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,

126 in turn, cause cellular biochemical stress, called oxidative stress, and consequent oxidative damage
127 to biomolecules (George and Gatehouse, 2013; Halliwell and Gutteridge, 2007). Previous studies in
128 animals have shown increased oxidative stress or alteration in antioxidant defense systems in relation
129 to various glyphosate-based herbicides (thereafter GBHs; Contardo-Jara et al., 2009; El-Shenawy,
130 2009; Glusczak et al., 2007; Modesto and Martinez, 2010; Rainio et al., 2019; Uren Webster and
131 Santos, 2015). Also, the breakdown products of glutathione (e.g. γ-glutamylglutamine and
132 cysteinylglycine), involved in the regulation of redox balance, have been shown to increase in rats
133 exposed to GBH (Mesnage et al. 2019). Moreover, GBHs have been shown to affect the survival rate,
134 development, and reproduction of invertebrates found in agroecosystems (Benamú et al., 2010;
135 Castilla et al., 2010; Evans et al., 2010; Saska et al., 2016; Schneider et al., 2009), though there are
136 also studies reporting little or no effects (Margus et al., 2019; Salvio et al., 2016; Thompson et al.,
137 2014). The impacts of GBHs on plants and non-target organisms may differ substantially depending
138 on the use of commercial formulations that differ in their surfactant and salts, which are added to
139 enhance the effectiveness of glyphosate. Some adjuvants used in GBHs may be even more toxic than
140 the glyphosate itself (Mesnage et al., 2014). Previous studies have shown that the consequences of
141 GBH use in target ecosystems and their surrounding areas are relatively poorly known and require
142 further studies from a multidisciplinary approach.
143
144 The increasing evidence of glyphosate toxicity on non-target organisms has caused growing concern
145 about the use of glyphosate as the primary weed management strategy (Helander et al., 2012; Torretta
146 et al., 2018; Van Bruggen et al., 2018). The environmental risks of glyphosate are likely to be
147 pronounced in northern ecosystems, which are characterized by long biologically inactive winters
148 and short growing seasons, limiting the time period of peak glyphosate degradation activity to the
149 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-

250 treated) were collected every day, weighed, sexed, and used for oxidative status analyses to study the
251 possible soil-mediated or carry-over effects of the GBH. To analyze potato plant glycoalkaloids,
252 α-solanine and α-chaconine, we took ca 5 leaves per potato plant a) before placing the larvae on the
253 plants (1st measurement) and b) when the larvae had pupated and the shoots had been cut down (2nd
254 measurement). Leaves were freeze-dried, ground (TissueLyser, Qiagen, Austin, TX, USA), and
255 stored in a freezer at -20°C until the chemical analyses. The licenses for rearing quarantine pest
256 species in laboratory conditions were given by the Finnish Food Authority, Finland (Ruokavirasto,
257 permission 4057/0614/2016). Licenses for conducting experiments with insects are not necessary in
258 Finland.
259
260 *2.2. Determination of potato plant defense chemicals*
261
262 For the quantitation of potato plant glycoalkaloids, α-chaconine and α-solanine, 5 mg of ground
263 potato plant leaf material was weighed in a 2 ml Eppendorf tube. Samples were extracted with 2 ml
264 of 5% aqueous acetic acid (5:95, v/v) utilizing overnight maceration in a cold room (4°C) and were
265 shaken with a planar shaker (280 min^{-1}) for 3 hours at room temperature. Extracts were centrifuged
266 (14,000 min^{-1}) for 10 min and decanted into new 2 ml Eppendorf tubes. 100 × dilutions were made
267 with the extraction solvent and samples were filtered via polytetrafluoroethylene filters (13 mm i.d.;
268 0.2 µm) and analyzed with a UHPLC-DAD-ESI-Orbitrap-MS instrument. One of the potato plant leaf
269 extracts was chosen as the quality control sample. It was analyzed before and after every 10 samples
270 to monitor the changes in the performance of the mass spectrometer. The ultrahigh performance liquid
271 chromatograph was coupled to a photodiode array detector (UHPLC-DAD, Waters Corporation,
272 Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher
273 Scientific, Bremen, Germany). ACQUITY UPLC BEH Phenyl (100*2.1 mm i.d., 1.7 µm, Waters
274 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 distributed, lognormal distribution was used in the models. The Spearman correlation coefficient was used to test the correlations between oxidative status parameters, body mass, and potato plant glycoalkaloids for larvae and adult beetles, separately in both treatment groups.

3. Results

3.1. Potato plant defense chemicals

The α-solanine levels were significantly reduced in the potato plants grown in the GBH-treated soil ($F_{df}=6.05_{1, 98}$, p=0.016), and the concentrations differed between the measurement times ($F_{df}=98.08_{1, 98}$, p= <0.001, Fig. 1), being clearly lower at the second measurement. The treatment × measurement time interaction was not significant ($F_{df}=0.44_{1, 97}$, p=0.509). The α-chaconine levels did not differ between the treatment groups ($F_{df}=0.36_{1, 98}$, p=0.552, Fig. 1), but the concentrations differed between the measurement time ($F_{df}=16.17_{1, 98}$, p=0.0001, Fig. 1), being likewise lower at the second measurement. There was no significant treatment × measurement time interaction ($F_{df}=0.02_{1, 97}$, p=0.880). The defense chemicals also correlated with each other. The first measurement of α-solanine correlated positively with the second measurement of α-solanine ($r_P^2=0.64$, p = <0.001) and with the first measurement of α-chaconine ($r_P^2=0.30$, p=0.036); whereas, the second measurement of α-solanine correlated positively with the first ($r_P^2=0.42$, p=0.002) and second measurement ($r_P^2=0.74$, p= <0.001) of α-chaconine. The first measurement of α-chaconine further correlated positively with the second measurement of α-chaconine ($r_P^2=0.61$, p= <0.001).

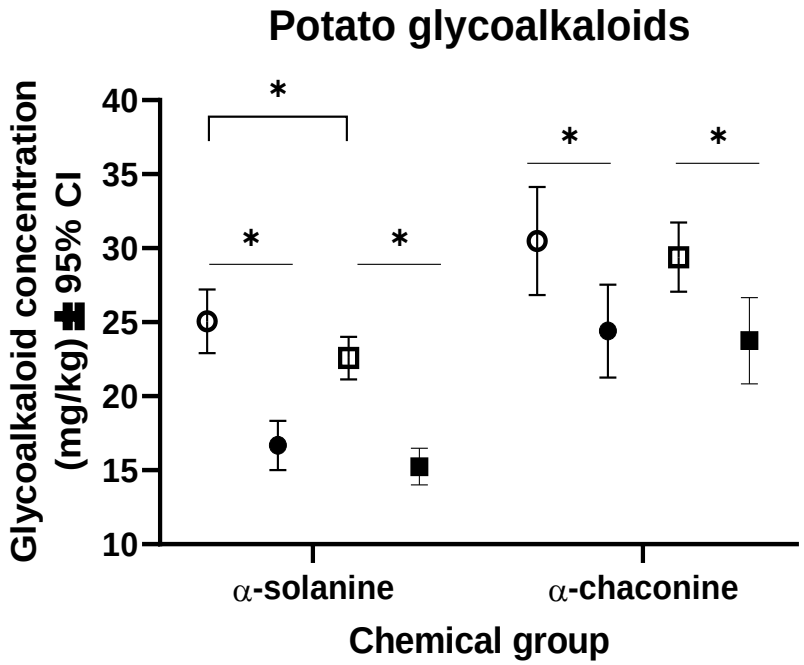
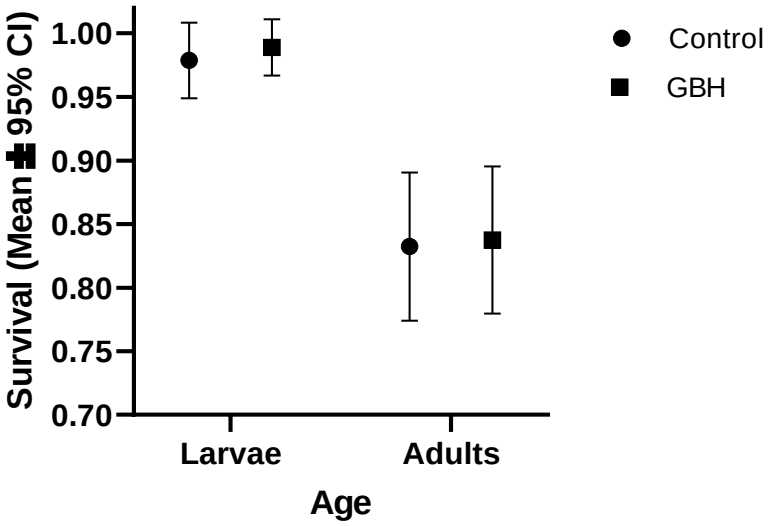


Figure 1. Potato glycoalkaloid (α-solanine and α-chaconine) concentrations (mean ± 95% CI) between the treatment groups (GBH treatment, control) at two measurement points (measurement 1, measurement 2). The color of the symbols indicates measurement time (white=measurement 1, black=measurement 2) and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

3.2. Survival rate and changes in developmental time

GBH treatment had no effect on the survival rate of the Colorado potato beetle larvae or the adult beetles (Fig. 2). The survival rate of the larvae and the adult beetles differed significantly from each other, but there was no treatment × age interaction (Table 1). Larval survival rate in the GBH and control groups was 98.9% and 97.9%, respectively; whereas, adult survival was 83.9% and 83.4%, respectively (Table 1). The body mass of the larvae or the adult beetles was not affected by GBH 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,

409 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)
410 differed between the treatment groups. However, the developmental time of the adult beetles was
411 significantly increased in the GBH-treated group compared to the control group (Table 1). Yet, the
412 estimated difference was only 0.56 days (marginal means: GBH-treated: 30.22, SE: 0.268; control:
413 29.66, SE: 0.268). Developmental time was not affected by sex or sex × treatment interaction (Table
414 1).



415

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

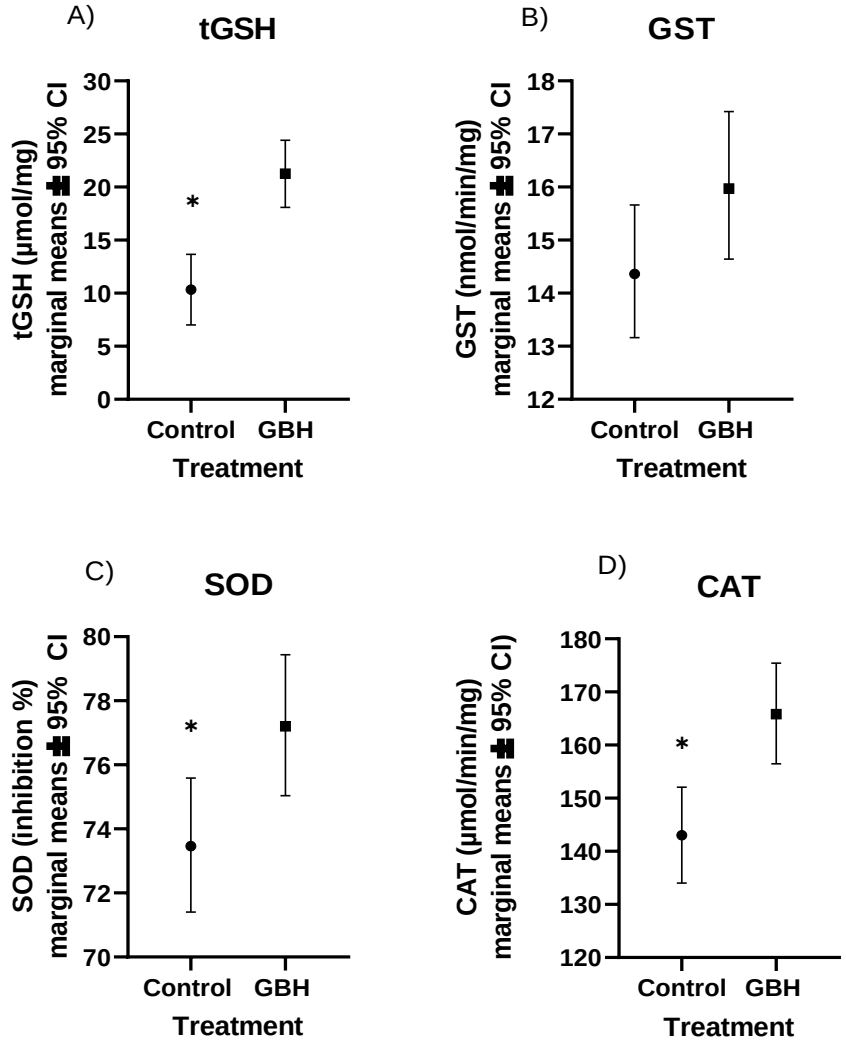
Table 1. The relationship between glyphosate-based herbicide (GBH) treatment and age (larvae and adults) on survival rate of the Colorado potato beetle (*L. decemlineata*). Significant results are indicated in bold.

	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.



436

437 **Figure 3.** Variation in A) total glutathione (tGSH) concentration, B) glutathione-S-transferase (GST),
438 C) superoxide dismutase (SOD), and D) catalase (CAT) activity in larvae of the Colorado potato
439 beetle (*L. decemlineata*) between treatment groups (control=black circle, GBH treatment=black
440 square). The bars represent the marginal means from the models (± 95% CI). The star above the bars
441 indicate significant difference between the treatment groups (generalized linear mixed model,
442 $p<0.05$).

443

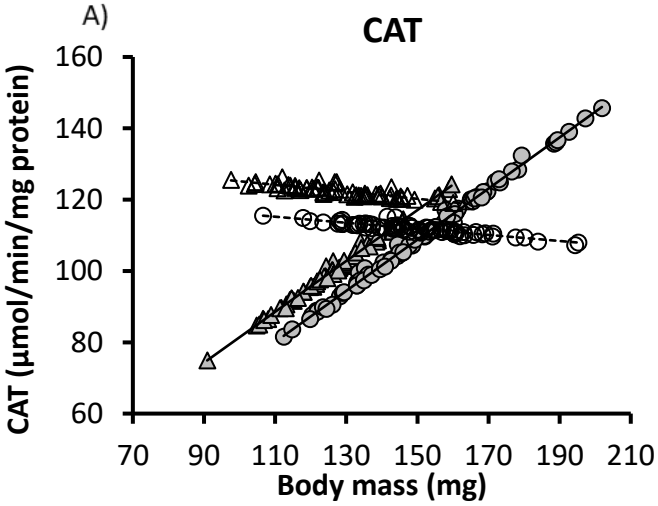
444

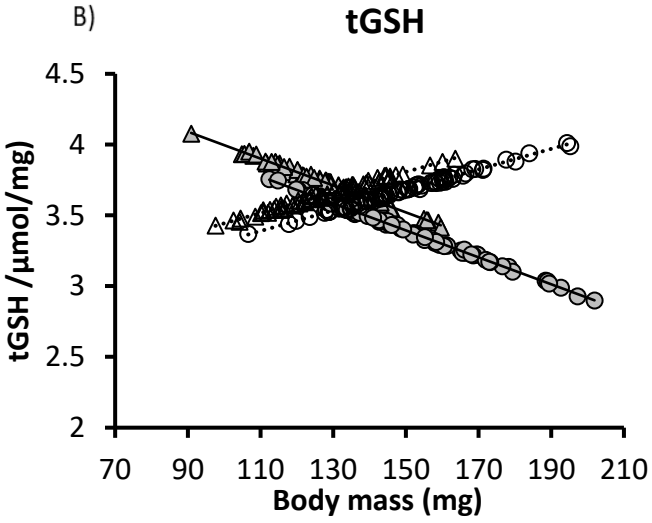
445

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.





458 **Figure 4.** The relationship between oxidative status parameters (CAT and tGSH) and body mass in
459 adult Colorado potato beetles (*L. decemlineata*) indirectly exposed to glyphosate (predicted values
460 from the model; tGSH log transformed values). Legend: white triangle = control male, white
461 circle = control female, grey triangle = GBH male, grey circle = GBH female.
462
463 We further examined the correlations between the oxidative status parameters and potato plant
464 glycoalkaloids in the larvae and the adult beetles separately in both treatment groups. We found that
465 in the GBH treatment group, the CAT activity of the larvae correlated negatively with both
466 measurements of α-chaconine (r_S^2=-0.606, p=0.028 and r_S^2=-0.628, p=0.022, respectively) and with
467 the second measurement of α-solanine (r_S^2=-0.694, p=0.009, Table A2 A). Also, the GST levels of
468 the larvae in the GBH treatment group correlated negatively with the second measurement of α-
469 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).
470 There was also a tendency for a negative correlation between GST and the first measurement of α-
471 chaconine (r_S^2=-0.513, p=0.061, Table A2 A). The GSH:GSSG ratio had a nearly significant negative
472 correlation with the first measurement of α-solanine (r_S^2=-0.592, =0.055, Table A2 A). The larvae in
473 the control group had a negative correlation between CAT and the second measurement of α-solanine
474 (r_S^2=-0.824, p=0.006), and a nearly significant negative correlation between CAT and the second
475 measurement of α-chaconine (r_S^2=-0.656, p=0.055, Table A2 B). There were no significant
476 correlations between the other parameters (p>0.05). In the adult beetles, no correlations between the
477 potato plant glycoalkaloids and oxidative status parameters were shown in the GBH treatment group
478 (p>.05, Table A2 C), but in the control group, LHP correlated negatively with the first measurement
479 of α-solanine (r_S^2=-0.558, p=0.031, Table A2 D). There were no significant correlations between the
480 body mass of the larvae and the adult beetles and the potato plant glycoalkaloids (p>0.05) in either
481 of the treatment groups.
482

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

508 Salminen). However, we cannot entirely rule out the influence of larval feeding or changes caused by
509 potato plant growth on the levels of defense chemicals. For example, Colorado potato beetles have
510 been shown to secrete symbiotic bacteria to suppress plant defenses in tomato plants (*Solanum*
511 *lycopersicum*; Chung et al., 2013), which may apply to potato plant defense chemicals as well.
512 Moreover, GBHs have been shown to affect the growth (Helander et al., 2019) and quality of plants,
513 such as nutrient accumulation (Zobiole et al., 2012) as well as antioxidant defense (Radwan and
514 Fayez, 2016). For example, glyphosate has been shown to lower photosynthesis and reduce protein-
515 and free amino acid levels as well as induce antioxidant enzyme activities (e.g. CAT, SOD and
516 peroxidases) in peanut (*Arachis hypogaea* L. cv. Giza; Radwan and Fayez, 2016). We did not monitor
517 potato plant growth in this study, but Helander et al. (2019) have shown in their greenhouse
518 experiment that potato plants growing in GBH-treated soil had shorter sprouts soon after planting,
519 but the height of the plants did not differ later during the growing season. However, in the field
520 experiment, the potato plant shoot and tuber biomass was 25% and 14% higher, respectively, from
521 plants grown in GBH-treated soil compared to those grown in control soil (Helander et al., 2019).
522
523 *4.2. Survival rate and developmental time*
524
525 Soil-mediated exposure to a GBH had no effect on the survival rate of the Colorado potato beetle
526 larvae or the adult beetles, indicating that the environmentally relevant concentrations used in the soil
527 did not increase mortality during the larval stage or show carry-over or soil-mediated effects in adult
528 beetles. The soil used in our experiment contained some glyphosate residues (glyphosate July: 0.41-
529 0.91 mg/kg, AMPA: 0.24-1.00 mg/kg, certified laboratory, Groen Agro Control, Delfgauw,
530 Netherlands, LC-MS/MS, with a detection limit of 0.01 mg/kg). The glyphosate concentrations of the
531 leaves from the present study were not measured, but potato plant leaves, measured from the potato
532 plants grown outside in the field, had no detectable residues (<0.01 mg/kg), unlike potato tubers

533 (glyphosate: 0.02-0.07 mg/kg, AMPA: 0.06-0.07 mg/kg). The adult beetles were also tested for GBH
534 residues to see whether the GBH accumulates in beetles via food at the larval stage or via soil during
535 the pupal phase. Low levels of AMPA were indeed detected in the beetles (AMPA: 0.11mg/kg,
536 glyphosate: 0.013mg/kg), but the residue levels were low and did not affect the survival rate of the
537 beetles at any developmental stage. Our results are in accordance with some other invertebrate
538 studies, which show no effects of GBHs on survival rate (Baker et al., 2014; Haughton et al., 2001;
539 Michalková and Pekár, 2009; Salvio et al., 2016; Thompson et al., 2014). On the other hand, several
540 studies of invertebrates (Benamú et al., 2010; Castilla et al., 2008; Evans et al., 2010; Janssens and
541 Stoks, 2017; Schneider et al., 2009) have shown either direct mortality effects or sublethal effects
542 when exposed to various GBHs, indicating temporal and dose-dependent effects, as well as species-
543 specific differences in insect susceptibility to GBHs. In our earlier study (Rainio et al., 2019), where
544 the Colorado potato beetle larvae were directly exposed to different concentrations of the GBH, low
545 (environmentally relevant) concentrations had no effect on larval survival rate, whereas high
546 concentrations increased larval mortality.
547
548 In the present study, neither the body mass of the larvae or the newly emerged adult beetles (neither
549 females nor males) was affected by GBH treatment, which was expected since the larvae never come
550 in direct contact with the GBH, supporting the finding that the GBH does not affect the beetles'
551 survival rate. However, the developmental time of the adult beetles increased significantly in the
552 GBH treatment group compared to the control group, but the difference (0.56 days) was rather low
553 in a biological sense and likely does not have notable effects on the overall survival rate of the beetles.
554 In general, the Colorado potato beetle tolerates pesticides relatively well, and has developed
555 resistance to several synthetic insecticides, including organophosphates (Kostic et al., 2016; Piiroinen
556 et al., 2013), used as a control method in potato farms. The metabolic adaptation is manifested by a
557 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

583 exposed to Roundup Ultra (Contardo-Jara et al., 2009), and increased CAT activity in teleost fish
584 exposed to GBHs (Samanta et al., 2014). Our previous direct exposure study of Colorado potato
585 beetles (Rainio et al. 2019) did not show any differences in those same markers of oxidative status,
586 which may be related to the exposure time or the absorption of the GBH by the beetles' bodies
587 (absorption through the cuticle and epidermis vs. via food or soil).
588
589 In addition to enzyme activity, tGSH concentrations in the larvae were elevated in the GBH treatment
590 group compared to the control group. GSH protects cells from oxidative stress by scavenging and
591 neutralizing ROS and simultaneously converting them to GSSG (Halliwell and Gutteridge, 2007;
592 Singh, 2002). The detoxification capacity of GSH is related to its reduced thiol group, and thus the
593 reduced form is the most important in resisting oxidative stress (Singh, 2002). Larsen et al. (2012)
594 reported elevated GSH concentrations in rats exposed to GBHs via drinking water, while some other
595 studies have shown the opposite trend (El-Shenawy, 2009). Increased GSH synthesis, as an adaptive
596 response during moderate oxidative stress, has been previously reported in aquatic organisms by
597 Slaninová et al. (2009). Furthermore, GSH has been suggested to be depleted after short periods of
598 oxidative stress, but elevated after long-term exposure to oxidants (Slaninová et al., 2009). The
599 contradictory results highlight the species- (see also Berglund et al., 2014; Rainio et al., 2013;) and
600 tissue-specificity (Yang et al., 2013) of antioxidant defense, but also the use of various GBHs, the
601 dose and the susceptibility of different species to GBH exposure may induce opposite results. In the
602 present study, the GSH:GSSG ratio and the LHP levels of the larvae did not differ between the
603 treatment groups, suggesting that the increased tGSH level, together with up-regulated enzyme
604 activities, has been effective enough in keeping the cellular redox balance (i.e., GSH:GSSG ratio)
605 stable (Lushchak, 2012). However, the long-term up-regulation of antioxidant enzyme activity is
606 energetically costly and may, in the long-term, increase oxidative stress, ultimately trading-off with
607 the overall survival rate and fitness of the beetles.

608

609 The effect of body mass on oxidative status parameters was further studied in the larvae and the adult
610 beetles, since it has been previously shown that the enzyme activity can be linked to body mass, which
611 is often associated with overall animal condition (Koivula et al., 2011; Rainio et al., 2015). In the
612 larvae (as also in the adults), the body mass had a negative association with GSTs, meaning that the
613 lighter larvae had higher GST activity compared to heavier larvae. It is possible that, in general, the
614 lighter larvae that are in poorer condition need to up-regulate GST activity more for detoxification
615 processes, which may be energy demanding, than the heavier ones that are in better condition. A
616 similar results between the antioxidant enzyme activities of GPx, SOD, and CAT and body mass have
617 been found in birds, such as the great tit (*Parus major*), when exposed to metal pollution (Rainio et
618 al., 2015). The larvae further showed a positive association between body mass and tGSH
619 concentrations, meaning that heavier larvae had higher tGSH levels, which is opposite to what we
620 found for GST. However, it may be that the heavier larvae can produce more GSH in their system,
621 reflecting better antioxidant capacity, compared to the lighter larvae that are in poorer condition.

622

623 In this study, we were able to follow the individuals from the larvae to the adult stage to examine the
624 long-term effects of early-life GBH exposure. The GBH directly decreased the oxidative status
625 parameters CAT and tGSH in the adult beetles, and there was a significant treatment × body mass
626 interaction. In the adult beetles, CAT activity (and GR activity to some extent) increased with body
627 mass in the GBH treatment group, but decreased in the control group. The opposite was shown for
628 tGSH, where the levels increased with body mass in the control group, but decreased in the GBH
629 treatment group. The higher CAT activity of the heavier adult beetles in the GBH treatment group
630 may be due to being in better condition, allowing them to allocate more resources for their defense in
631 case of increased ROS production compared to lighter ones that are in poorer condition. However, in
632 the controls, the body mass may not be so critical since their activities stay rather constant.

The increased tGSH levels may reflect the better condition of heavier adults in the control group; whereas, in the GBH treatment group, the decreased tGSH levels may suggest either lesser need of tGSH (e.g. due to up-regulated enzyme activities) or more rapid transformation of GSH to GSSG to cope with the potential increase in ROS production. This is further supported by the higher GR activity in the bigger adults than the smaller ones in the GBH treatment group, since the main function of GR is to transform oxidized GSH (i.e. GSSG) back to its reduced form (GSH; Halliwell and Gutteridge, 2007). The results suggest that the early-life indirect GBH exposure via diet may show some long-term effects on the adult beetles. On the other hand, the pupa may also be directly exposed to GBH residues during their 2-week pupal stage in the soil, which can partly explain the observed effects on the adults' physiology and developmental time between the treatment groups. In future, it would be important to concentrate more on the plant-mediated effects and separate them from the soil-mediated effects at the pupal stage, and, moreover, extend the studies to observe the following breeding season to see whether the GBH affects the overwintering and reproduction success of the adult beetles later in life.

We also examined the relationships between oxidative status parameters and potato plant glycoalkaloids separately in larvae and the adult beetles to see whether these chemicals affect the beetle's oxidative status. We found that for the larvae in the GBH treatment group (as also in the control group), the activity of CAT and GST correlated negatively with α-solanine and α-chaconine levels, either with both of the measurements (before and after larval feeding) or with only one of the measurements. Interestingly, these are the same parameters that were affected by GBH treatment in larvae, but in the opposite direction. The GST and CAT activity decreased with increased α-solanine and α-chaconine levels, but increased with GBH treatment. The results are logical, since the lower α-solanine levels were shown in the GBH treatment group with higher antioxidant enzyme activity. The

observed changes in antioxidant defense of the beetles can be derived from the GBH itself or from the GBH-mediated effects on potato glycoalkaloid levels, in case the glycoalkaloids affect the potato quality as food items. The α-solanine has been previously shown to increase lipid peroxidation (measured as malondialdehyde [MDA] concentration) and GST activity in the mid-gut, but decrease the GST activity in body fat in Lepidoptera, such as *G. mellonella*, indicating the oxidative activity of glycoalkaloids (Adamski et al., 2014). Furthermore, GSH:GSSG ratio had a similar tendency for a negative correlation with only the first measurement of α-solanine (see table S2), reflecting the increased oxidation of GSH to GSSG in the higher concentrations of glycoalkaloids. In the adult beetles, on the other hand, none of the oxidative status parameters correlated with potato plant glycoalkaloids. Even though both potato plant defense chemicals and GBH treatment seemed to affect the same oxidative status parameters of the beetle larvae (e.g., GST, CAT), we cannot say for sure whether they show additive or synergistic effects on the beetles. More experimental studies with different concentrations of glycoalkaloids and GBHs would be needed to understand the complex combined effects of glycoalkaloids and GBHs on the oxidative status parameters of the beetles.

4.4. Conclusions

The reduction of α-solanine levels in potato plants grown in GBH-treated soil suggests the potential reduction of potato plant defense against the Colorado potato beetle, but more dose-dependent 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,

683 which could in turn delay the developmental time. In the adult beetles, CAT activity and tGSH levels
684 were affected by the interactive effect of GBH treatment and body mass of the adult beetles,
685 suggesting that the early-life glyphosate treatment or soil-mediated effects at the pupal stage may
686 have long-term effects on the adult beetles. Our results highlight the importance of measuring the
687 physiological parameters, such as oxidative status, along with life-history traits in sublethal herbicide
688 studies, since they may be important factors in affecting the health and survival of animals. In future,
689 it would be important to extend the monitoring of the adult beetles to the following breeding season,
690 to study the effects of GBHs on fertility, reproductive success, and overwinter survival rate of the
691 adult beetles.
692
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698
699 **Conflicts of Interest**
700
701 The authors declare no conflict of interest.
702
703 **Credit Author Statement**
704
705 **Miia J. Rainio:** Study design, conducting experiment, biochemical analyses, statistical analyses,
706 manuscript writing. **Aigi Margus:** Study design, experiment preparation, manuscript editing.
707 **Valtteri Virtanen:** Glycoalkaloid analyses, manuscript editing. **Leena Lindström:** Study design,

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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

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

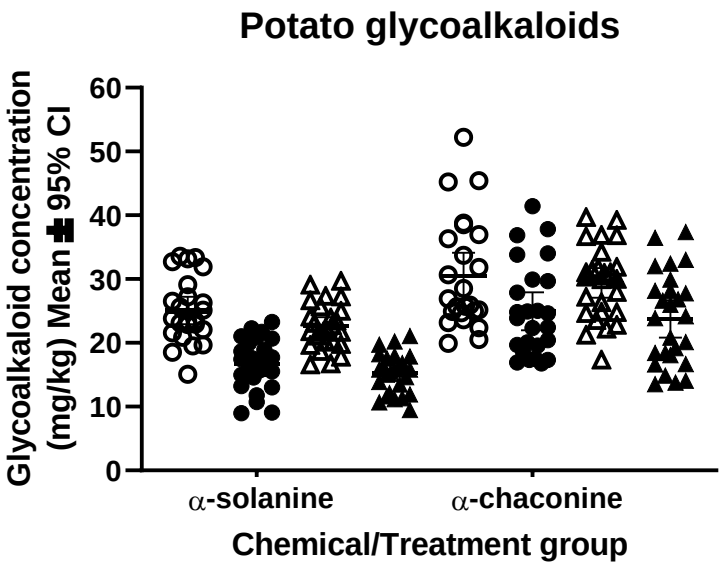

Figure A1. Potato glycoalkaloid (α-solanine and α-chaconine) concentrations (raw data mean ± 95% CI) between the treatment groups (control=circle, GBH treatment=triangle) at two measurement points (measurement 1=white, measurement 2=black).

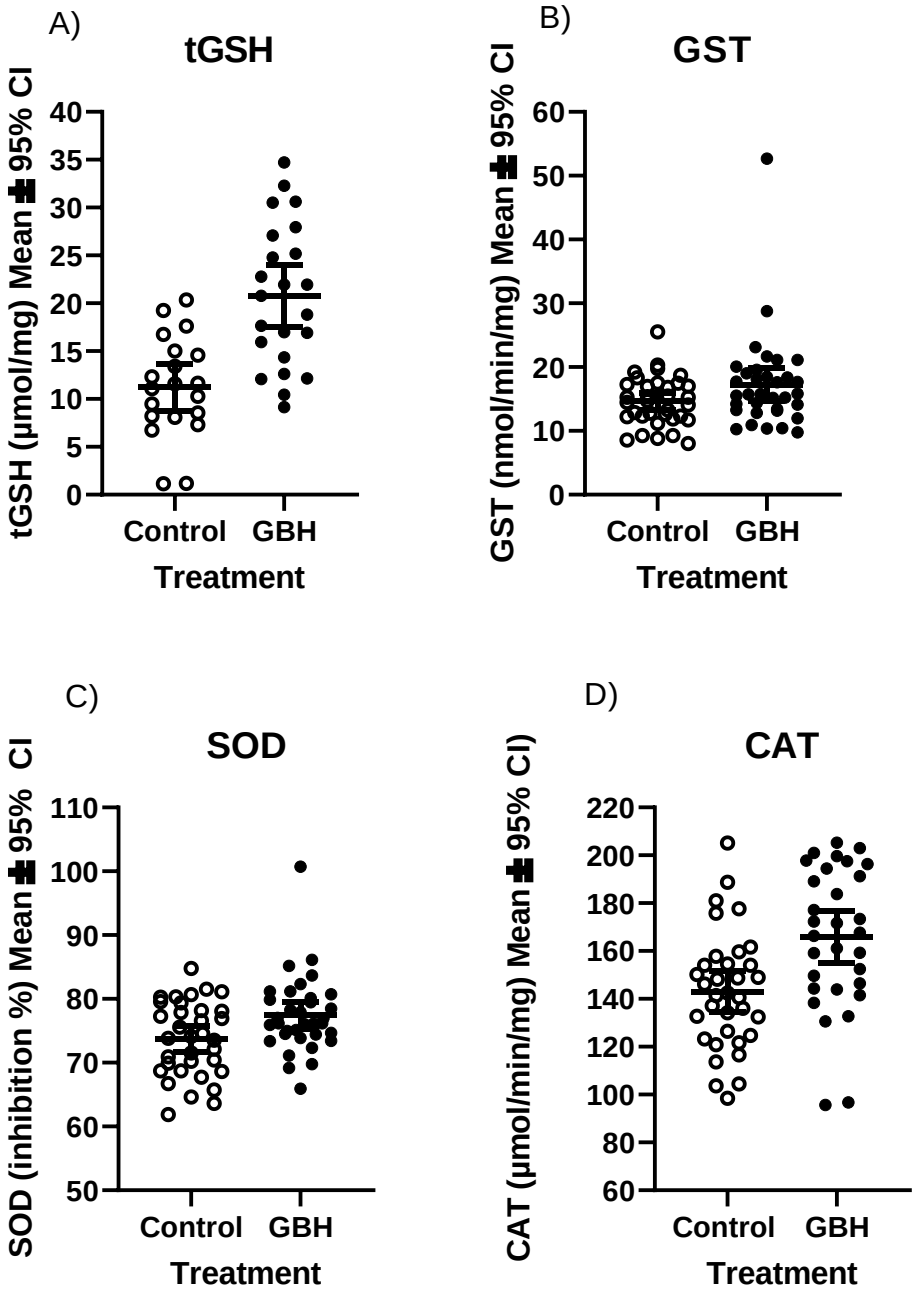

Figure A2. Variation in A) total glutathione (tGSH) concentration, B) glutathione-S-transferase (GST), C) superoxide dismutase (SOD), and D) catalase (CAT) activity in larvae of the Colorado potato beetle (*L. decemlineata*) between treatment groups (control=white circle, GBH treatment=black circle).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