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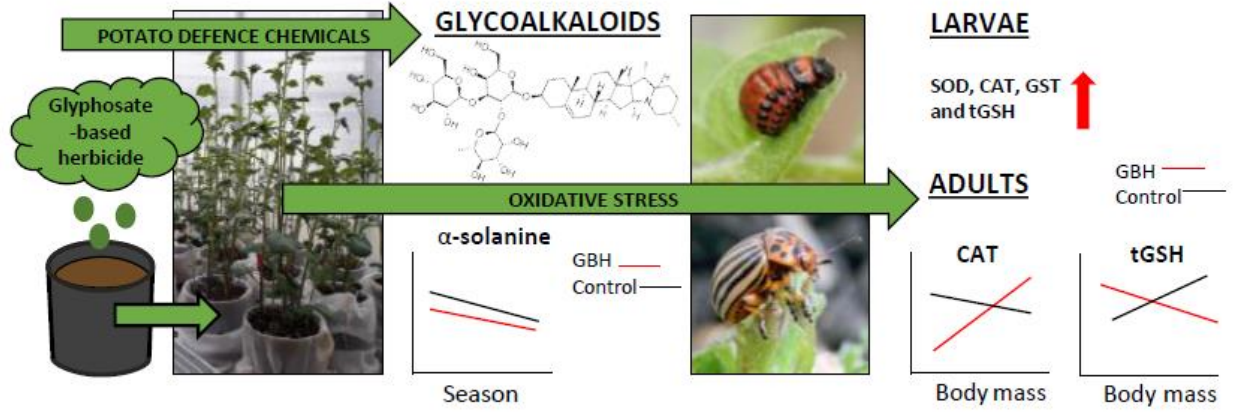
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Credit Author Statement

Miia J. Rainio: Study design, conducting experiment, biochemical analyses, statistical analyses, manuscript writing. **Aigi Margus:** Study design, experiment preparation, manuscript editing.

Valteri 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.



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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

26 **Highlights**

27

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

29

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

31

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

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

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51 **Abstract**

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53 Glyphosate is the most used herbicide worldwide, targeting physiological pathways in plants.
54 Recent studies have shown that glyphosate can also cause toxic effects in animals. We investigated
55 the glyphosate-based herbicide (GBH)-induced changes in potato (*Solanum tuberosum*) plant
56 chemistry and the effects of a GBH on the survival rate and oxidative status of the Colorado potato
57 beetle (*Leptinotarsa decemlineata*). The beetles were reared on potato plants grown in pots
58 containing soil treated with a GBH (Roundup Gold, 450 g/l) or untreated soil (water control). The
59 2nd instar larvae were introduced to the potato plants and then collected in 2 phases: as 4th instar
60 larvae and as adults. The main glycoalkaloids of the potato plants, α -solanine and α -chaconine,
61 were measured twice during the experiment. The α -solanine was reduced in potato plants grown in
62 GBH-treated soil, which can be detrimental to plant defenses against herbivores. GBH treatment
63 had no effect on the survival rate or body mass of the larvae or the adult beetles. In the larvae, total
64 glutathione (tGSH) concentration and the enzyme activity of catalase (CAT), superoxide dismutase,
65 and glutathione-S-transferase were increased in the GBH treatment group. In the adult beetles, CAT
66 activity and tGSH levels were affected by the interactive effect of GBH treatment and the body
67 mass. To conclude, environmentally relevant concentrations of a GBH can affect the potato plant's
68 glycoalkaloid concentrations, but are not likely to directly affect the survival rate of the Colorado
69 potato beetle, but instead, modify the antioxidant defense of the beetles via diet.

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

75

76 **1. Introduction**

77

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

91

92 Glyphosate is also the most important herbicide directly affecting the synthesis of secondary
93 compounds in plants (Duke and Powles, 2008). The glyphosate-based reduction of secondary
94 compounds in plants (i.e., defense chemicals) may expose plants to herbivore attacks; influence the
95 flavor-producing chemicals important in herbivore behavior or food quality (El-keltawi and
96 Croteau, 1987); and reduce plant resistance to pathogens and fungal infections (Lydon and Duke,
97 1989). On the other hand, glyphosate may also increase the production of plant secondary
98 metabolites (Ossipov et al. 2003). Overall, the sub-lethal effects of herbicides on non-target plants
99 may affect agricultural ecosystems by altering the synthesis of compounds that are important in
100 inter- and intraspecific interactions (Lydon and Duke, 1989). Plant-herbivore interactions are

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

103

104 Glyphosate is the only herbicide affecting the inactivation of the 5-enolpyruvylshikimate-3-
105 phosphate synthase (EPSPS) enzyme (Duke and Powles, 2008; Steinrücken and Amrhein, 1980).

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

122

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

126 (Annett et al., 2014; Hultberg, 2007; Modesto and Martinez, 2010; Uren Webster and Santos,
127 2015), which can, in turn, cause cellular biochemical stress, called oxidative stress, and consequent
128 oxidative damage to biomolecules (George and Gatehouse, 2013; Halliwell and Gutteridge, 2007).
129 Previous studies in animals have shown increased oxidative stress or alteration in antioxidant
130 defense systems in relation to various glyphosate-based herbicides (thereafter GBHs; Contardo-Jara
131 et al., 2009; El-Shenawy, 2009; Gluszczak et al., 2007; Modesto and Martinez, 2010; Rainio et al.,
132 2019; Uren Webster and Santos, 2015). Also, the breakdown products of glutathione (e.g. γ -
133 glutamylglutamine and cysteinylglycine), involved in the regulation of redox balance, have been
134 shown to increase in rats exposed to GBH (Mesnage et al. 2019). Moreover, GBHs have been
135 shown to affect the survival rate, development, and reproduction of invertebrates found in
136 agroecosystems (Benamú et al., 2010; Castilla et al., 2010; Evans et al., 2010; Saska et al., 2016;
137 Schneider et al., 2009), though there are also studies reporting little or no effects (Margus et al.,
138 2019; Salvio et al., 2016; Thompson et al., 2014). The impacts of GBHs on plants and non-target
139 organisms may differ substantially depending on the use of commercial formulations that differ in
140 their surfactant and salts, which are added to enhance the effectiveness of glyphosate. Some
141 adjuvants used in GBHs may be even more toxic than the glyphosate itself (Mesnage et al., 2014).
142 Previous studies have shown that the consequences of GBH use in target ecosystems and their
143 surrounding areas are relatively poorly known and require further studies from a multidisciplinary
144 approach.

145

146 The increasing evidence of glyphosate toxicity on non-target organisms has caused growing
147 concern about the use of glyphosate as the primary weed management strategy (Helander et al.,
148 2012; Torretta et al., 2018; Van Bruggen et al., 2018). The environmental risks of glyphosate are
149 likely to be pronounced in northern ecosystems, which are characterized by long biologically
150 inactive winters and short growing seasons, limiting the time period of peak glyphosate degradation

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

154

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

166

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

176 plant glycoalkaloids have been previously shown to reduce the growth rate and food consumption
177 rate in the khapra beetle (*Trogoderma granarium*; Nenaah, 2011), decrease reproduction rates in the
178 potato aphid (*Macrosiphum euphorbiae*; Güntner et al., 1997); decrease fertility, survival rate, and
179 hatchability in the greater wax moth (*Galleria mellonella*; Adamski et al., 2014); and increase
180 mortality in peach potato aphids (*Myzus persicae*; Fragoyiannis et al., 1998). On the other hand, it is
181 possible that under a certain threshold level of foliage glycoalkaloids, the herbivores may still feed
182 and reproduce (Khan et al., 2013). Colorado potato beetle larvae have shown either negative (Hare,
183 1987) or no response (Kowalski et al., 1999) in relation to glycoalkaloids, suggesting that the
184 effects of glycoalkaloids may vary with the life-stage of the beetle or the length of exposure
185 (Lyytinen et al., 2007).

186
187 To examine the soil-mediated effects of the GBH on the oxidative status of the beetles, we
188 measured antioxidant glutathione (total glutathione, tGSH) and the ratio of its reduced and oxidized
189 form (GSH:GSSG). Glutathione (GSH) is one of the most important small antioxidant molecules in
190 almost all organisms (Andrews, 2000) and the GSH:GSSG ratio, which indicates the overall redox
191 status of cells, is commonly used as an indicator of oxidative stress (Halliwell and Gutteridge, 2007;
192 Isaksson et al., 2005; Rainio et al., 2013). In addition, we measured the activity of insect homologs'
193 antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR), as well as
194 glutathione-S-transferases (GSTs) related to GSH metabolism (Halliwell and Gutteridge, 2007).
195 GSTs are a ubiquitous and important family of enzymes (isozymes) participating in detoxification
196 processes by catalyzing the conjugation of GSHs on xenobiotics (Alghamdi and Frey, 2017;
197 Halliwell and Gutteridge, 2007) and showing the peroxidative activity function in insects (Corona
198 and Robinson, 2006; Farjan et al., 2012). ROS regulation enzymes, superoxide dismutase (SOD)
199 and catalase (CAT), were measured to study first-line antioxidant defense (Fridovich, 1974), where
200 superoxides are transformed to hydrogen peroxide (H₂O₂) by SOD and further catalyzed to water

201 (H₂O) and molecular oxygen by CAT (Finkel and Holbrook, 2000; Pinto et al., 2003). To determine
202 oxidative damage, we measured lipid hydroperoxides (LHP), which have been suggested to increase
203 with ROS production. Lipid peroxidation can be harmful in insects, because, in addition to being
204 essential components in cell membranes, they also have unique physiological functions (e.g., in
205 developmental and reproductive physiology; Downer, 1985).

206

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

219

220 **2. Materials and methods**

221

222 *2.1. Study design*

223

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

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

242

243 We used the United States (Vermont) Colorado potato beetle population collected from the field
244 ($44^{\circ}43' \text{N}$, $73^{\circ}20' \text{W}$) in 2010, which had been since grown in laboratory conditions (see Lehmann et
245 al., 2015). Altogether, 500 Colorado potato beetle larvae (250 larvae/treatment group, 30
246 larvae/family) from 16 families (full-sib design) were used in this experiment. After 3.5 weeks of
247 the potato planting, small 2-day-old larvae (2st instar) were randomly introduced to the potato plants
248 (5 larvae to each plant), which were covered by light-permeable fabric bags. After 9 days, when the
249 larvae were at their 4th instar, 184 larvae (94 controls, 90 GBH-treated) were collected, weighed,
250 and stored in a freezer at -80°C for oxidative status analyses. The remaining larvae were grown

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

262

263 *2.2. Determination of potato plant defense chemicals*

264

265 For the quantitation of potato plant glycoalkaloids, α -chaconine and α -solanine, 5 mg of ground
266 potato plant leaf material was weighed in a 2 ml Eppendorf tube. Samples were extracted with 2 ml
267 of 5% aqueous acetic acid (5:95, v/v) utilizing overnight maceration in a cold room (4°C) and were
268 shaken with a planar shaker (280 min⁻¹) for 3 hours at room temperature. Extracts were centrifuged
269 (14,000 min⁻¹) for 10 min and decanted into new 2 ml Eppendorf tubes. 100 × dilutions were made
270 with the extraction solvent and samples were filtered via polytetrafluoroethylene filters (13 mm i.d.;
271 0.2 μ m) and analyzed with a UHPLC-DAD-ESI-Orbitrap-MS instrument. One of the potato plant
272 leaf extracts was chosen as the quality control sample. It was analyzed before and after every 10
273 samples to monitor the changes in the performance of the mass spectrometer. The ultrahigh
274 performance liquid chromatograph was coupled to a photodiode array detector (UHPLC-DAD,
275 Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q

276 Exactive, Thermo Fisher Scientific, Bremen, Germany). ACQUITY UPLC BEH Phenyl (100*2.1
277 mm i.d., 1.7 μ m, Waters Technologies Ireland, Wexford, Ireland) columns were utilized. The
278 mobile phase consisted of acetonitrile (A) and 0.1% aqueous formic acid (99.9:0.1, v/v) (B): 0-0.5
279 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
280 heated electrospray ionization (ESI) source (H-ESI, Thermo Fisher Scientific, Bremen, Germany)
281 was operated in the positive ion mode. Source parameters were as follows: spray voltage, +3.8 kV;
282 sheath gas (N₂) flow rate, 60 (arbitrary units); auxiliary gas (N₂) flow rate, 20 (arbitrary units);
283 sweep gas flow rate, 0 (arbitrary units); capillary temperature, 380°C. The Orbitrap spectrometer
284 was operated with a resolution of 35,000 and a mass range of m/z 150-2250. Data processing was
285 done using Thermo Xcalibur Quan Browser software (Version 4.1.31.9, Thermo Fisher Scientific,
286 Waltham, MA, USA). Concentrations of α -chaconine and α -solanine in samples were quantified
287 using external calibration curves made from the commercial standards of both α -chaconine and α -
288 solanine (Carbosynth, Compton, UK).

289

290 2.3. Oxidative status analyses

291

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

300

301 Samples were homogenized individually (TissueLyser, Qiagen, Austin, TX, USA) with 180 μ l
302 (larvae) or 150 μ l (adults) KF buffer (0.1 M K_2HPO_4 + 0.15 M KCl, pH 7.4). The protein
303 concentration (mg/ml) was measured with bicinchoninic acid (BCA) protein assay (Smith et al.,
304 1985) using bovine serum albumin (BSA) as a standard (Sigma-Aldrich Finland, Espoo, Finland)
305 with an EnVision[®] microplate reader at an absorbance of 570 nm.

306
307 GST assay (Sigma-Aldrich CS0410) was adjusted from a 96- to 384-well plate. We used 2 μ l of
308 each sample in triplicate and our own reagents: Dulbecco's phosphate-buffered saline (DPBS), 200
309 mM GSH (Sigma G4251), and 100 mM 1-Chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich
310 C6396) in ethanol. The change in absorbance was measured at 340 nm. GPx assay (Sigma-Aldrich
311 CGP1) was adjusted from a cuvette to a 384-well plate and the activity was measured according to
312 kit instructions, using 2 mM H_2O_2 instead of t-Bu-OOH as a substrate (see details in Rainio et al.,
313 2019). The change in absorbance was measured at 340 nm. GR-assay (Sigma-Aldrich GR-SA) was
314 adjusted from a cuvette to a 384-well plate and modified from the kit instructions by using our own
315 reagents: assay buffer (100 mM potassiumphosphate buffer + 1 mM EDTA, pH 7.5), 2 mM GSSG
316 (Sigma-Aldrich GG4626), 3 mM DTNB (Sigma-Aldrich D8130), and 2 mM NADPH (Sigma-
317 Aldrich N1630). The change in absorbance was measured at 412 nm. SOD assay (Sigma-Aldrich
318 19160) was adjusted from 96- to 384-well plate and measured according to kit instructions. We
319 used 0.3 mg/ml sample dilution and the activity was expressed as inhibition % at an absorbance of
320 450 nm. CAT-assay (Sigma-Aldrich CAT100) was adjusted from a cuvette to a 96-well plate. We
321 used 0.6 mg/ml sample dilution and tested each sample in triplicate. We made our own reagents: 10
322 \times CAT assay buffer (500 mM KF, pH 7.0), CAT dilution buffer (50 mM KF + 0.1% TritonX, pH
323 7.0), chromogen reagent (0.25 mM 4-aminoantipyrine + 2 mM 3,5-dicloro-2-
324 hydroxybenzenesulfonic acid in 150 mM potassium phosphate buffer, pH 7.0), peroxidase solutions
325 (from horseradish), stop solution (15 mM NaN_3 , Sigma-Aldrich), and 200 mM and 10 mM H_2O_2

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

333

334 For the LHP measurement, the larvae were first weighed and then homogenized with 125 μ l
335 methanol. LHP were measured using the FOX-II method, modified from Nourooz-Zadeh et al.
336 (1995) and Bou et al. (2008). We used 45 μ l of the sample, 5 μ l 10 mM thiamine pyrophosphate
337 (TPP) or methanol, and 950 μ l of FOX reagent (see also Vuori et al., 2015). Cumene hydroperoxide
338 (0/8/16/32/64/96/128/160 mM, Sigma-Aldrich, USA) was used as a standard (see more details in
339 Rainio et al. 2019). The absorbance was measured at 570 nm. The results were set against the
340 weight of the body mass of the beetles.

341

342 *2.4. Statistics*

343

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

351

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

357

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

365

366 To examine the effects of GBH treatment on the oxidative status of the beetles, we performed a
367 GLMM (with lognormal distribution and identity link function, except for CAT and tGSH [for
368 larvae only], in which we used Gaussian distribution and identity link function) for each parameter,
369 separately for larvae and the adult beetles, using treatment (GBH treatment, control), body mass,
370 treatment \times body mass, sex (female, male, adults only), and treatment \times sex (adults only) as
371 explanatory variables. Family was used as a random factor in the models when applicable (larvae:
372 GST, GR, SOD, tGSH, LHP; adults: GP, CAT, tGSH). Non-significant terms were dropped
373 sequentially from the final model, but the main effect of treatment was always kept in the model, as
374 this was our main study question. Degrees of freedom were calculated as mentioned above. Prior to
375 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**

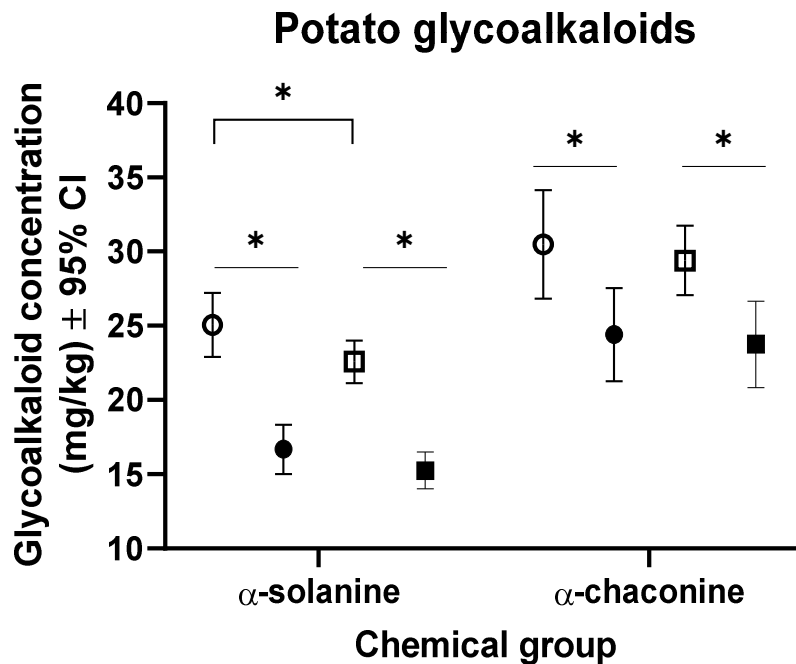
381

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 \times
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 \times 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 \pm 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, $p < 0.05$).

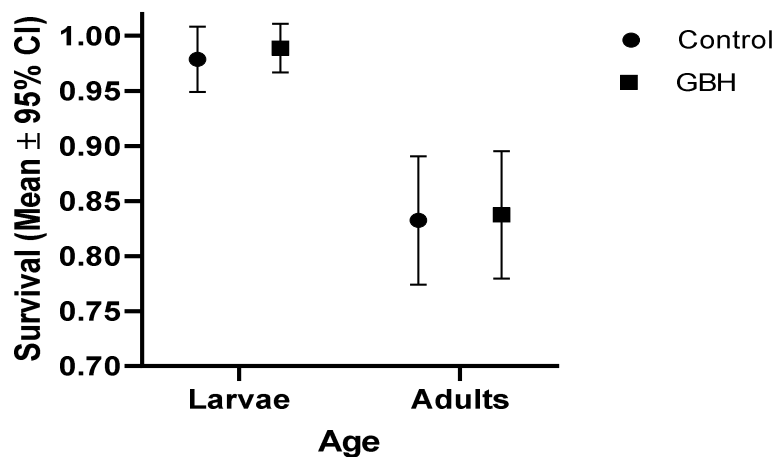
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406 3.2. Survival rate and changes in developmental time

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 \times 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 \times treatment interaction
 419 (Table 1).



420
 421 **Figure 2.** Survival of the Colorado potato beetle (*L. decemlineata*) larvae (2nd instar to 4th instar)
 422 and adults (2nd instar to adult) between the treatment groups (control=black circle, GBH
 423 treatment=black square). The bars represent mean survival (\pm 95% CI) 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 (<i>L. decemlineata</i>). 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 \times 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 \times sex	0.19 _{1, 252.9}	0.667

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

427 ** GLMM with Gaussian distribution and identity link function, family used as a random factor in the
428 model.

429

430 3.3. Oxidative status

431

432 Oxidative status parameters (GR and GPx homologs, GST, tGSH, GSH:GSSG, CAT, SOD and
433 LHP) were analyzed separately between the developmental stages (larvae, adults, Table A1).

434 Oxidative status parameters of the larvae were associated with GBH treatment and body mass, but

435 the body mass \times treatment interaction was not associated with any of the oxidative status

436 parameters (Table 2). In the larvae, tGSH concentration and the activity of GST, CAT, and SOD

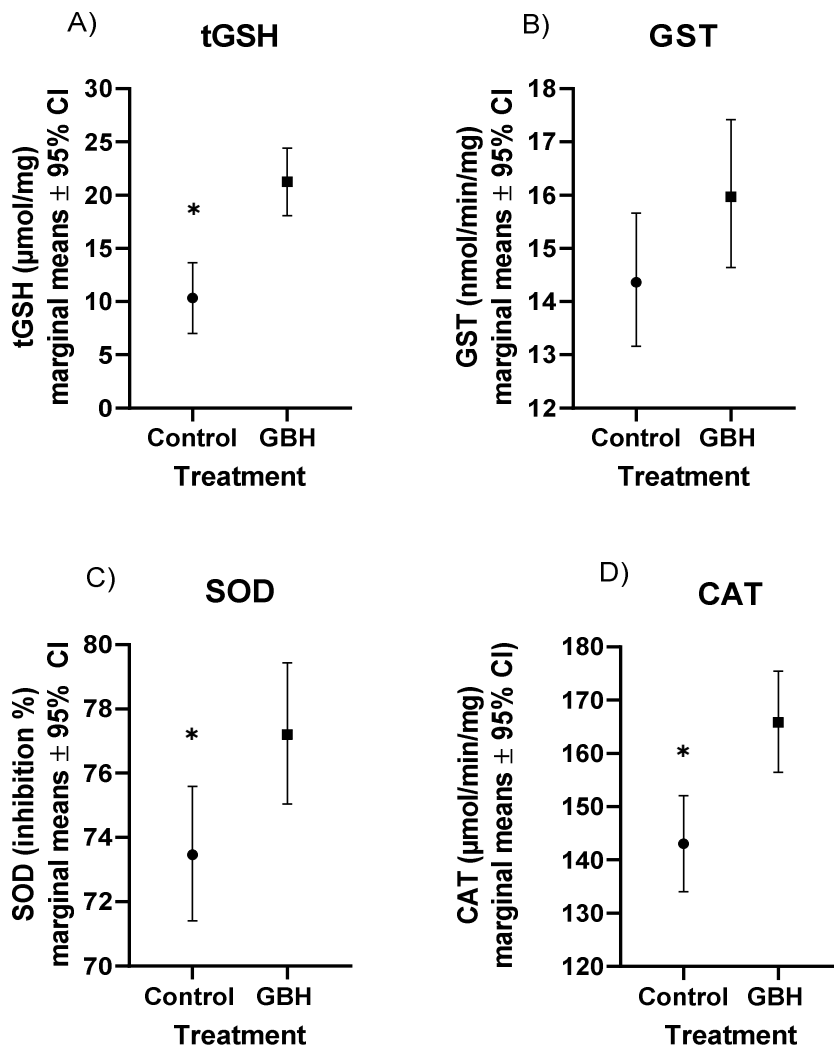
437 were up-regulated in the GBH treatment group compared to the control group (Table 2, Fig 3.). The

438 other oxidative status parameters (GPx, GR, GSH:GSSG, and LHP) were not associated with GBH

439 treatment. In addition, GST activity was negatively associated with larval body mass, while tGSH

440 concentrations had a positive association with body mass (Table 2). No association between body

441 mass and other oxidative status parameters were found.



442

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

449

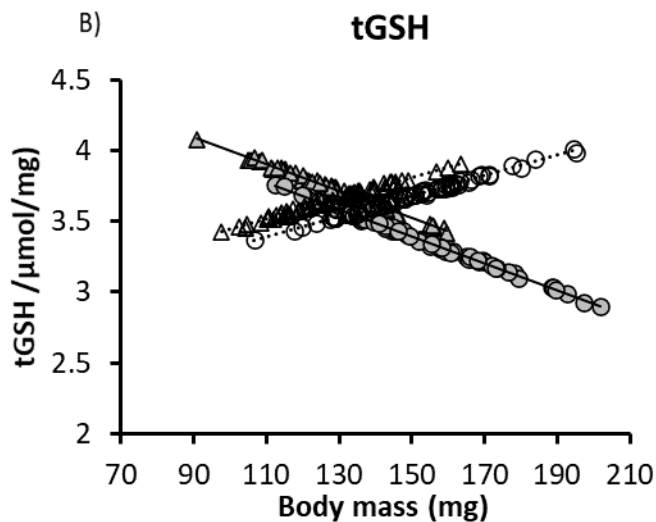
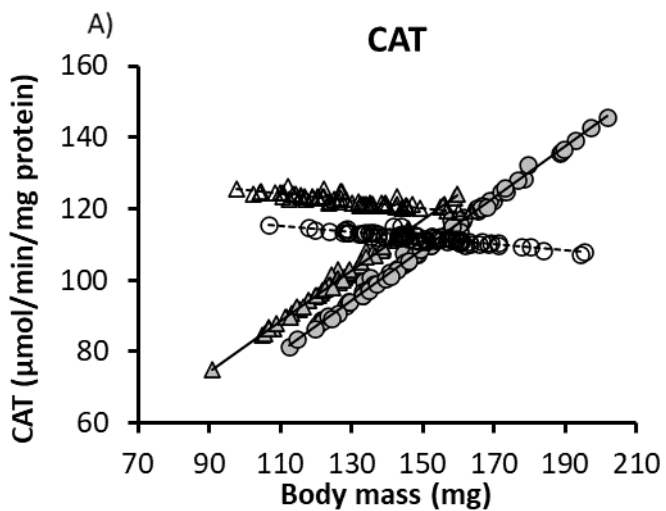
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451

Table 2. The effects of glyphosate treatment (GBH, control), body mass (bm), sex (female, male), body mass \times treatment, and sex \times 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
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
	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
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
	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
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
	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
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	

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



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

468

469 We further examined the correlations between the oxidative status parameters and potato plant
470 glycoalkaloids in the larvae and the adult beetles separately in both treatment groups. We found that
471 in the GBH treatment group, the CAT activity of the larvae correlated negatively with both
472 measurements of α -chaconine ($r_s^2=-0.606$, $p=0.028$ and $r_s^2=-0.628$, $p=0.022$, respectively) and with
473 the second measurement of α -solanine ($r_s^2=-0.694$, $p=0.009$, Table A2 A). Also, the GST levels of
474 the larvae in the GBH treatment group correlated negatively with the second measurement of α -
475 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).
476 There was also a tendency for a negative correlation between GST and the first measurement of α -
477 chaconine ($r_s^2=-0.513$, $p=0.061$, Table A2 A). The GSH:GSSG ratio had a nearly significant
478 negative correlation with the first measurement of α -solanine ($r_s^2=-0.592$, $p=0.055$, Table A2 A). The
479 larvae in the control group had a negative correlation between CAT and the second measurement of
480 α -solanine ($r_s^2=-0.824$, $p=0.006$), and a nearly significant negative correlation between CAT and
481 the second measurement of α -chaconine ($r_s^2=-0.656$, $p=0.055$, Table A2 B). There were no
482 significant correlations between the other parameters ($p>0.05$). In the adult beetles, no correlations
483 between the potato plant glycoalkaloids and oxidative status parameters were shown in the GBH
484 treatment group ($p>.05$, Table A2 C), but in the control group, LHP correlated negatively with the
485 first measurement of α -solanine ($r_s^2=-0.558$, $p=0.031$, Table A2 D). There were no significant
486 correlations between the body mass of the larvae and the adult beetles and the potato plant
487 glycoalkaloids ($p>0.05$) in either of the treatment groups.

488

489 4. Discussion

490

491 4.1. Potato plant defense chemicals

492

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

510

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

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

529

530 4.2. Survival rate and developmental time

531

532 Soil-mediated exposure to a GBH had no effect on the survival rate of the Colorado potato beetle
533 larvae or the adult beetles, indicating that the environmentally relevant concentrations used in the
534 soil did not increase mortality during the larval stage or show carry-over or soil-mediated effects in
535 adult beetles. The soil used in our experiment contained some glyphosate residues (glyphosate July:
536 0.41-0.91 mg/kg, AMPA: 0.24-1.00 mg/kg, certified laboratory, Groen Agro Control, Delfgauw,
537 Netherlands, LC-MS/MS, with a detection limit of 0.01 mg/kg). The glyphosate concentrations of
538 the leaves from the present study were not measured, but potato plant leaves, measured from the

539 potato plants grown outside in the field, had no detectable residues (<0.01 mg/kg), unlike potato
540 tubers (glyphosate: 0.02-0.07 mg/kg, AMPA: 0.06-0.07 mg/kg). The adult beetles were also tested
541 for GBH residues to see whether the GBH accumulates in beetles via food at the larval stage or via
542 soil during the pupal phase. Low levels of AMPA were indeed detected in the beetles (AMPA:
543 0.11mg/kg, glyphosate: 0.013mg/kg), but the residue levels were low and did not affect the survival
544 rate of the beetles at any developmental stage. Our results are in accordance with some other
545 invertebrate studies, which show no effects of GBHs on survival rate (Baker et al., 2014; Haughton
546 et al., 2001; Michalková and Pekár, 2009; Salvio et al., 2016; Thompson et al., 2014). On the other
547 hand, several studies of invertebrates (Benamú et al., 2010; Castilla et al., 2008; Evans et al., 2010;
548 Janssens and Stoks, 2017; Schneider et al., 2009) have shown either direct mortality effects or
549 sublethal effects when exposed to various GBHs, indicating temporal and dose-dependent effects,
550 as well as species-specific differences in insect susceptibility to GBHs. In our earlier study (Rainio
551 et al., 2019), where the Colorado potato beetle larvae were directly exposed to different
552 concentrations of the GBH, low (environmentally relevant) concentrations had no effect on larval
553 survival rate, whereas high concentrations increased larval mortality.

554

555 In the present study, neither the body mass of the larvae or the newly emerged adult beetles (neither
556 females nor males) was affected by GBH treatment, which was expected since the larvae never
557 come in direct contact with the GBH, supporting the finding that the GBH does not affect the
558 beetles' survival rate. However, the developmental time of the adult beetles increased significantly
559 in the GBH treatment group compared to the control group, but the difference (0.56 days) was
560 rather low in a biological sense and likely does not have notable effects on the overall survival rate
561 of the beetles. In general, the Colorado potato beetle tolerates pesticides relatively well, and has
562 developed resistance to several synthetic insecticides, including organophosphates (Kostic et al.,
563 2016; Piironen et al., 2013), used as a control method in potato farms. The metabolic adaptation is

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

568

569 4.3. Oxidative status

570

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

589 been found in bullfrog (*Lithobates catesbeiana*) tadpoles exposed to Roundup Original (Costa et al.,
590 2008), increased SOD activity in blackworm exposed to Roundup Ultra (Contardo-Jara et al.,
591 2009), and increased CAT activity in teleost fish exposed to GBHs (Samanta et al., 2014). Our
592 previous direct exposure study of Colorado potato beetles (Rainio et al. 2019) did not show any
593 differences in those same markers of oxidative status, which may be related to the exposure time or
594 the absorption of the GBH by the beetles' bodies (absorption through the cuticle and epidermis vs.
595 via food or soil).

596

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

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

703

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709

710 **Conflicts of Interest**

711

712 The authors declare no conflict of interest.

713

714 **Credit Author Statement**

715

716 **Miia J. Rainio:** Study design, conducting experiment, biochemical analyses, statistical analyses,717 manuscript writing. **Aigi Margus:** Study design, experiment preparation, manuscript editing.718 **Valtteri Virtanen:** Glycoalkaloid analyses, manuscript editing. **Leena Lindström:** Study design,719 experiment preparation, manuscript editing. **J-P Salminen:** Glycoalkaloid analyses, manuscript720 editing. **Kari Saikkonen:** manuscript editing. **Marjo Helander:** Study design, manuscript editing.

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1186 **Appendices:**

Table A1. Mean (\pm 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*).

Biomarker	Larvae				Adults			
	Control		GBH		Control		GBH	
	Mean (\pm 95% CI)	n	Mean (\pm 95% CI)	n	Mean (\pm 95% CI)	n	Mean (\pm 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 (μ mol/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 (μ mol/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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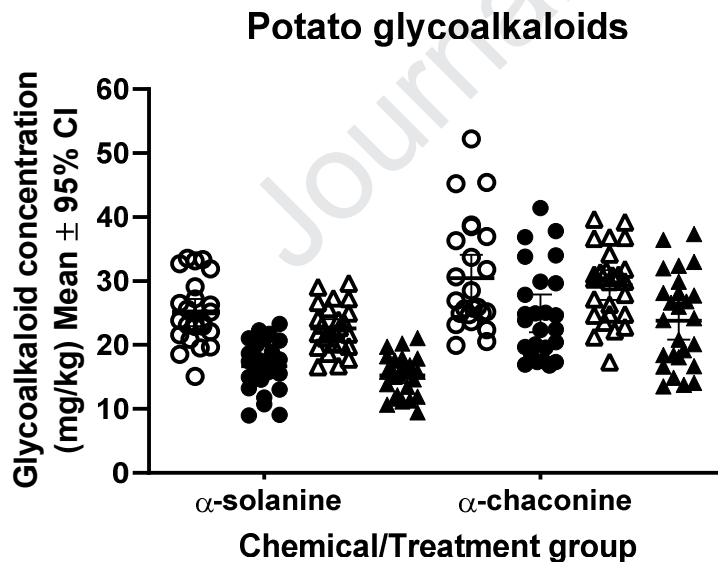
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Table A2 D. Spearman correlation coefficients (r^2 , p-value, n) between the potato glycoalkaloids (α -solanine and α -chaconine) and oxidative status biomarkers glutathione-S-transferase (GST), glutathione oxidase (GPx), glutathione reductase (GR), 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

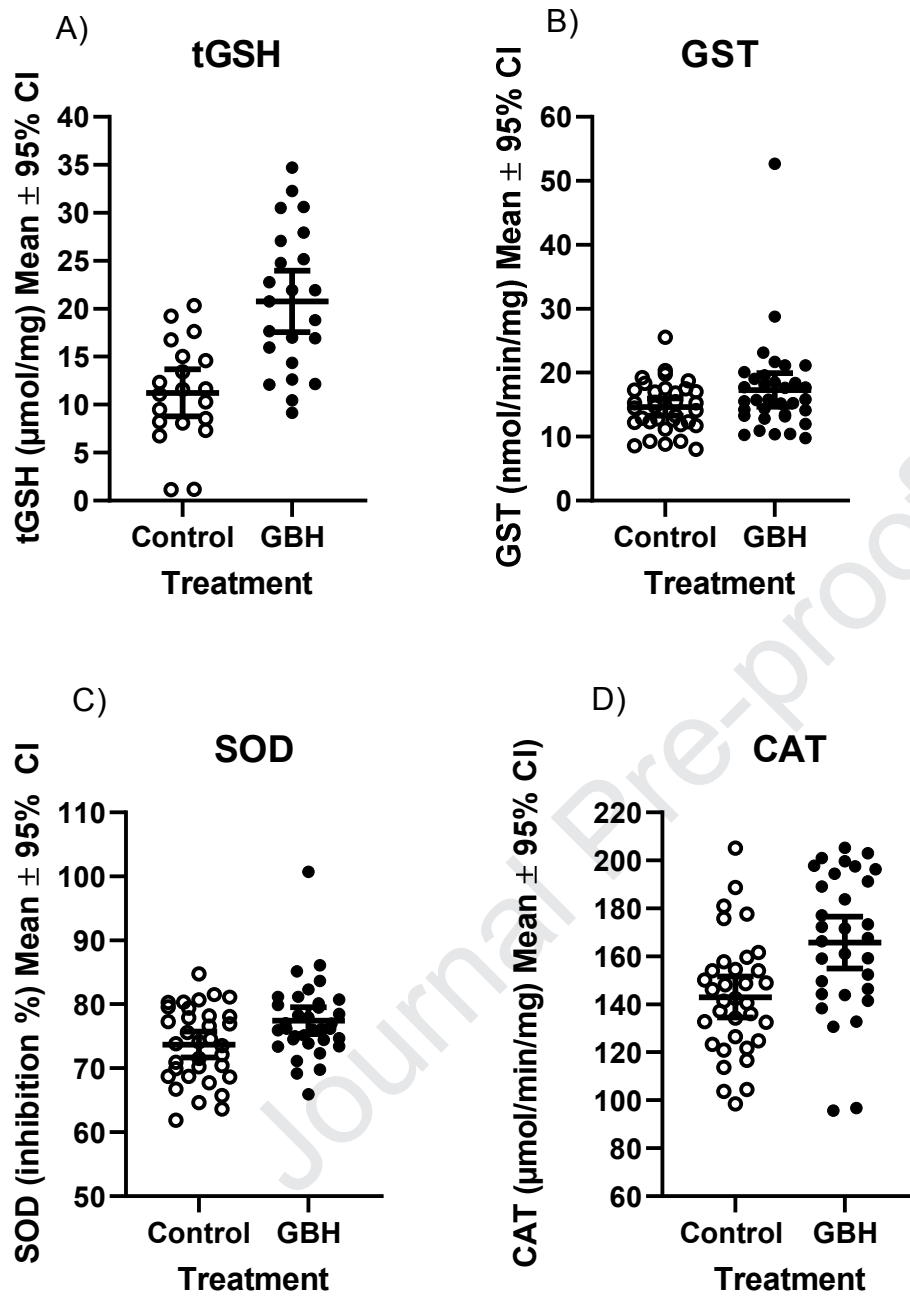
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Figure A1. Potato glycoalkaloid (α -solanine and α -chaconine) concentrations (raw data mean \pm 95% CI) between the treatment groups (control=circle, GBH treatment=triangle) at two measurement points (measurement 1=white, measurement 2=black).

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1202 **Figure A2.** Variation in A) total glutathione (tGSH) concentration, B) glutathione-S-transferase
 1203 (GST), C) superoxide dismutase (SOD), and D) catalase (CAT) activity in larvae of the Colorado
 1204 potato beetle (*L. decemlineata*) between treatment groups (control=white circle, GBH
 1205 treatment=black circle). The dots represent the raw data (mean \pm 95% CI).

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

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: