Effects of a glyphosate-based herbicide on survival and oxidative status of a non-target herbivore, the Colorado potato beetle (*Leptinotarsa decemlineata*)

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Abstract

Glyphosate is the globally most used herbicide against a wide range of weeds. Glyphosate has been considered safe to animals as it mainly targets physiological pathways in plants. However, recent toxicological studies have revealed that glyphosate can cause various toxic effects also on animals. In this study, we investigated the direct toxic effects of a glyphosate-based herbicide (GBH, Roundup® Bio) on **1)** survival and **2)** oxidative status of a non-target herbivore by using Colorado potato beetles (*Leptinotarsa decemlineata*), originating from Poland and USA, as model species. Larvae were randomly divided into three groups: **1)** high concentration (100% Roundup Bio, 360 g/l), **2)** low concentration (1.5% Roundup Bio) and **3)** control group (water). Larvae were exposed to Roundup for different time periods: 2h, 24h, 48h, 72h and 96h. Larval survival decreased in the group treated with high concentration of GBH compared to controls, whereas the low concentration group did not differ from the control group. GBH treatment had no association with oxidative status biomarkers (i.e. catalase, superoxide dismutase, glutathione-S-transferase, glutathione and glutathione related enzymes), but increased lipid hydroperoxide levels after 2h exposure, suggesting increased oxidative damage soon after the exposure. Larvae of different origin also differed in their oxidative status, indicating population-dependent differences in antioxidant defence system. Environmentally relevant concentrations of GBH are not likely to affect larval survival, but high concentrations can reduce survival and increase oxidative damage of non-target herbivores. Also, populations of different origin and pesticide usage history can differ in their tolerance to GBH.

Key words: Antioxidant defence, Glyphosate, Insects, Organophosphate, Origin, Pesticide, Roundup

Introduction

Herbicides are actively used to control weeds and to improve crop production in agri- and horticultural fields and forestry (Duke and Powles, 2008; Giesy et al., 2000; Samanta et al., 2014). A growing body of literature suggests that plant-protective agents used in agriculture, i.e. pesticides (including herbicides) may have also negative effects on non-target species (Freemark and Boutin, 1995; Myers et al., 2016; Van Bruggen et al., 2018). Indeed, heavy usage of pesticides has resulted in a non-desired loss of overall herbivore biodiversity, as pesticides generally are not species-specific. Further, as species usually interact with many other species (e.g. as food source), pesticides can also influence species at higher trophic levels (Freemark and Boutin, 1995). Even though herbicides mainly target physiological pathways in plants, minor changes in plant physiology can have fitness consequences also on non-target herbivorous species. The results of these changes are often difficult to observe as they are not necessarily direct toxic mortality effects, but rather subtle physiological changes.

Glyphosate (*N*-(phosphonomethyl)glycine) is the globally most used herbicide against a wide range of weeds that efficiently and non-selectively kills nearly all herbaceous plants (Myers et al., 2016; Woodburn Allan, 2000). The function of glyphosate is based on inactivation of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme belongs to the shikimate metabolic pathway and is essential for the synthesis of aromatic amino acids and several other aromatic compounds. The ESPSP enzyme is found in plants and some microbes, but not in animals (if not considering their microbiota) (Bentley, 1990; Haslam, 1993; Kishore and Shah, 1988).

Glyphosate is proclaimed safe to non-target organisms due to its low accumulation and rapid inactivation in soils (Giesy et al., 2000; Vereecken, 2005). However, recent toxicological studies

have revealed that glyphosate and its metabolites (e.g. aminomethylphosphonic acid, AMPA) can enter living organisms and have various toxic effects, such as changes in the function of the cells, tissues, physiology, survival and behaviour (reviewed in Mesnage et al., 2015). Glyphosate is also known to interfere with the antioxidant system and/or increase the production of reactive oxygen species (ROS) (Annett et al., 2014; Modesto and Martinez, 2010; Uren Webster and Santos, 2015). These can in turn cause cellular biochemical stress, called oxidative stress and consequent oxidative damage to biomolecules, such as DNA, lipids and proteins (Georg and Gatehouse, 2013; Halliwell and Gutteridge, 2007). Although organisms have developed effective antioxidant defence systems (consisting of vitamins, cellular antioxidants and antioxidant enzymes) to minimize the effects of oxidative metabolism (Halliwell and Gutteridge, 2007), it is possible that the additional ROS production caused by glyphosate cannot be handled by the antioxidant defence, resulting to increased oxidative stress (Modesto and Martinez, 2010; Uren Webster and Santos, 2015). Earlier studies in animals have indeed shown increased oxidative stress, variation in antioxidant enzyme activities or alteration in antioxidant defence system in relation to various glyphosate products (Astiz et al., 2009; Cavalcante et al., 2008; Cavas and Konen, 2007; Contardo-Jara et al., 2009; El-Shenawy, 2009; Glusczak et al., 2007; Modesto and Martinez, 2010; Mottier et al., 2015; Sinhorin et al., 2014; Uren Webster and Santos, 2015). However, to our knowledge GBH induced oxidative stress has not been studied in non-target herbivorous insects.

The increasing evidence of accumulation and transportation of glyphosate in soils as well as interaction of glyphosate with target plants, non-target plants and other organisms (e.g. humans, animals and microbes) have given rise to serious concerns about the increasing use of glyphosate as the main weed management strategy (Helander et al., 2012). Effective crop production requires the usage of plant-protective agents, yet we should also know their full effects to improve the sustainability of crop production.

In this study, we investigated the direct toxic effects of a glyphosate-based herbicide (hereafter GBH, using the commercial product Roundup® Bio) on **1)** survival and **2)** oxidative status of a non-target herbivore by using the Colorado potato beetles (*Leptinotarsa decemlineata*), originating from different countries (Poland and USA), as model species. Since the different countries have different pesticide history (Alyokhin, 2008) the populations may show variation in resistance against several pesticides, such as organophosphates (see also Piiroinen, 2010).

The Colorado potato beetle is a serious invasive pest of potato (Casagrande, 1987) and is able to destroy large potato crops with a major reduction in the yield for potato farmers. The Colorado potato beetle was first identified as a pest in the Midwestern United States (1859), after it expanded from its native host plant, buffalobur nightshade (*Solanum rostratum)*, onto potato (*Solanum tuberosum*) (Schoville et al., 2018; Walsh, 1865). It has spread to Europe via North-America in the beginning of the 20th century (Casagrande, 1987; Grapputo et al., 2005). The current northern range margin of the Colorado potato beetle lies around 60ºN in Russia, but it occurs annually also in Finland (based on the data collected by Finnish Food and Safety Authority), where it is classified as a quarantine pest species. Potato field are not a direct target for GBH, but since the Colorado potato beetle can overwinter in the edges of agricultural fields, it may occasionally become exposed to GBH residues (e.g. via spray drifts).

Our study system simulates the effects of GBH sprayings (i.e. spray drifts), since besides it is widely used in the crop fields, it is also used to synchronize and accelerate the ripening of forage cereals (Helander et al., 2012), to control for invasive species in the context of nature conservation (Weidenhamer and Callaway, 2010) and as a defoliant in forestry (Tanney and Hutchison, 2010). Due to the widespread and frequent use of GBH in agri- and horticulture, it is likely that animals are

sprayed directly, exposed via spray drifts by the wind (Ucar and Hall, 2001) or may end up contact a sprayed surface shortly after spraying (Evans et al., 2010). Earlier studies have shown evidence of detrimental effects of GBH exposure on the development and reproduction in invertebrates common in agroecosystems (Benamu et al., 2010; Castilla et al., 2010; Castilla et al., 2008; Evans et al., 2010; Saska et al., 2016; Schneider et al., 2009), though there are also studies reporting little or no effects (Salvio et al., 2016; Thompson et al., 2014). Therefore, further studies are needed with new species and a broader array of responses to predict how the repeated GBH application might affect the community structure of animals and what the role of glyphosate and its formulated products may be in food webs (Benamu et al., 2010; Michalková and Pekár, 2009; Schneider et al., 2009). So far, only a few comprehensive studies investigating the global mechanisms of toxicity of GBH have been performed in terrestrial invertebrates.

To study the oxidative status of the Colorado potato beetle larvae in relation to GBH exposure, we measured enzymatic (glutathione peroxidase GPx and glutathione reductase GR like enzymes, glutathione-S-transferase GST, glucose-6-phosphate dehydrogenase G6PDH, catalase CAT and superoxide dismutase SOD) and non-enzymatic (total glutathione, tGSH and the ratio of reduced and oxidized glutathione, GSH:GSSG) antioxidants. Of these, the ratio of GSH:GSSG represents the overall oxidative state of the cells and consequently, deviations in this ratio are often used as an indicator of oxidative stress (Halliwell and Gutteridge, 2007; Hoffman, 2002; Isaksson et al., 2005; Lilley et al., 2013; Rainio et al., 2013). The enzymes GPx and GR participate in the glutathione cycle (Fridovich, 1975), whereas CAT and SOD directly regulate the level of ROS (Ercal et al., 2001; Fridovich, 1978; Halliwell and Gutteridge, 2007). GST participates in biotransformation processes by catalysing the conjugation of GSH to xenobiotic substrates for the detoxification purposes (Halliwell and Gutteridge, 2007). In insects, GST’s have also peroxidase activity functions (Corona and Robinson, 2006; Farjan et al., 2012). G6PDH acts in the pentose phosphate pathway

158 by maintaining the cellular level of NADPH (nicotinamide adenine dinucleotide phosphate)
159 (Thomas et al., 1991). NADPH provides the reducing equivalents for biosynthetic reactions and the
160 oxidation-reduction involved in protecting against the toxicity of reactive oxygen species (ROS),
161 allowing the regeneration of glutathione (Rush et al., 1985). To estimate oxidative damage, we
162 measured lipid hydroperoxides, which are suggested to increase together with oxidative stress and
163 ROS production.

164

165 We hypothesise the following: **1)** High concentrations of GBH increase the mortality of the larvae
166 via direct toxic effects. Low concentrations of GBH may not show direct toxic effects, but possible
167 changes in physiology may indirectly decrease larval survival. **2)** We expect to see differences in
168 antioxidant enzyme activities, reflecting the effectiveness of antioxidant defence system, between
169 the treatment groups, since GBH can increase ROS production, alter levels of antioxidant enzymes,
170 and induce oxidative damage of lipids. **3)** We further expect to see differences in antioxidant
171 enzyme activities between beetles originated from different countries, if they differ in their
172 sensitivity to GBH. Beetle populations that are more resistant to organophosphate insecticides could
173 possibly develop resistance to GBH as well, since glyphosate also belongs to the organophosphate
174 chemical group.

175

176 **2. Materials and Methods**

177

178 *2.1. GBH experiment*

179

180 GBH (Roundup® Bio containing 360 g/l glyphosate, Monsanto, Missouri, USA) treatment was
181 conducted during the summer of 2014 in a licensed quarantine laboratory in the University of
182 Jyväskylä, Finland (62°13´48``N 25°44`34``E). In this experiment, we used Colorado potato beetles

183 originated from Poland (Belchow) and USA (Vermont). The Vermont beetles were field-collected
184 (44°43´N, 73°20`W) in 2010, after which it has been grown in the laboratory conditions in
185 Jyväskylä (more detailed description of laboratory conditions in Lehmann et al., 2015). The beetles
186 from Belchow were field-collected (53°01´N, 20°34`E) in 2010 and thereafter kept in laboratory
187 conditions. By using beetles from different continents, we were able to compare the susceptibility of
188 beetles originated from different populations to glyphosate-exposure.
189
190 We used altogether 1848 beetle larvae from 13-15 families (Belchow: 15 families, Vermont: 13
191 families) in our experiment. The newly hatched larvae were reared in petri dishes (with fresh potato
192 leaves provided *ad libitum*) for 3-6 days, to make sure that the larvae are big enough to handle
193 without unintentional mortality. Then they were randomly divided into three groups: **1)** high
194 concentration (3µl of 100% Roundup Bio, 360 g/l of glyphosate isopropylamine salt), **2)** low
195 concentration (3µl of 1.5% Roundup Bio, 5.4 g/l of glyphosate isopropylamine salt) and **3)** control
196 group (3µl of distilled water). The commercial product Roundup® Bio was used, because
197 glyphosate is seldom used alone as a pure glyphosate, but applied as part of a formulated products
198 (Mesnage et al., 2015). Commercial glyphosate products are generally made of around 36-48%
199 glyphosate, water, salts and adjuvants, which enhance the herbicidal properties (e.g. cellular uptake)
200 of glyphosate (Mesnage et al., 2015). The recommended volume of formulated glyphosate solution
201 (Roundup Bio) for field application is 1.5-3.0 l/ha (spring, before sowing) and 3.0-8.0 l/ha (autumn,
202 after harvesting) for weed (Finnish Food and Safety Authority). The 1.5 % Roundup concentration
203 was chosen to represent the commonly used spring concentration in fields in Finland. The high
204 concentration (100% Roundup Bio) was used to probe the physiological limits of the Colorado
205 potato beetle. However, beetles are seldom exposed to so high concentrations of GBH in nature.
206 Therefore, the lower concentration reflects more relevant doses to which beetles in general are
207 likely to be exposed in the field. To simulate direct glyphosate spraying in crop fields, the treatment

208 groups were treated with Roundup (high and low concentration) by pipetting a small drop (3µl) on
209 top of the larvae, which were placed in a petri dish covered with filter paper. After the exposure, the
210 larvae were kept without food for two hours to ensure that they did not get the exposure via food.
211 The control group was treated similarly but using water instead of Roundup. The larvae were
212 exposed to Roundup and then tracked for different time periods: 2h, 24h, 48h, 72h and 96h (with
213 different larvae at each time point). Larvae from each treatment group were checked for mortality
214 and immediately frozen at -80ºC for subsequent physiological analyses.
215
216 For oxidative status measurements, we only chose larvae from three time points; 2h, 24h and 96h.
217 The first two time points were chosen because they cover the timeframe when major physiological
218 changes in the antioxidant system occur. The 96h time point is of interest since it can indicate if the
219 antioxidant system is already balanced after GBH treatment. We used pooled larval samples within
220 families for oxidative status analyses, since the larvae were too small for individual testing.
221 Altogether 144 pooled samples from ten families (73 samples from Belchow and 71 from Vermont
222 origin) were randomly chosen for the oxidative status analyses.
223
224 *2.3. Biochemical analyses*
225
226 Larval homogenates were used to measure oxidative status biomarkers (GST, GPx and GR
227 homologs, G6PDH, CAT, SOD, tGSH and GSH:GSSG) and oxidative damage (lipid
228 hydroperoxides) of the beetles. Samples were homogenized (TissueLyser, Qiagen, Austin, USA)
229 with 150µl KF buffer (0.1 M K_2HPO_4 + 0.15 M KCl, pH 7.4) by pooling 2-3 larvae/family per
230 homogenate to enlarge the sample volume.
231

All antioxidant and enzyme activities were pipetted in triplicate (intra-assay coefficient of variability [CV] < 15% in all cases) using 96- (CAT) or 384-well (GPx, GR, GST, G6PDH, SOD, tGSH and GSH:GSSG) microplates, which in most cases required reducing reagent volumes as compared to kit instructions. All analyses were measured with EnVision microplate reader (PerkinElmer, Finland). Three control samples were used within each plate to be able to correct inter-assay precision with the ratio specific to the particular plate (range 0.8-1.2).

The protein concentration (mg/ml) was measured with BCA (bicinchoninic acid) protein assay (Smith et al., 1985) using BSA (bovine serum albumin) as a standard (Sigma Chemicals, USA) with EnVision microplate reader at an absorbance of 570 nm.

GPx-assay (Sigma CGP1) was adjusted from cuvette to 384-well plate. GPx was measured with kit instruction, but instead of t-Bu-OOH, we used 2 mM H_2O_2, which is a substrate for GPx and CAT. To block CAT, 1 mM NaN_3 was added, and the pH was adjusted to 7.0 with HCl in the buffer provided with the kit (Deisseroth and Dounce, 1970). The change in absorbance was measured at 340 nm.

GR-assay (Sigma GR-SA) was adjusted from cuvette to 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 GG4626), 3 mM DTNB (Sigma D8130) and 2 mM NADPH (Sigma N1630). The change in absorbance was measured at 412 nm.

GST-assay (Sigma CS0410) was likewise adjusted from 96- to 384-well plate using our own reagents; Dulbecco's Phosphate Buffered Saline –buffer (DPBS), 200 mM GSH (Sigma G4251)

and 100 mM 1-Chloro-2,4-dinitrobenzene (CDNB) (Sigma C6396) in ethanol. The assay description can be found in Habig et al. (1974). The change in absorbance was measured at 340 nm.

G6PDH activity was measured according to Noltmann et al. (1961), adjusted to 384-well plate. We used our own reagents; 250 mM glycylglycine (Sigma), 60 mM D-glucose-6-phosphate (Sigma), 20mM $NADP^+$ (Sigma) and 300mM $MgCl_2 \cdot 6\ H_2O$ (Sigma). The absorbance was measured at 340 nm.

Total GSH and the ratio of GSH:GSSG were measured with the ThioStar® Glutathione Fluorescent Detection Kit (K005-FI, Arbor Assays, USA) according to kit instructions and the fluorescence was measured (excitation 405 nm, emission 510 nm). Prior to the analyses, the sample homogenates were deproteinized with 5% sulfosalicylix acid (SSA), incubated on ice for 10 min and centrifuged for 10 min at 10 000g in +4°C.

SOD assay (kit Sigma-Aldrich 19160) was adjusted for 384-well plate. For SOD we used 0.6 mg/ml sample dilution. The measurement was done according to kit instructions at an absorbance of 450 nm. The activity was expressed as inhibition %.

CAT-assay (Sigma CAT100) was adjusted from cuvette to 96-well plate. We used 1.2 mg/ml sample dilution and 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 (0.25mM 4-aminoantipyrene + 2mM 3,5-dicloro-2-hydroxybenzenesulfonic acid in 150 mM potassium phosphate buffer, pH 7.0), peroxidase solutions (from Horse radish), stop solution (15 mM NaN_3, Sigma) and 200mM 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.

For the lipid hydroperoxide measurement the larvae were first weighed and then homogenized with 125µl methanol using 1-2 larvae per homogenate (depending on the weight of the larvae). Lipid hydroperoxides were measured using FOX-II method, modified from Nourooz-Zadeh et al. (1995) and Bou et al. (2008), using 45 µl of the sample, 5 µl 10 mM thiamine pyrophosphate (TPP) or methanol and 950 µl of FOX reagent (2.5mM ammonium(II)sulfate hexahydrate in 0.25 M H2SO4 + 0.111 mM xylenol orange in methanol, see also Vuori et al., 2015). Cumene hydroperoxide (Sigma) was used as a standard (0/8/16/32/64/96/128/160 mM). The absorbance was measured at 570 nm. The results were set against the weight of the body mass.

2.4. Statistics

All statistical analyses were performed with the SAS statistical software 9.4. (SAS, 2013). The survival of the larvae among the treatment groups (high dose, low dose and control), time points (2h, 24h, 48h, 72h and 96h) and origins (Belchow and Vermont) was analysed with a generalized linear mixed model (GLMM) with binary distribution and logit link function (events/trials syntax in GLIMMIX procedure). 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 Kenward–Roger method and post-hoc pairwise comparisons were performed using Tukey's test. Differences in survival between the treatment groups in each time point separately were analysed likewise with GLMM (binary distribution and logit link function) separately for both populations using treatment as dependent variable and family as a random factor. Degrees of freedom and post-hoc tests were carried out as above. Differences in larval survival (using only control larvae) between the larvae of

different origin in each time point were analysed likewise with GLMM (binary distribution and logit link function) with similar degrees of freedom method as mentioned above.

The differences in larval oxidative status parameters between time points (2h, 24h and 96h) were first tested with GLMM (lognormal distribution and identity link function) using family as a random factor. Lipid hydroperoxides were analysed similarly, but using Gaussian distribution and identity link function) instead. Since the larvae at different time points differed from each other in their oxidative status parameters, further analyses were conducted separately for each time point (2h, 24h and 96h). To investigate the effects of GBH treatment on oxidative status and lipid hydroperoxides we performed GLMM (lognormal distribution and identity link function, but Gaussian distribution and identity link function for lipid hydroperoxides) separately for all antioxidants, antioxidant enzymes and lipid hydroperoxides, using treatment, origin and treatment × origin interaction as explanatory variables. Model residuals were used as a random factor. 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. Each variable was further added separately to the reduced model. Degrees of freedom and post-hoc pairwise comparisons were calculated as mentioned above.

Differences in larval oxidative status among developmental stages were analysed separately for beetles from different origin by using the control larvae only (Belchow population: GLMM with Gaussian distribution and identity link function, but GR and GSH:GSSG with lognormal distribution, Vermont population: GLMM with Gaussian distribution and identity link function, but SOD and GSH:GSSG with lognormal distribution). Degrees of freedom and post-hoc pairwise comparisons were calculated as mentioned above.

3. Results

3.1. Survival

GBH treatment significantly affected survival of the Colorado potato beetle larvae (Table 1). Survival was lowest in the high concentration group (81.9%), followed by the control group (92.8%) and low concentration group (93.1%). The low concentration and control groups did not differ from each other. The beetles originated from Belchow and Vermont populations did not differ from each other and there was no origin × treatment interaction (Table 1), whereas time points had significant association to larval survival as also origin × time point interaction (Table 1).

Further analyses (separately for each time point) showed that larval survival between treatment groups differed also in each time point depending on time point and origin (Fig. 1). In the larvae originating from the Belchow population, treatment groups differed at the 48h ($F_{df}=4.93_{2,\ 162}$, p=0.008), 72h ($F_{df}=5.31_{2,\ 159}$, p=0.006) and 96h ($F_{df}=7.86_{2,\ 195}$, p=0.0005) time points, the high concentration group having significantly lower survival compared to the low concentration and the control group (see also Table A1). However, at the 2h ($F_{df}=0.00_{2,\ 171}$, p=0.999) and 24h ($F_{df}=1.76_{2,\ 159}$, p=0.176) time points, no treatment differences were seen ($p < 0.005$). The larvae originating from the Vermont population, on the other hand, had no differences between the treatment groups in any of the studied time points (2h: $F_{df}=0.00_{2,\ 192}$, p=0.999, 24h: $F_{df}=0.00_{2,\ 186}$, p=0.999, 48h: $F_{df}=0.71_{2,\ 183}$, p=0.494, 72h: $F_{df}=2.44_{2,\ 189}$, p=0.090, 96h: $F_{df}=1.09_{2,\ 222}$, p=0.339, see also Table A1). When comparing the survival of the larvae of different origin in each time point by using only control larvae, only larvae at 96h time point ($F_{df}=4.32_{1,\ 109}$, p=0.042) differed significantly between the larvae of different origin, but the other time point showed no differences ($p > 0.05$) in relation to larval origin.

355

356 *3.2. Oxidative status*

357

358 Oxidative status parameters (GST, GR, GPx, tGSH, GSH:GSSG, CAT, SOD, G6PDH and lipid
359 hydroperoxides) were analysed separately among the time points, since larvae in each time point
360 have different developmental stage (Table A2, Table A3). At the 2h time point, lipid hydroperoxide
361 levels were significantly higher in the high concentration group compared to the low concentration
362 and control groups (Table 2, Fig. 2). However, none of the other oxidative status parameters were
363 associated with GBH treatment (Table 2). At the 24h time point, GR activities differed between the
364 treatment groups, but post-hoc pairwise comparisons showed only marginally higher GR activities
365 in the low concentration group compared to the control group (t_{df}= -2.32$_{46}$, p= 0.063, Table 2). The
366 other groups did not differ from each other (Table 2). The GSH:GSSG ratio was significantly higher
367 in the high concentration group compared to the low concentration and control groups (Table 2),
368 whereas the other oxidative status parameters had no association with GBH treatment. At the 96h
369 time point, only GR activities differed between the treatment groups, the high concentration group
370 showing significantly lower activities compared to the low concentration group (t_{df}=2.78$_{45}$,
371 p=0.021, Table 2). However, neither of the groups differed from the control group (Table 2).

372

373 Larvae originating from the two different populations differed from each other in several oxidative
374 status parameters, depending on time point. The activities of GST and GR and the GSH:GSSG ratio
375 were significantly higher in larvae of Vermont origin than in those of Belchow origin among 2h
376 time point, whereas GPx activities were significantly lower in larvae of Vermont origin compared
377 to larvae of Belchow origin (Table 2). Also CAT activities and lipid hydroperoxide levels were
378 higher, though only marginally, in larvae of Vermont origin compared to larvae of Belchow origin
379 at 2h time point (Table 2). The other oxidative status parameters did not show any association with

GBH at 2h time point. The GR, CAT and SOD activities and GSH:GSSG ratio were higher in larvae originating from Vermont population compared to larvae of Belchow origin among 24h time point, whereas the other parameters had no association with GBH (Table 2). Among 96h time point, tGSH levels and GSH:GSSG ratio were significantly higher in larvae of Vermont origin than in Belchow origin, whereas lipid hydroperoxide levels were significantly higher in larvae of Belchow origin compared to larvae of Vermont origin (Table 2).

3.3. Oxidative status and developmental stage

Differences in oxidative status among time points were studied to examine the differences in oxidative status parameters between the developmental stages using the control larvae only. In the larvae originated from Belchow population, the GPx activities differed between 2h and 96h time points, the larvae at 2h time point showing significantly higher activities than the larvae at 96h time point (Table 3, Table A4). G6PDH activities were higher in larvae at 2h time point compared to larvae at 24h time point, but the other time points did not differ from each other (Table 3, Table A4). In the larvae originated from Vermont population, the GPx activities differed significantly between 2h and 24h time points, the 2h time point showing lower activities than 24h time point (Table 3, Table A4), but none of the groups did differ from 96h time point. Lipid hydroperoxide levels were significantly lower in larvae at 96h time point compared to larvae at 2h and 24h time points, but 2h and 24h time points did not differ from each other (Table 3, Table A4).

4. Discussion

4.1. Survival

High GBH concentration increased larval mortality in the Colorado potato beetle larvae, but the low concentration group did not differ from the controls. Our results show that GBH can be toxic at very high concentrations, but it needs to be highlighted that herbivores are rarely exposed to such concentrations (100% Roundup Bio) in nature. The high concentration mainly tests the physiological limits of the system and the antioxidant enzyme capacity of the beetles against this GBH. Several studies in both invertebrates (Benamu et al., 2010; Castilla et al., 2010; Castilla et al., 2008; Contardo-Jara et al., 2009; Evans et al., 2010; Janssens and Stoks, 2017; Schneider et al., 2009) and vertebrates (Cauble and Wagner, 2005; Lajmanovich et al., 2003) have shown either direct mortality effects or sublethal effects, when exposed to various GBH, indicating temporal and dose-dependent effects as well as species-specific differences in insect susceptibility to GBH. Also other simultaneous stress factors (e.g. pathogens), available resources (e.g. food quality) or the overall quality of animals may cause the opposing effects observed in different studies. On the other hand, there is also many studies in invertebrates showing no direct toxic effects of GBH (Baker et al., 2014; Haughton et al., 2001; Michalková and Pekár, 2009; Salvio et al., 2016; Thompson et al., 2014).

Larval survival differed between the treatment groups among the time points, suggesting exposure time and developmental stage differences in larval tolerance to GBH. Overall, survival was highest in the 2h time point in all treatment groups and decreased towards the 96h time point. The exposure time is essential in herbicide studies, thus it is possible that the relatively short exposure time (e.g. 2h) to GBH may not be long enough to show any effects on larval survival thus underestimating the negative effects of GBH (see also Relyea, 2005). Several studies have shown increased mortality within longer exposure periods, which could be related to the slower breakdown of Roundup under low pH conditions (Giesy et al., 2000). De Aguiar et al. (2016; 2018), for example, reported increased mortality in the fruit fly *Drosophila melanogaster* when exposed to Roundup (10 g/l) and

the mortality increased temporally from 24h to 96h period. A study using tadpoles of the wood frog (*Rana sylvatica*) by Relyea (2005) showed 100% survival after Roundup treatment (1 mg AI/l) until day 4, after which the survival decreased to 65% after 16 days of exposure. The spider *Pardosa milvina* also showed temporal increase in mortality 60 days post-exposure to GBH (Evans et al., 2010). Our study tested only short-term effects of GBH on larval survival. Long-term studies would be needed to see whether GBH exposure have carry-over effects e.g. on overwintering survival and reproduction success later in life.

On the other hand, larval developmental stage (instars) varies among the time points, which could also affect the observed treatment differences among time points. However, this is not a likely explanation, since the older larvae with lower oxidative damage (i.e. lipid hydroperoxides) showed higher mortality compared to younger ones. On the other hand, the LdAChE1 gene, which is suggested to be the main target of organophosphate pesticides, has been shown to be more expressed in the earlier larval stages (Revuelta et al., 2011), suggesting that the younger larvae could be more tolerant to organophosphate pesticides (e.g. glyphosate) that are suggested to inhibit the acetylcholinesterase enzyme (Annett et al., 2014 and references therein). We did not measure the exact Roundup Bio levels of the beetle larvae in this study, so we do not know how much Roundup was absorbed by the tissues via topical application. Further studies would be needed to confirm the levels of GBH in the beetle larvae to know the exact exposure levels in the larvae to be able to study the dose-dependent effects more specifically.

The survival between treatment groups among each time point varied between the larvae of different origin as well. The larvae of Belchow origin showed higher mortality in the high treatment group compared to the other groups from the 48h to 96h time points, whereas the larvae of Vermont origin had no differences between the treatment groups in any of the studied time points. However,

455 the mortality of control group was significantly higher in larvae of Vermont origin compared to
456 larvae of Belchow origin at the 96h time point (but not in the other time points), suggesting
457 different overall survival rates between larvae from different origin in later instars. The beetles have
458 been reared in the laboratory conditions for four generations, which may have potentially affected
459 the viability of the beetles. However, the survival differences are similar also in our previous studies
460 (unpublished data by Lindström et al.) when using the beetles from the same origin. If there were
461 viability problems in beetles originated from the Vermont population, we would expect to see
462 higher mortality also in high concentration group due to more stressful conditions. However, the
463 larvae at 96h time point did not show treatment differences and the larval survival in high
464 concentration group was very similar to beetles originated from Belchow population. However, it is
465 possible that the differences in energetically costly antioxidant defence machinery between the
466 larvae of different origin can be reflected in larval survival.
467
468 In general, the Colorado potato beetle has been shown to tolerate pesticides relatively well and it
469 has developed resistance against several insecticides used to control them in the potato fields
470 (Alyokhin et al., 2008). Organophosphate insecticides are one of the chemical groups towards
471 which the Colorado potato beetle can develop resistance (Piiroinen et al., 2013). Populations from
472 the US in general have higher frequencies of resistance-associated mutations than European
473 populations (including Polish populations) (Piiroinen et al., 2013). Glyphosate belongs to the same
474 organophosphate chemical group and thus it is possible that the Colorado potato beetles are less
475 susceptible to GBH as well, though this has not been investigated in detail. Differences in resistance
476 against organophosphate insecticides could also explain the slightly higher mortality in the larvae of
477 Belchow origin compared to the larvae of Vermont origin when exposed to high concentrations of
478 GBH. However, similar studies with other leaf beetles, having no resistance against pesticides,
479 would be needed to confirm our results.

480

481 *4.2. Oxidative status*

482

483 GBH had only minor effects on the oxidative status parameters of the Colorado potato beetle larvae.
484 The effects varied depending on time point and larval origin. None of the antioxidant enzymes or
485 antioxidants were associated with GBH at 2h time point. However, the increased lipid
486 hydroperoxide levels in the high concentration group after 2h GBH exposure indicate higher
487 oxidative damage soon after the exposure, but this effect was not visible within the 24h and 96h
488 time points. It may be that increased hydroperoxide levels can be seen only shortly after GBH
489 exposure or it may be that the larvae with high lipid hydroperoxide levels have already died before
490 reaching the 96h time point. Our results are consistent with the study of Modesto and Martines
491 (2010), where the fish *Prochilodus lineatus* showed increased lipid hydroperoxide concentrations
492 after 6h of exposure to Roundup, but the concentrations returned to control levels after 24h and 96h
493 exposure, suggesting that the antioxidant defence may be insufficient at 6h of exposure in the
494 presence of Roundup. Increased lipid peroxidation levels (TBARS method) in relation to GBH have
495 been reported earlier in several fish species (Samanta et al., 2014; Sinhorin et al., 2014), rats (El-
496 Shenawy, 2009) and bullfrog tadpoles (Costa et al., 2008). However, studies in tadpoles of
497 *Pelopates cultripes* (Burraco et al., 2013), honey bees *Apis mellifera* (Helmer et al., 2015) and fruit
498 flies *Drosophila melanogaster* (de Aguiar et al., 2016) have not shown any effects on lipid
499 peroxidation when exposed to GBH.

500

501 The oxidative status parameters did not show consistent change depending on time point of the
502 experiment highlighting the complexity of these traits. After 24h of GBH exposure, the GSH:GSSG
503 ratio was highest in high concentration group compared to low concentration and control groups,
504 suggesting lower oxidative stress levels in high concentration group compared to the other groups.

505 This difference levelled off at the 96h time point. From the enzyme activities, only GR showed
506 lower activities in high concentration group compared to low concentration group at both 24h and
507 96h time points, but neither of the groups did differ from controls. The results suggest that despite
508 some minor effects of GBH on glutathione metabolism of the larvae, the GBH exposure did not
509 increase oxidative stress or show major differences in ROS regulating (CAT and SOD) and
510 biotransformation (GST) enzyme activities, which could be related to the effective antioxidant
511 defence or other detoxification mechanisms of the larvae. However, since we did not measure ROS
512 levels, we do not know the level of increase in ROS production during the GBH exposure. Also, the
513 highest glyphosate concentration used in this experiment may still be within the range that the
514 larvae are able to tolerate without showing major intracellular toxic effects. Our results mirror the
515 earlier studies of GBH induced oxidative status, which have revealed contradictory results
516 depending on species, used glyphosate products and different measures of oxidative status. Some
517 studies showed variation in antioxidant enzyme activities, whereas some studies did not find any
518 treatment effects on antioxidant enzymes (Burraco et al., 2013; Tarouco et al., 2017). Samanta et al.
519 (2014) have shown increased CAT activities, but decreased GST activities in teleost fishes exposed
520 to GBH (trade name Excel Mera), whereas El-Shenawy (2009) has reported reduced GSH levels in
521 rats after glyphosate and Roundup treatments, the levels being lowest in the Roundup group.
522 Studies in the blackworm *Lumbriculus variegatus* (Contardo-Jara et al., 2009) have revealed
523 elevated SOD and soluble GST activities, whereas elevated hepatic SOD and CAT activities were
524 found in bullfrog tadpoles *Lithobates catesbeiana* exposed to Roundup (Costa et al., 2008), again
525 suggesting increased oxidative stress following herbicide exposure. Common to the previous
526 studies has been the elevation of either SOD or CAT activities (or both). The removal of superoxide
527 has been suggested to be one key antioxidant defence mechanisms (Fridovich, 1974), thus it is logic
528 that SOD is one of the first enzymes upregulated after exposure to contaminants. The contradictory
529 results, on the other hand, highlight the species- (see also Berglund et al., 2014, Rainio et al., 2013)

and tissue-specificity (Yang et al., 2013) of antioxidant defence, but also the susceptibility of different species to various GBH may at least partly explain the opposite results.

There was suggestion that the antioxidant defence machinery might be different for larvae originating from different populations. The larvae of Vermont origin had higher activities of GST, GR and CAT and higher GSH:GSSG ratio and lipid hydroperoxide levels at 2h time point together with the higher activities of GR, CAT and SOD and higher ratio of GSH:GSSG at 24h time point compared to larvae of Belchow origin. These difference levelled off as the experiment progressed as at the 96h time point, only tGSH levels and GSH:GSSG ratio were higher in larvae of Vermont origin, whereas lipid hydroperoxide levels were higher in larvae of Belchow origin. The higher GSH:GSSG ratio among all time points indicate lower oxidative state in larvae of Vermont origin compared to those of Belchow origin, which may be due to higher enzyme activities that keep the redox balance at lower level. The higher lipid peroxidation levels together with lower GSH:GSSG ratio in larvae of Belchow origin at 96h time point suggest less powerful antioxidant defence machinery in larvae of Belchow origin compared to larvae of Vermont origin, which could be related to differences in their pesticide history or some other environmental factors complicating the interpretation of the results.

Oxidative status showed only minor differences between the developmental stages, when only control larvae were used in the analyses. From the studied oxidative status parameters only GPx, G6PDH and lipid hydroperoxides varied along the developmental stages, but there was no clear pattern in direction of antioxidant enzymes. Previous studies have suggested that the effectiveness of antioxidant enzymes vary with the developmental stage of organism (Halliwell and Gutteridge 2007, Livingstone, 2001), often increasing with age, but the levels can vary between tissues and species (Ahmed 2005, Hussain et al., 1995, L´vova and Abaeva, 1996). The formation of

antioxidant enzymes during development is also suggested to be related to the changes in the levels of free radicals, which is why it would be important to measure ROS levels as well (Ahmed 2005). The lipid hydroperoxide levels, however, were lowest at the 96h time point (control larvae only) compared to the 2h and 24h time points, meaning that the older larvae had less oxidative damage compared to younger ones. This could be related to more effective antioxidant defence of older larvae or higher oxidative stress of younger larvae. Several studies in other species have indeed suggested that during early development the oxidative stress levels are high due to the link between high metabolic activities required for growth and ROS generation (Monaghan, Metcalfe & Torres 2009).

5. Conclusions

The high GBH concentration had negative effects on larvae of the Colorado potato beetle, but environmentally relevant concentrations of GBH are not likely to have a major effect on survival. Our results show that GBH is dose-dependent and linked to exposure time. GBH had only minor effects on the antioxidant enzyme activities and glutathione metabolism of the Colorado potato beetle larvae, but lipid peroxidation increased after the 2h exposure to high GBH concentration, suggesting increased oxidative damage soon after the exposure. Our data also suggests that populations of different origin and pesticide usage history can differ in their tolerance to GBH. It also needs to be highlighted that even thought this study did not show any short-term effects on oxidative status or survival when using environmentally relevant doses, the carry-over effects (e.g. for fertility, reproduction success or overwintering survival) cannot be entirely ruled out, since the larvae were not monitored for longer periods.

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Figure captions:

Figure 1. (A+B) Survival of the Colorado potato beetle *(Leptinotarsa decemlineata)* larvae between the treatment groups in each time point separately in larvae of different origin (Belchow and Vermont). Bars represent survival (means ± 95% Cl) in each time point separately between treatment groups (white=control, striped=low, grey=high). Asterisks above the bars indicate significant differences between the groups in each time point (GLMM, $p < 0.05$).

Figure 2. (A+B) Variation in lipid hydroperoxide levels (nmol/min/mg body mass) of the Colorado potato beetle (*Leptinotarsa decemlineata*) larvae in each time point (2h, 24h and 96h) in larvae of different origin (Belchow and Vermont). Bars represent the predicted means (± 95% CI) from the model. Colour of the bars indicate treatment group (white=control, striped=low, grey=high). Star above bars indicate significant difference between the treatment groups (GLMM, $p < 0.05$).

945 **Figures:**

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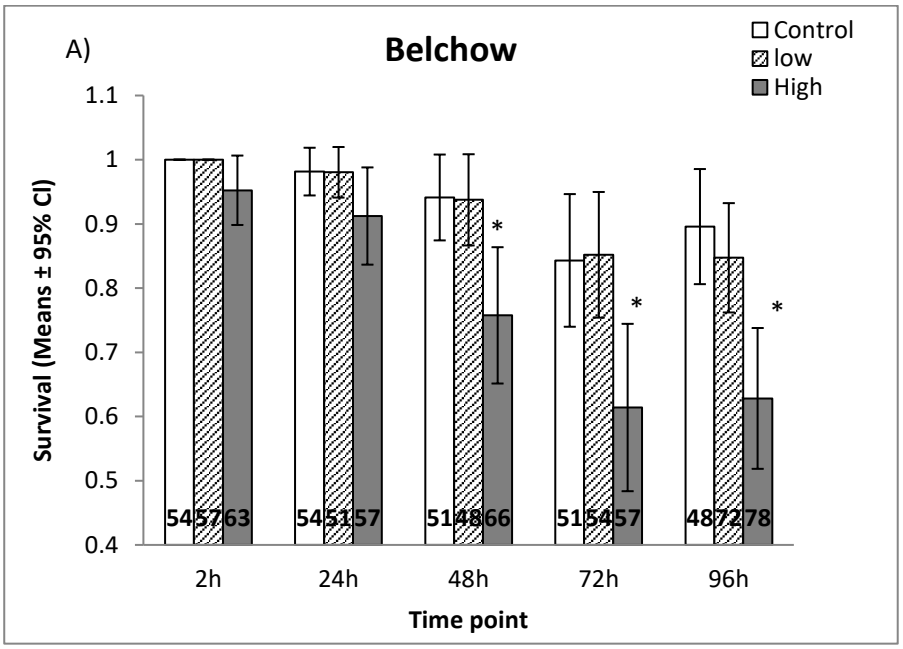
947 **Figure 1. (A+B)**



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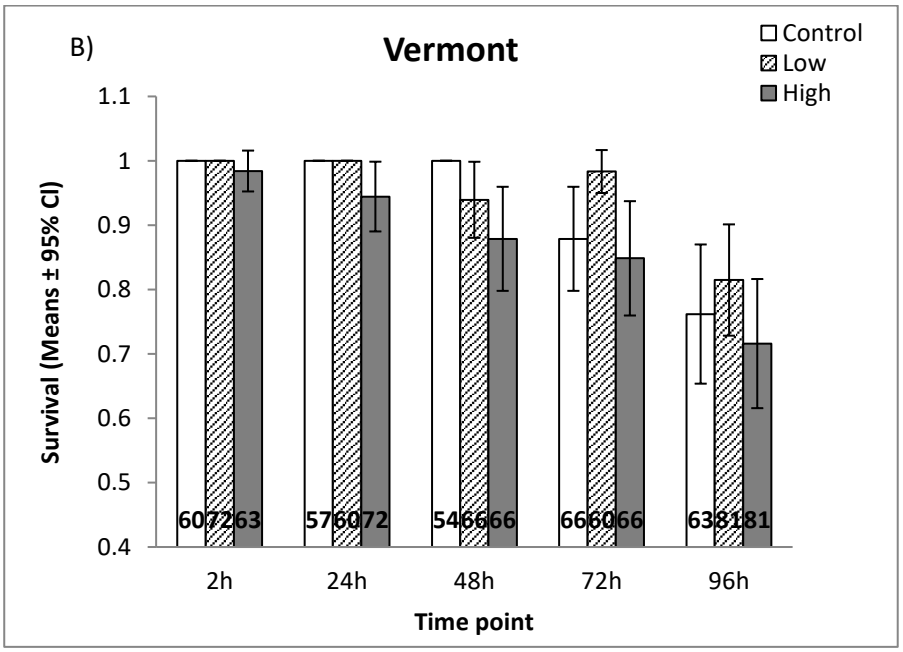

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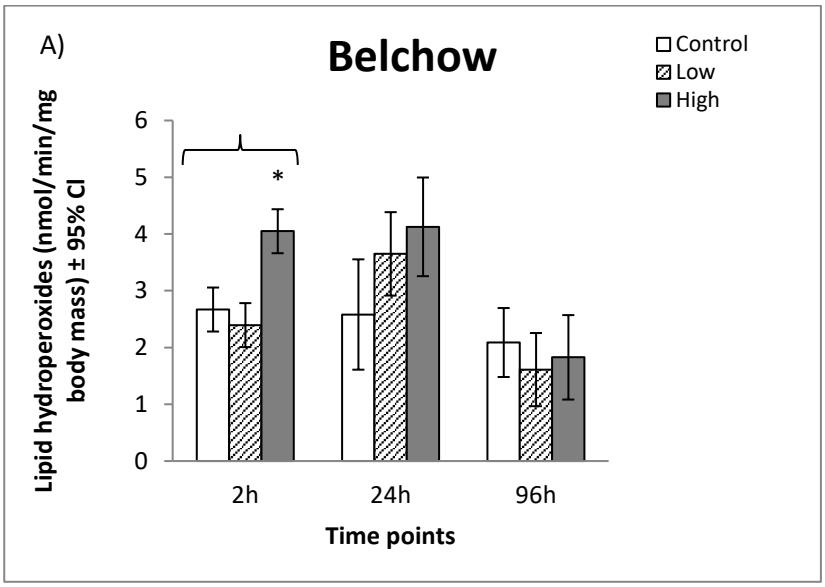
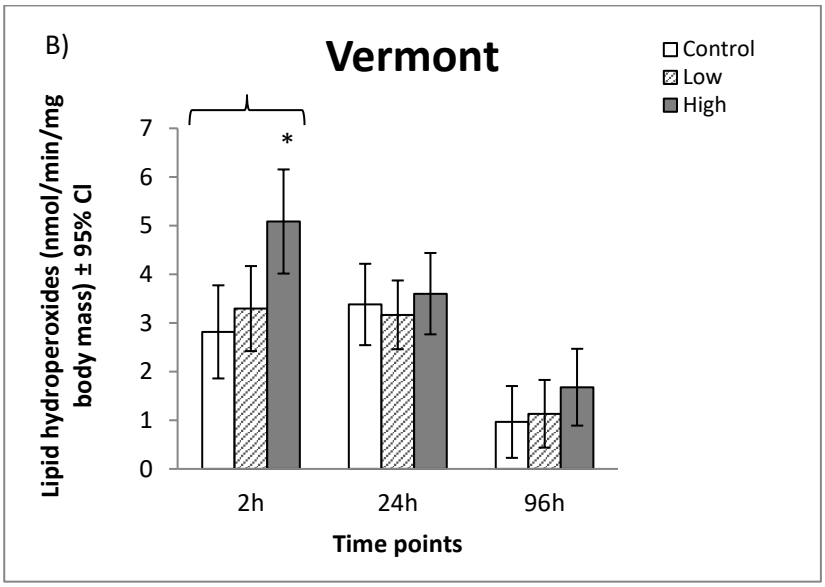
Figure 2. (A+B)


A)
Belchow
Control
Low
High
Lipid hydroperoxides (nmol/min/mg body mass) ± 95% CI
0
1
2
3
4
5
6
*
2h
24h
96h
Time points


B)
Vermont
Control
Low
High
Lipid hydroperoxides (nmol/min/mg body mass) ± 95% CI
0
1
2
3
4
5
6
7
*
2h
24h
96h
Time points

970 **Tables:**

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Table 1. The relationship between glyphosate treatments (high, low, control), origin (Belchow and Vermont) and time points (2h, 24h, 48h, 72h and 96h) on survival of the Colorado potato beetle (*Leptinotarsa decemlineata*) larvae. Significant results are shown in bold.

	Survival	
Model*	**F_{df}**	**p**
Treatment	$26.06_{2, 1836}$	< **0.0001**
Timepoint	$21.51_{4, 1836}$	< **0.0001**
Origin	$3.70_{1, 53.97}$	0.060
Origin × timepoint	$2.53_{4, 1836}$	**0.039**
Origin × treatment	$2.48_{2, 1834}$	0.084

972 * GLMM with binary distribution and logit link function,
973 family used as a random factor in the model.

974

Table 2. The effects of glyphosate treatment and origin of population on oxidative status parameters (GST, GR, GPx, tGSH, GSH:GSSG, CAT, SOD, G6PDH and lipid hydroperoxides, LHP) in Colorado potato beetle (*Leptinotarsa decemlineata*) larvae among three different time points (2h, 24h and 96h). Non-significant terms were dropped sequentially from each model, starting from interactions (GLM with lognormal distribution and identity link function). Significant results are shown in bold.

		Time points								
Biomarkers	**Model**	**2h**			**24h**			**96h**		
		F_{df}	**p**	**n**	**F_{df}**	**p**	**n**	**F_{df}**	**p**	**n**
GST*	treatment	$0.25_{2, 39}$	0.781	43	$1.58_{2, 47}$	0.216	50	$0.75_{2, 48}$	0.476	51
	origin	**$5.78_{1, 39}$**	**0.021**		$1.10_{1, 46}$	0.300		$2.40_{1, 47}$	0.128	
	origin*treatment	$0.34_{2, 37}$	0.712		$1.00_{2, 44}$	0.375		$0.09_{2, 45}$	0.915	
GR*	treatment	$0.07_{2, 39}$	0.937	43	**$3.22_{2, 46}$**	**0.049**	50	**$3.85_{2, 45}$**	**0.029**	48
	origin	**$5.30_{1, 39}$**	**0.027**		**$6.72_{1, 46}$**	**0.013**		$0.10_{1, 44}$	0.754	
	origin*treatment	$0.60_{2, 37}$	0.554		$0.02_{2, 44}$	0.982		$1.09_{2, 42}$	0.344	
GP*	treatment	$1.35_{2, 38}$	0.272	42	$0.25_{2, 47}$	0.779	50	$2.56_{2, 42}$	0.090	45
	origin	**$5.36_{1, 38}$**	**0.026**		$0.00_{1, 46}$	0.968		$2.41_{1, 41}$	0.128	
	origin*treatment	$2.86_{2, 36}$	0.070		$0.09_{2, 44}$	0.910		$2.95_{2, 39}$	0.064	
tGSH*	treatment	$0.91_{2, 39}$	0.410	43	$1.62_{2, 45}$	0.209	48	$1.28_{2, 29}$	0.293	33
	origin	$0.13_{1, 39}$	0.717		$2.00_{1, 44}$	0.165		**$4.32_{1, 29}$**	**0.047**	
	origin*treatment	$0.21_{2, 37}$	0.811		$0.57_{2, 42}$	0.571		$0.55_{2, 27}$	0.583	
GSH:GSSG*	treatment	$1.05_{2, 38}$	0.360	42	**$5.50_{2, 42}$**	**0.008**	46	$0.38_{2, 43}$	0.685	47
	origin	**$6.72_{1, 38}$**	**0.014**		**$7.43_{1, 42}$**	**0.009**		**$7.80_{1, 43}$**	**0.008**	
	origin*treatment	$0.42_{2, 36}$	0.659		$0.02_{2, 40}$	0.981		$0.13_{2, 41}$	0.878	
CAT*	treatment	$0.09_{2, 39}$	0.912	43	$0.76_{2, 46}$	0.473	50	$1.40_{2, 47}$	0.256	50
	origin	**$3.89_{1, 39}$**	**0.056**		**$4.36_{1, 46}$**	**0.042**		$0.20_{1, 46}$	0.658	
	origin*treatment	$1.57_{2, 37}$	0.222		$0.55_{2, 44}$	0.582		$0.53_{2, 44}$	0.593	
SOD*	treatment	$0.22_{2, 40}$	0.803	43	$1.23_{2, 46}$	0.303	50	$1.06_{2, 47}$	0.354	50
	origin	$3.61_{1, 39}$	0.065		**$6.47_{1, 46}$**	**0.014**		$0.26_{1, 46}$	0.611	
	origin*treatment	$0.12_{2, 37}$	0.884		$0.07_{2, 44}$	0.934		$0.66_{2, 44}$	0.522	
G6PDH*	treatment	$0.30_{2, 31}$	0.746	34	$0.81_{2, 34}$	0.454	37	$0.13_{2, 35}$	0.875	38
	origin	$0.11_{1, 30}$	0.744		$1.19_{1, 33}$	0.283		$0.07_{1, 34}$	0.788	
	origin*treatment	$2.92_{2, 28}$	0.071		$0.46_{2, 31}$	0.638		$0.02_{2, 32}$	0.982	
LHP**	treatment	**$12.67_{2, 20}$**	**0.0003**	24	$2.09_{2, 30}$	0.141	33	$0.65_{2, 43}$	0.529	47
	origin	**$4.12_{1, 20}$**	**0.056**		$0.22_{1, 29}$	0.640		**$5.06_{1, 43}$**	**0.030**	
	origin*treatment	$0.63_{2, 18}$	0.542		$1.75_{2, 27}$	0.194		$1.06_{2, 41}$	0.357	

975 * GLM with lognormal distribution and identity link function
976 ** GLM with Gaussian distribution and identity link function

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Table 3. Variation of oxidative status biomarkers (GST, GR, GPx, tGSH, GSH:GSSG, CAT, SOD; G6PDH and lipid hydroperoxides, LHP) in the control larvae of Colorado potato beetles (*Leptinotarsa decemlineata*) among developmental stages. Significant results are shown in bold.

		Belchow		**Vermont**	
Biomarkers	**Model**	**F_{df}**	**p**	**F_{df}**	**p**
GST	timepoint*	$2.47_{2,24}$	0.106	$0.52_{2, 18}$	0.600
GR	timepoint**	$1.59_{2, 24}$	0.224	$0.46_{2, 17}$	0.639
GP	timepoint*	**$4.30_{2, 24}$**	**0.025**	**$4.31_{2, 16}$**	**0.032**
tGSH	timepoint*	$0.75_{2,22}$	0.484	$0.79_{2, 11}$	0.480
GSH:GSSG	timepoint**	$3.12_{2, 21}$	0.065	$1.49_{2, 16}$	0.255
CAT	timepoint*	$1.46_{2, 24}$	0.252	$3.04_{2, 18}$	0.073
SOD	timepoint*	$1.09_{2, 24}$	0.351	$2.16_{2, 18}$	0.144
G6PDH	timepoint*	**$3.78_{2, 15}$**	**0.047**	$3.33_{2, 17}$	0.060
LHP	timepoint*	$0.73_{2, 13}$	0.499	**$19.29_{2, 15}$**	**< 0.0001**

978 * GLM with Gaussian distribution and identity link function
979 ** GLM with lognormal distribution and identity link function