a metabolic tree with many branches. C*ritical Reviews*
755 *in Biochemistry and Molecular Biology* 25(5), 307-384.
756
757 Berglund, Å.M.M., Rainio, M.J., Kanerva, M., Nikinmaa, M., Eeva, T., 2014. Antioxidant status in
758 relation to age, condition, reproductive performance and pollution in three passerine species.
759 *Journal of Avian Biology* 45(3), 235-246.
760
761 Blumenthal, D., Augustine, D., 2009. Plant interactions with herbivores. *Encyclopedia of the Life*
762 *Sciences*.
763
764 Bou, R., Codony, R., Tres, A., Decker, E.A., Guardicila, F., 2008. Determination of hydroperoxides
765 in foods and biological samples by the ferrous oxidation-xylenol orange method: A review of the
766 factors that influence the method's performance. *Analytical Biochemistry* 377(1), 1-15.
767
768 Casagrande, R.A., 1987. The Colorado potato beetle: 125 years of mismanagement. *Bulletin of the*
769 *Entomological Society of America* 33(3), 142-150.
770
771 Castilla, A.M., Dauwe, T., Mora, I., Malone, J., Guitart, R., 2010. Nitrates and herbicides cause
772 higher mortality than the traditional organic fertilizers on the grain beetle, *Tenebrio molitor*.
773 *Bulletin of Environmental Contamination and Toxicology* 84(1), 101-105.
774
775 Castilla, A.M., Dauwe, T., Mora, I., Palmer, M., Guitart, R., 2008. Mortality of the yellow
776 mealworm *Tenebrio molitor* exposed to fertilizers and herbicides commonly used in agriculture.
777 Vie Et Milieu-Life and Environment 58(3-4), 243-247.
778
779 Che-Mendoza, A., Penilla, R., Rodríguez, D., 2009. Insecticide resistance and glutathione S-
780 transferases in mosquitoes: A review. *African Journal of Biotechnology* 8(8), 1386-1397.
781
782 Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K.,
783 Falabella, P., Scrano, L., Ventrella, E., Lelario, F., Bufo, S., 2016. A review of bioinsecticidal
784 activity of Solanaceae alkaloids. *Toxins (Basel)* 8(3), 60.
785
786 Chung, S.H., Rosa, C., Hoover, K., Luthe, D.S., Felton, G.W., 2013. Colorado potato beetle
787 manipulates plant defenses in local and systemic leaves. *Plant Signaling & Behavior* 8(12), e27592.
788
789 Claus, S.P., Guillou, H., Ellero-Simatos, S., 2016. The gut microbiota: a major player in the toxicity
790 of environmental pollutants? *npj Biofilms and Microbiomes* 2, 16003.
791
792 Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its
793 formulation Roundup Ultra in Lumbriculus variegatus and its effects on biotransformation and
794 antioxidant enzymes. *Environmental Pollution* 157(1), 57-63.
795
796 Corona, M., Robinson, G.E., 2006. Genes of the antioxidant system of the honey bee: annotation
797 and phylogeny. *Insect Molecular Biology* 15(5), 687-701.
798
799 Costa, M.J., Monteiro, D.A., Oliveira-Neto, A.L., Rantin, F.T., Kalinin, A.L., 2008. Oxidative
800 stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original®.
801 *Ecotoxicology* 17(3), 153-163.
802

Deisseroth, A., Dounce, A.L., 1970. Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. Physiological Reviews 50(3), 319-375.

Dewick, P., 2009. Medicinal Natural Products: A Biosynthetic Approach, 3rd ed. John Wiley and Sons Ltd, United Kingdom.

Diepens, N., Pfennig, S., Van den Brink, P., Gunnarsson, J., Ruepert, C., Castillo, L., 2014. Effect of pesticides used in banana and pineapple plantations on aquatic ecosystems in Costa Rica. *Journal of Environmental Biology* 35(1), 73-84.

Downer, R.G.H., 1985. Lipid metabolism, in: Kerkut, G.A., Gilbert L. I. (Eds.), Comprehensive insect physiology, biochemistry and pharmacology, Pergamon Press, Oxford, United Kingdom, pp. 77-113.

Duke, S.O., Powles, S.B., 2008. Glyphosate: A once-in-a-century herbicide. *Pest Management Science* 64(4), 319-325.

El-keltawi, N.E., Croteau, R., 1987. Influence of foliar applied cytokinins on growth and essential oil content of several members of the lamiaceae. *Phytochemistry* 26(4), 891-895.

El-Shenawy, N.S., 2009. Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. *Environmental Toxicology and Pharmacology* 28(3), 379-385.

Evans, S.C., Shaw, E.M., Rypstra, A.L., 2010. Exposure to a glyphosate-based herbicide affects agrobiont predatory arthropod behaviour and long-term survival. *Ecotoxicology* 19(7), 1249-1257.

Farjan, M., Dmitryjuk, M., Lipinski, Z., Biernat-Lopienska, E., Zoltowska, K., 2012. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera carnica* brood at different stages. *Journal of Apicultural Research* 51(3), 263-270.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408(6809), 239-247.

Fossati, P., Prencipe, L., Berti, G., 1980. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric-acid in serum and urine. *Clinical Chemistry* 26(2), 227-231.

Fragoyiannis, D., McKinlay, R., D'Mello, J., 1998. Studies of the growth, development and reproductive performance of the aphid shape Myzus persicae on artificial diets containing potato glycoalkalois. *Entomologia Experimentalis et Applicata* 88, 59-66.

Friedman, M., 2006. Potato glycoalkaloids and metabolites: roles in the plant and in the diet. *Journal of Agricultural and Food Chemistry* 54(23), 8655-8681.

Friedman, M., McDonald, G.M., Filadelfi-Keszi, M., 1997. Potato Glycoalkaloids: Chemistry, Analysis, Safety, and Plant Physiology. *Critical Reviews in Plant Sciences* 16(1), 55-132.

Funke, T., Han, H., Healy-Fried, M.L., Fischer, M., Schönbrunn, E., 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. *Proceedings of the National Academy of Sciences of the United States of America* 103(35), 13010-13015.

George, D.G.M., Gatehouse, A.M.R., 2013. Oxidative stress enzymes in *Busseola fusca*. *International Journal of Current Microbiology and Applied Sciences* 2(10), 485-495.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup (R) Herbicide. In: Ware, G.W. (Ed.). *Reviews of Environmental Contamination and Toxicology*, Vol 167, Springer, New York, 35-120.

Glass, R.L., 1984. Metal complex formation by glyphosate. *Journal of Agricultural and Food Chemistry* 32(6), 1249-1253.

Glusczak, L., Miron, D.D., Moraes, B.S., Simoes, R.R., Schetinger, M.R.C., Morsch, V.M., Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* 146(4), 519-524.

Gomes, M.P., Le Manac'h, S.G., Maccario, S., Labrecque, M., Lucotte, M., Juneau, P., 2016. Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants. *Pesticide Biochemistry and Physiology* 130, 65-70.

GraphPad Prism, 2020. User Guide. GraphPad Software, LLC.

Grapputo, A., Boman, S., Lindstrom, L., Lyytinen, A., Mappes, J., 2005. The voyage of an invasive species across continents: genetic diversity of North American and European Colorado potato beetle populations. *Molecular Ecology* 14(14), 4207-4219.

Güntner, C., Gonzalez, A., Dos Reis, R., Usubillanga, A., Ferreira, F., Moyna, P., 1997. Effect of Solanum glycoalkaloids on potato aphid, *Macrosiphum euphorbiae*. *Journal of Chemical Ecology* 23, 1651-1659.

Hagner, M., Mikola, J., Saloniemi, I., Saikkonen, K., Helander, M., 2019. Effects of a glyphosate-based herbicide on soil animal trophic groups and associated ecosystem functioning in a northern agricultural field. *Scientific Reports* 9, 8540.

Halliwell, B., Gutteridge, J., 2007. Free Radicals in Biology and Medicine, Fourth ed. Oxford University Press, New York.

Hare, J.D., 1987. Growth of *Leptinotarsa decemlineata* larvae in response to simultaneous variation in protein and glycoalkaloid concentration. *Journal of Chemical Ecology* 13, 39-46.

Harvey, J.A., van Nouhuys, S., Biere, A., 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *Journal of Chemical Ecology* 31(2), 287-302.

Haslam, E., 1993. Shicimic Acid: Metabolism and Metabolites, 1 ed. Wiley, New York.

Haughton, A.J., Bell, J.R., Wilcox, A., Boatman, N.D., 2001. The effect of the herbicide glyphosate on non-target spiders: Part I. Direct effects on *Lepthyphantes tenuis* under laboratory conditions. *Pest Management Science* 57(11), 1033-1036.

Helander, M., Pauna, A., Saikkonen, K., Saloniemi, I., 2019. Glyphosate residues in soil affect crop plant germination and growth. *Scientific Reports* 9, 19653.

Helander, M., Saloniemi, I., Omacini, M., Druille, M., Salminen, J.-P., Saikkonen, K., 2018. Glyphosate decreases mycorrhizal colonization and affects plant-soil feedback. *Science of the Total Environment* 642, 285-291.

Helander, M., Saloniemi, I., Saikkonen, K., 2012. Glyphosate in northern ecosystems. *Trends in Plant Science* 17(10), 569-574.

Hultberg, M., 2007. Cysteine turnover in human cell lines is influenced by glyphosate. *Environmental Toxicology and Pharmacology* 24(1), 19-22.

Isaksson, C., Oernborg, J., Stephensen, E., Andersson, S., 2005. Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in great tits. *EcoHealth* 2(2), 138-146.

Janssens, L., Stoks, R., 2017. Stronger effects of Roundup than its active ingredient glyphosate in damselfly larvae. *Aquatic Toxicology* 193, 210-216.

Khan, M., Munir, I., Khan, I., 2013. The potential of unintended effects in potato glycoalkaloids. *African*
Journal of Biotechnology 12(8), 754-766.

Kishore, G.M., Shah, D.M., 1988. Amino-acid biosynthesis inhibitors as herbicides. *Annual Review of Biochemistry* 57, 627-663.

Koivula, M.J., Kanerva, M., Salminen, J.P., Nikinmaa, M., Eeva, T., 2011. Metal pollution indirectly increases oxidative stress in great tit (*Parus major*) nestlings. *Environmental Research* 111(3), 362-370.

Kostic, M., Stankovic, S., Kuzevski, J., 2016. Role of AChE in Colorado potato beetle (*Leptinotarsa decemlineata* Say) Resistance to Carbamates and Organophosphates. InTechOpen. Retrieved from https://www.intechopen.com/books/insecticides-resistance/role-of-ache-in-colorado-potato-beetle-leptinotarsa-decemlineata-say-resistance-to-carbamates-and-or

Kowalski, S.P., Domek, J.M., Deahl, K.L., Sanford, L.L., 1999. Performance of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say), reared on synthetic diets supplemented with *Solanum* glycoalkaloids. *American Journal of Potato Research* 76, 305-312.

Laanest, L., 1987. Incorporation of exogenous tyrosine and phenylalanine into C-glycosylflavones in glyphosate-treated barley seedlings. *Eesti NSV Teaduste Akadeemia Toimetised Bioloogia* 36(3), 204-209.

Lachman, J, Hamouz, K., Orsak, M., Pivec, V., 2001. Potato glycoalkaloids and their significance in plant protection and human nutrition - review. *Rostlinna Výroba* 47(4), 181-191.

Laitinen, P., 2009. Glyphosate and phosphorus leaching and residues in boreal sandy soil. *Plant and Soil* 323(1), 267-283.

Larsen, K., Najle, R., Lifschitz, A., Virkel, G., 2012. Effects of sub-lethal exposure of rats to the herbicide glyphosate in drinking water: Glutathione transferase enzyme activities, levels of reduced glutathione and lipid peroxidation in liver, kidneys and small intestine. E*nvironmental Toxicology and Pharmacology* 34(3), 811-818.

Latunde-Dada, A.O., Lucas, J.A., 1985. Involvement of the phytoalexin medicarpin in the differential response of callus lines of lucerne (*Medicago sativa*) to infection by *Verticillium albo-atrum*. *Physiological Plant Pathology* 26(1), 31-42.

Lehmann, P., Lyytinen, A., Piiroinen, S., Lindstrom, L., 2015. Latitudinal differences in diapause related photoperiodic responses of European Colorado potato beetles (*Leptinotarsa decemlineata*). *Evolutionary Ecology* 29(2), 269-282.

Liu, X., Williams, C.E., Nemacheck, J.A., Wang, H., Subramanyam, S., Zheng, C., Chen, M.-S., 2010. Reactive oxygen species are involved in plant defense against a gall midge. *Plant Physiology* 152(2), 985-999.

Lushchak, V.I., 2012. Glutathione homeostasis and functions: potential targets for medical interventions. *Journal of Amino Acids* 2012, 1-26.

Lydon, J., Duke, S.O., 1989. Pesticide effects on secondary metabolism of higher-plants. *Pesticide Science* 25(4), 361-373.

Lyytinen, A., Lindstrom, L., Mappes, J., Julkunen-Tiitto, R., Fasulati, S.R., Tiilikkala, K., 2007. Variability in host plant chemistry: behavioural responses and life-history parameters of the Colorado potato beetle (*Leptinotarsa decemlineata*). *Chemoecology* 17, 51-56.

Margus, A., Rainio, M., Lindström, L., 2019. Can indirect herbicide exposure modify the response of the Colorado potato beetle to an organophosphate insecticide? *Journal of Economic Entomology* 112(5), 2316-2323.

Martinez, D.A., Loening, U.E., Graham, M.C., 2018. Impacts of glyphosate-based herbicides on disease resistance and health of crops: a review. *Environmental Sciences Europe* 30(1), 2.

Matthews, D., Jones, H., Gans, P., Coates, S., Smith, L.M.J., 2005. Toxic secondary metabolite production in genetically modified potatoes in response to stress. *Journal of Agricultural and Food Chemistry* 53(20), 7766-7776.

Mesnage, R., Teixeira, M., Madrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D., Amiel, C., Panoff, J-M., Belpoggi, F., Antoniou, M.N. 2019. Shotgun metagenomics and metabolomics reveal glyphosate alters the gut microbiome of Sprague-Dawley rats by inhibiting the shikimate pathway. *BioRxiv* preprint. doi: https://doi.org/10.1101/870105.

Mesnage, R., Defarge, N., de Vendômois, J.S., Séralini, G.E., 2015. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. *Food and Chemical Toxicology* 84, 133-153.

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.-E., 2014. Major pesticides are more toxic to human cells than their declared active principles. *BioMed Research International* 2014, 179691.

Michalková, V., Pekár, S., 2009. How glyphosate altered the behaviour of agrobiont spiders (Araneae: Lycosidae) and beetles (Coleoptera: Carabidae). *Biological Control* 51(3), 444-449.

Modesto, K.A., Martinez, C.B.R., 2010. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere* 78(3), 294-299.

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health* 15, 19.

Nenaah, G., 2011. Toxic and antifeedant activities of potato glycoalkaloids against *Trogoderma granarium* (Coleoptera: Dermestidae). *Journal of Stored Products Research* 47(3), 185-190.

Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., McCarthy, S., Betteridge, D.J., Wolff, S.P., 1995. Elevated levels of authentic plasma hydroperoxides in NIDDM. *Diabetes* 44(9), 1054.

Nylin, S., Janz, N., 1993. Oviposition preference and larval performance in Polygonia c-album (Lepidoptera: Nymphalidae): the choice between bad and worse. *Ecological Entomology* 18(4), 394-398.

Oruc, E., 2011. Effects of diazinon on antioxidant defense system and lipid peroxidation in the liver of *Cyprinus carpio* (L.). *Environmental Toxicology* 26(6), 571-578.

Ossipov, V., Salminen, J.-P., Ossipova, S., Haukioja, E., Pihlaja, K., 2003. Gallic acid and hydrolysable tannins are formed in birch leaves from an intermediate compound of the shikimate pathway. *Biochemical Systematics and Ecology* 31(1), 3-16.

Piiroinen, S., Lindstrom, L., Lyytinen, A., Mappes, J., Chen, Y.H., Izzo, V., Grapputo, A., 2013. Pre-invasion history and demography shape the genetic variation in the insecticide resistance-related acetylcholinesterase 2 gene in the invasive Colorado potato beetle. *BMC Evolutionary Biology* 13, 13.

Pinto, E., Sigaud-Kutner, T.C.S., Leitao, M.A.S., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. *Journal of Phycology* 39(6), 1008-1018.

Radwan, D.E.M., Fayez, K.A., 2016. Photosynthesis, antioxidant status and gas-exchange are altered by glyphosate application in peanut leaves. *Photosynthetica* 54, 307–316.

Rainio, M.J., Eeva, T., Lilley, T., Stauffer, J., Ruuskanen, S., 2015. Effects of early-life lead exposure on oxidative status and phagocytosis activity in great tits (*Parus major*). *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* 167, 24-34.

Rainio, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M., Eeva, T., 2013. Oxidative status in nestlings of three small passerine species exposed to metal pollution. *Science of the Total Environment* 454-455, 466-473.

Rainio, M.J., Margus, A., Lehmann, P., Helander, M., Lindström, L., 2019. Effects of a glyphosate-based herbicide on survival and oxidative status of a non-target herbivore, the Colorado potato beetle (*Leptinotarsa decemlineata*). *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* 215, 47-55.

Salvio, C., Menone, M.L., Rafael, S., Iturburu, F.G., Manetti, P.L., 2016. Survival, reproduction, avoidance behavior and oxidative stress biomarkers in the earthworm *Octolasion cyaneum* exposed to glyphosate. *Bulletin of Environmental Contamination and Toxicology* 96(3), 314-319.

Samanta, P., Pal, S., Mukherjee, A.K., Ghosh, A.R., 2014. Biochemical effects of glyphosate based herbicide, Excel Mera 71 on enzyme activities of acetylcholinesterase (AChE), lipid peroxidation (LPO), catalase (CAT), glutathione-S-transferase (GST) and protein content on teleostean fishes. *Ecotoxicology and Environmental Safety* 107, 120-125.

Santos-Sánchez, N.F., Salas-Coronado, R., Hernández-Carlos, B., Villanueva-Cañongo, C., 2019. Shikimic acid pathway in biosynthesis of phenolic compounds. *InTechOpen*. Retrieved from https://www.intechopen.com/books/plant-physiological-aspects-of-phenolic-compounds/shikimic-acid-pathway-in-biosynthesis-of-phenolic-compounds.

SAS, 2013. Base SAS 9.4 Procedures Guide: Statistical Procedures. SAS Institute Inc.

Saska, P., Skuhrovec, J., Lukas, J., Chi, H., Tuan, S.J., Honek, A., 2016. Treatment by glyphosate-based herbicide alters life history parameters of the rose-grain aphid *Metopolophium dirhodum*. *Scientific Reports* 6, 27801.

Schneider, M.I., Sanchez, N., Pineda, S., Chi, H., Ronco, A., 2009. Impact of glyphosate on the development, fertility and demography of *Chrysoperla externa* (Neuroptera: Chrysopidae): ecological approach. *Chemosphere* 76(10), 1451-1455.

Shilo, T., Zygier, L., Rubin, B., Wolf, S., Eizenberg, H., 2016. Mechanism of glyphosate control of *Phelipanche aegyptiaca*. *Planta* 244(5), 1095-1107.

Siehl, D.L., 1997. Inhibitors of EPSP synthase, glutamine synthethase and histidine synthesis. IOS Press, Amsterdam. Retrieved from https://www.researchgate.net/publication/292667029_Inhibitors_of_EPSP_synthase_glutamine_synthetase_and_histidine_synthesis

Sihtmäe, M., Blinova, I., Künnis-Beres, K., Kanarbik, L., Heinlaan, M., Kahru, A., 2013. Ecotoxicological effects of different glyphosate formulations. *Applied Soil Ecology* 72, 215-224.

Silva, V., Montanarella, L., Jones, A., Fernández-Ugalde, O., Mol, H.G.J., Ritsema, C.J., Geissen, V., 2018. Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. *Science of the Total Environment* 621, 1352-1359.

Singh, R.J., 2002. Glutathione: A marker and antioxidant for aging. J*ournal of Laboratory and Clinical Medicine* 140(6), 380-381.

Slaninová, A., Smutna, M., Modra, H., Svobodova, Z., 2009. A review: oxidative stress in fish induced by pesticides. *Neuroendocrinology Letters* 30, 2-12.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* 150(1), 76-85.

Steinrücken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochemical and Biophysical Research Communications 94, 1207-1212.

Thompson, H.M., Levine, S.L., Doering, J., Norman, S., Manson, P., Sutton, P., von Merey, G., 2014. Evaluating Exposure and Potential Effects on Honeybee Brood (Apis mellifera) Development Using Glyphosate as an Example. Integrated Environmental Assessment and Management 10, 463-470.

Torretta, V., Katsoyiannis, A.I., Viotti, P., Rada, C.E., 2018. Critical Review of the Effects of Glyphosate Exposure to the Environment and Humans through the Food Supply Chain. *Sustainability* 10.

Tzin, V., Galili, G., 2010. New Insights into the Shikimate and Aromatic Amino Acids Biosynthesis Pathways in Plants. *Molecular Plant* 3, 956-972.

Uren Webster, T.M., Santos, E.M., 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup. *BMC Genomics* 16(32), 1254-1255.

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K.C., Finckh, M.R., Morris, J.G., 2018. Environmental and health effects of the herbicide glyphosate. *Science of the Total Environment* 616-617, 255-268.

Vereecken, H., 2005. Mobility and leaching of glyphosate: a review. *Pest Management Science* 61(12), 1139-1151.

Vuori, K.A., Lehtonen, K.K., Kanerva, M., Peltonen, H., Nikinmaa, M., Berezina, N.A., Boikova, E., 2015. Oxidative stress biomarkers in the copepod *Limnocalanus macrurus* from the northern Baltic Sea: effects of hydrographic factors and chemical contamination. *Marine Ecology Progress Series* 538, 131-144.

Vänninen, I, Worner, S., Huusela-Veistola, E., Tuovinen, T., Nissinen, A., Saikkonen, K., 2011. Recorded and potential alien invertebrate pests in Finnish agriculture and horticulture. *Agricultural and Food Science* 20(1), 96-114.

Walsh, B.D., 1865. The new potato bug and its natural history. *The Practical Entomology* 1, 1-4.

Ward, E., 1984. Suppression of metalaxyl activity by glyphosate: evidence that host defence mechanisms contribute to metalaxyl inhibition of *Phytophthora megasperm f. sp. glycinea* in soybeans. *Physiological Plant Pathology* 25(3), 381-386.

Woodburn, A.T., 2000. Glyphosate: production, pricing and use worldwide. *Pest Management Science* 56(4), 309-312.

Yang, D.-B., Xu, Y.-C., Wang, D.-H., Speakman, J.R., 2013. Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *Journal of Experimental Biology* 216, 4242-4250.

Zobiole, L.H.S., Kremer, R.J., de Oliveira Jr., R.S., Constantin, J., 2012. Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. *Journal of Plant Nutrition and Soil Science* 175(2), 319-330.

1186 **Appendices:**

Table A1. Mean (± 95% CI) activities of oxidative status parameters: glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG), and lipid hydroperoxides (LHP) in control and GBH treatment groups of larval and adult Colorado potato beetles (*L. decemlineata*).

	Larvae				**Adults**			
	Control		**GBH**		**Control**		**GBH**	
Biomarker	**Mean (± 95% CI)**	**n**	**Mean (± 95% CI)**	**n**	**Mean (± 95% CI)**	**n**	**Mean (± 95% CI)**	**n**
GST (nmol/min/mg)	14.62 (13.26-15.97)	34	17.28 (14.65-19.91)	34	35.76 (32.12-39.41)	32	35.15 (31.60-38.71)	32
GPx (nmol/min/mg)	5.31 (4.90-5.72)	34	5.59 (5.06-6.13)	34	2.71 (1.67-3.75)	30	3.17 (1.75-4.58)	31
GR (nmol/min/mg)	4.93 (3.94-5.92)	33	5.37 (3.86-6.88)	33	4.03 (3.30-4.77)	32	3.78 (3.09-4.46)	32
CAT (µmol/min/mg)	143.04 (134.46-151.63)	34	165.80 154.98-176.63)	31	116.90 (107.74-126.06)	32	103.14 (90.67-115.61)	32
SOD (inhibition %)	73.70 (71.65-75.74)	34	77.42 (75.31-79.54)	34	80.94 (78.84-83.04)	32	78.12 (75.71-80.53)	32
tGSH (µmol/mg)	11.23 (8.78-13.68)	20	20.77 (17.55-23.99)	23	41.88 (35.88-47.89)	31	40.35 (33.98-46.72)	25
GSH:GSSG (ratio)	0.45 (0.082-0.83)	20	0.62 (0.23-1.01)	23	3.51 (2.39-4.62)	29	4.37 (1.84-6.89)	25
LHP (nmol/mg bm)	0.57 (0.11-1.04)	16	0.40 (-0.01-0.82)	17	0.018 (0.014-0.023)	27	0.017 (0.014-0.020)	30

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Table A2 A. Spearman correlation coefficients (r^2, p-value, n) between the potato glycoalkaloids (α-solanine and α-chaconine) and oxidative status biomarkers glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG), lipid hydroperoxides (LHP) and body mass (g) in the Colorado potato beetle larvae *(L. decemlineata)* in the GBH treatment.

		GST	**GPx**	**GR**	**CAT**	**SOD**	**tGSH**	**GSH: GSSG**	**LHP**	**Body mass**
α-solanine (1)	**r^2**	0.111	-0.243	-0.163	***-0.517***	-0.126	-0.326	-0.041	-0.476	-0.387
	p	0.707	0.402	0.594	***0.070***	0.668	0.328	0.904	0.233	0.171
	n	14	14	13	***13***	14	11	11	8	14
α-solanine (2)	**r^2**	**-0.558**	0.053	-0.202	**-0.694**	-0.268	-0.436	***-0.592***	-0.167	0.144
	p	**0.038**	0.857	0.508	**0.009**	0.355	0.180	***0.055***	0.693	0.624
	n	**14**	14	13	**13**	14	11	***11***	8	14
α-chaconine (1)	**r^2**	***-0.513***	-0.226	-0.147	**-0.606**	-0.285	-0.454	-0.537	-0.286	0.002
	p	***0.06***	0.438	0.632	**0.028**	0.323	0.161	0.089	0.493	0.994
	n	***14***	14	13	**13**	14	11	11	8	14
α-chaconine (2)	**r^2**	**-0.593**	0.199	-0.091	**-0.628**	-0.215	-0.087	-0.500	-0.048	0.400
	p	**0.025**	0.495	0.767	**0.022**	0.461	0.799	0.117	0.911	0.156
	n	**14**	14	13	**13**	14	11	11	8	14

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Table A2 B. Spearman correlation coefficients (r^2, p-value, n) between the potato glycoalkaloids (α-solanine and α-chaconine) and oxidative status biomarkers glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG), lipid hydroperoxides (LHP) and body mass (g) in the Colorado potato beetle larvae *(L. decemlineata)* in the control treatment.

		GST	GP	GR	CAT	SOD	tGSH	GSH: GSSG	LHP	Body mass
α-solanine (1)	**r^2**	-0.193	0.067	-0.034	0.269	0.168	0.154	0.410	-0.257	0.269
	p	0.618	0.864	0.932	0.484	0.666	0.805	0.493	0.623	0.484
	n	9	9	9	9	9	5	5	6	9
α-solanine (2)	**r^2**	0.193	0.185	-0.135	**-0.824**	-0.572	0.667	-0.205	-0.371	-0.303
	p	0.618	0.634	0.730	**0.006**	0.108	0.219	0.741	0.469	0.429
	n	9	9	9	**9**	9	5	5	6	9
α-chaconine (1)	**r^2**	-0.126	0.252	-0.118	0.017	0.168	0.154	0.410	-0.257	0.168
	p	0.747	0.513	0.763	0.966	0.666	0.805	0.493	0.623	0.666
	n	9	9	9	9	9	5	5	6	9
α-chaconine (2)	**r^2**	0.261	0.387	0.151	***-0.656***	-0.454	0.667	-0.205	0.029	-0.437
	p	0.498	0.304	0.698	***0.055***	0.220	0.219	0.741	0.957	0.240
	n	9	9	9	***9***	9	5	5	6	9

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Table A2 C. Spearman correlation coefficients (r^2, p-value, n) between the potato glycoalkaloids (α-solanine and α-chaconine) and oxidative status biomarkers glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG), lipid hydroperoxides (LHP) and body mass (g) in the Colorado potato beetle adults *(L. decemlineata)* in the GBH treatment.

		GST	GPx	GR	CAT	SOD	tGSH	GSH: GSSG	LHP	Body mass
α-solanine (1)	**r^2**	-0.062	-0.061	0.064	-0.021	0.054	-0.050	-0.177	-0.102	0.341
	p	0.807	0.810	0.801	0.932	0.832	0.859	0.528	0.687	0.167
	n	18	18	18	18	18	15	15	18	18
α-solanine (2)	**r^2**	0.068	0.131	0.019	0.199	0.180	0.032	-0.134	0.331	0.250
	p	0.788	0.604	0.942	0.428	0.476	0.909	0.634	0.179	0.317
	n	18	18	18	18	18	15	15	18	18
α-chaconine (1)	**r^2**	0.165	0.049	0.015	-0.018	-0.025	-0.093	-0.120	0.084	-0.066
	p	0.512	0.848	0.955	0.945	0.922	0.742	0.671	0.741	0.795
	n	18	18	18	18	18	15	15	18	18
α-chaconine (2)	**r^2**	0.235	0.179	-0.079	0.129	-0.006	0.004	-0.216	0.206	0.145
	p	0.347	0.478	0.757	0.610	0.981	0.990	0.439	0.413	0.567
	n	18	18	18	18	18	15	15	18	18

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Table A2 D. Spearman correlation coefficients (r^2, p-value, n) between the potato glycoalkaloids (α-solanine and α-chaconine) and oxidative status biomarkers glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), total glutathione (tGSH), ratio of reduced and oxidized glutathione (GSH:GSSG), lipid hydroperoxides (LHP) and body mass (g) in the Colorado potato beetle adults *(L. decemlineata)* in the control treatment.

		GST	GPx	GR	CAT	SOD	tGSH	GSH: GSSG	LHP	Body mass
α-solanine (1)	**r^2**	-0.385	-0.005	-0.218	-0.096	0.039	0.010	-0.282	**-0.558**	0.437
	p	0.127	0.985	0.400	0.715	0.881	0.970	0.273	**0.031**	0.070
	n	17	17	17	17	17	17	17	**15**	18
α-solanine (2)	**r^2**	-0.128	-0.135	-0.306	-0.230	-0.326	-0.289	-0.24	0.075	0.385
	p	0.626	0.606	0.232	0.374	0.202	0.260	0.353	0.790	0.115
	n	17	17	17	17	17	17	17	15	18
α-chaconine (1)	**r^2**	-0.299	-0.164	-0.015	-0.341	0.005	0.159	-0.326	-0.329	0.270
	p	0.244	0.529	0.955	0.181	0.985	0.541	0.202	0.231	0.280
	n	17	17	17	17	17	17	17	15	18
α-chaconine (2)	**r^2**	-0.103	-0.174	0.034	-0.279	-0.081	-0.015	-0.123	-0.021	-0.038
	p	0.694	0.504	0.896	0.277	0.758	0.955	0.639	0.940	0.880
	n	17	17	17	17	17	17	17	15	18

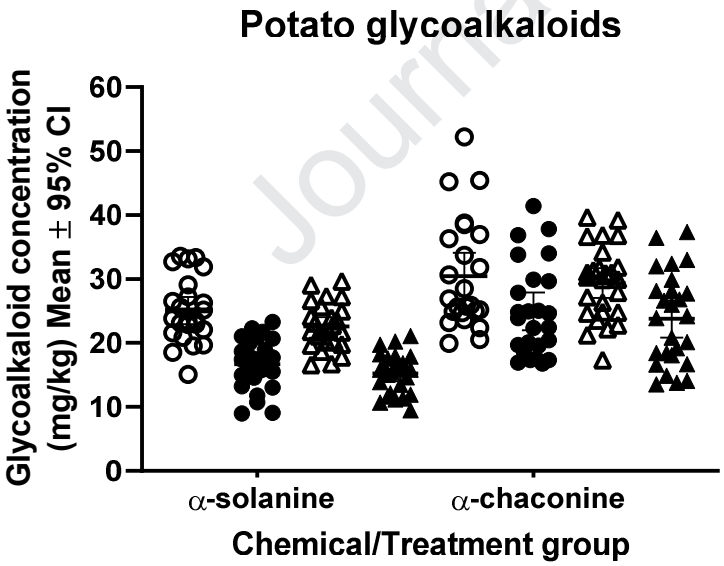

Figure A1. Potato glycoalkaloid (α-solanine and α-chaconine) concentrations (raw data mean ± 95% CI) between the treatment groups (control=circle, GBH treatment=triangle) at two measurement points (measurement 1=white, measurement 2=black).

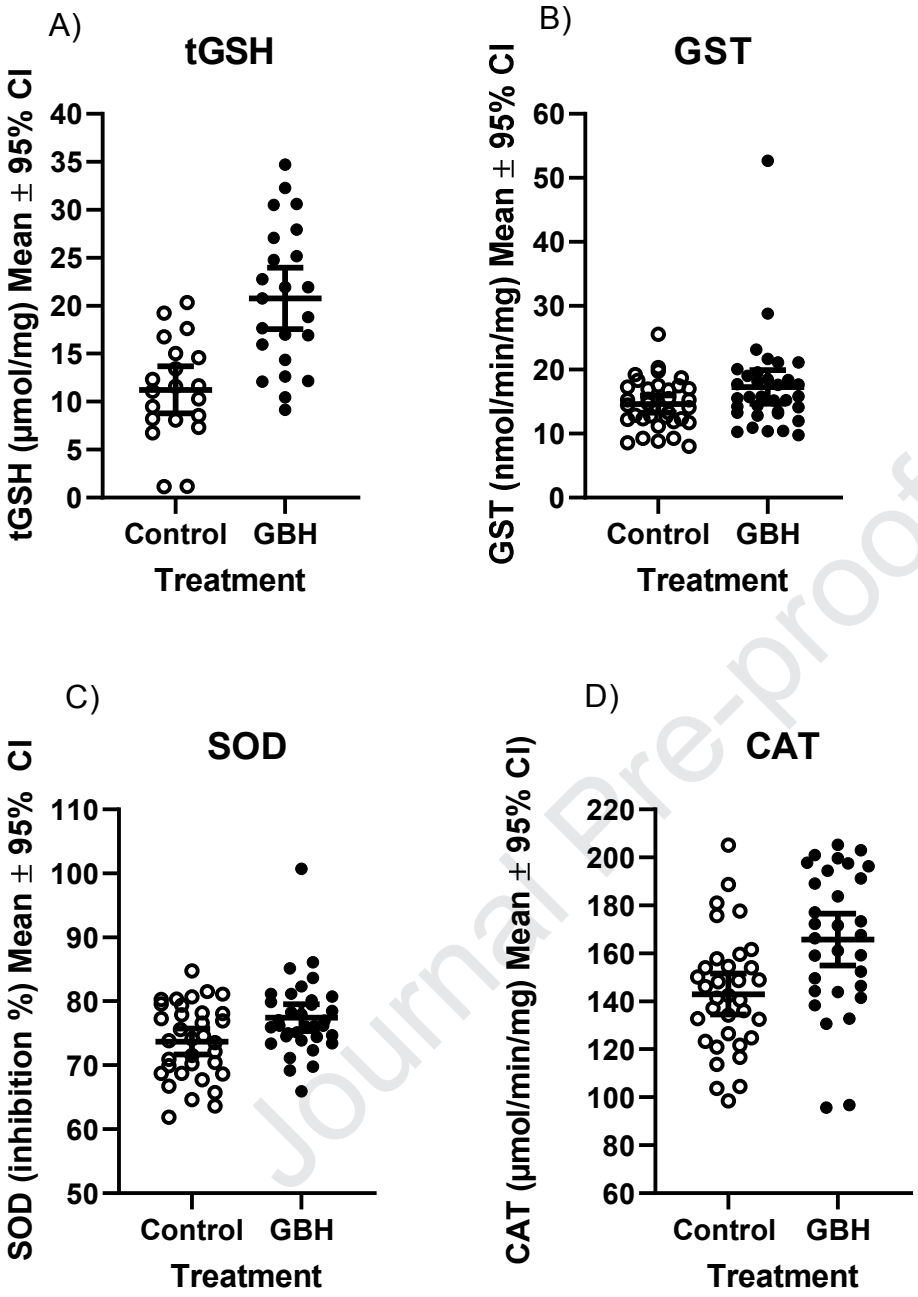

Figure A2. Variation in A) total glutathione (tGSH) concentration, B) glutathione-S-transferase (GST), C) superoxide dismutase (SOD), and D) catalase (CAT) activity in larvae of the Colorado potato beetle (*L. decemlineata*) between treatment groups (control=white circle, GBH treatment=black circle). The dots represent the raw data (mean ± 95% CI).

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Highlights

The α-solanine levels were reduced in potato plants grown in GBH-treated soil.

The survival of the beetles was not affected by the soil-mediated GBH treatment.

Indirect GBH treatment modify the antioxidant defense of the Colorado potato beetle larvae.

Soil-mediated GBH treatment at larval stage may have long-term effects on the adult beetles.

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: