

1 Title: Distinct defense strategies allow different grassland species to cope with root herbivore  
2 attack

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21 **Abstract**

- 22 1. Root-feeding insect herbivores are of substantial evolutionary, ecological and economical  
23 importance. Plants can resist insect herbivores through a variety of tolerance and resistance  
24 strategies. To date, few studies have systematically assessed the prevalence and importance  
25 of these strategies for root-herbivore interactions across different plant species.
- 26 2. Here, we characterize the defense strategies used by three different grassland species to  
27 cope with a generalist root herbivore, the larvae of the European cockchafer *Melolontha*  
28 *melolontha*.
- 29 3. Our results reveal that the different plant species rely on distinct sets of defense strategies.  
30 The spotted knapweed (*Centaurea stoebe*) resists attack by dissuading the larvae through  
31 the release of repellent chemicals. White clover (*Trifolium repens*) does not repel the  
32 herbivore, but reduces feeding, most likely through structural defenses and low nutritional  
33 quality. Finally, the common dandelion (*Taraxacum officinale*) allows *M. melolontha* to  
34 feed abundantly but compensates for tissue loss through induced regrowth.
- 35 4. Synthesis: Three co-occurring plant species have evolved different solutions to defend  
36 themselves against attack by a generalist root herbivore. The different root defense  
37 strategies may reflect distinct defense syndromes.

38

39 **Keywords:** belowground herbivores, chemical and structural defenses, generalist herbivores,  
40 host resistance and tolerance, plant - insect interactions

## 41 **Introduction**

42 Belowground, root-feeding herbivore insects have long been known for their importance in  
43 structuring agroecosystems (Hunter, 2001). More recently, their effects on host plant  
44 interactions with aboveground insects (Biere & Goverse, 2016; Papadopoulou & van Dam,  
45 2017), on host plant defense evolution (van Dam, 2009) and plant communities (Van der Putten,  
46 2003) were unraveled. Given the prevalence and importance of root herbivores, an important  
47 question is how plants cope with root herbivore attack (Erb, Glauser, & Robert, 2012; Rasmann  
48 & Agrawal, 2008).

49 Direct plant defense strategies against root herbivores encompass resistance and tolerance  
50 (Johnson, Erb, & Hartley, 2016). Resistance can be achieved by exuding soluble or volatile  
51 repellent chemicals in the rhizosphere, and/or by producing deterrent or toxic compounds at the  
52 surface or internally (Erb et al., 2013). It can also rely on structural traits that act as deterrents  
53 or digestibility reducers (Hanley, Lamont, Fairbanks, & Rafferty, 2007). Tolerance to root  
54 herbivory has mostly been associated with the ability for compensatory growth that is  
55 accompanied by a reconfiguration of plant metabolism (Johnson, Erb, et al., 2016). Finally,  
56 indirect defense strategies work through plant-mediated reinforcement of top-down control of  
57 herbivores by the third trophic level (Turlings & Erb, 2018). Over the last years, mechanistic  
58 studies have provided detailed examples of these different traits in root-herbivore interactions  
59 (Erb et al., 2015; Johnson, Hallett, Gillespie, & Halpin, 2010; Lu et al., 2015; Rasmann et al.,  
60 2005; Robert et al., 2014). Several studies also compared defenses of different plant species  
61 against root-herbivore insects, mostly focusing on chemical resistance traits (e.g. Rasmann &  
62 Agrawal, 2011; Tsunoda, Krosse, & van Dam, 2017). However, we currently lack systematic,  
63 integrated studies that compare different direct defense traits in root-herbivore interactions  
64 across different plant species. Assessing the relative importance of different types of defenses  
65 and their combination within individual plant species into so-called plant defense-syndromes

66 (Agrawal & Fishbein, 2006) is an important next step towards a better understanding of the  
67 ecology and evolution of root-herbivore interactions.

68 In the present study, we combine different experimental approaches to understand the root-  
69 defense strategies of three different, co-occurring European grassland species: the common  
70 dandelion *Taraxacum officinale* agg. (Asteraceae), the spotted knapweed *Centaurea stoebe*  
71 (Asteraceae) and white clover *Trifolium repens* (Fabaceae). All three species co-occur with a  
72 generalist root herbivore, the larva of the European cockchafer *Melolontha melolontha*  
73 (Coleoptera: Scarabeidae). *Melolontha melolontha* is native to Europe and occurs abundantly  
74 in grasslands. Its larvae develop best on this species (Hauss, 1975; Hauss & Schütte, 1976). The  
75 reasons for this preference and host suitability are unknown. Recently, it was shown that *C.*  
76 *stoebe* is a bad host for *M. melolontha* larvae (Huang, Zwimpfer, Hervé, Bont, & Erb, 2018).  
77 The host suitability of *T. repens* is less clear (Huang et al., 2018; Sukovata, Jaworski,  
78 Karolewski, & Kolk, 2015). Regarding potential defense strategies of the three species against  
79 root-herbivores, mechanistic work so far has mostly focused on *T. officinale*. Upon damage, *T.*  
80 *officinale* releases a bitter latex sap containing high amount of the sesquiterpene lactone  
81 taraxinic acid  $\beta$ -D-glucopyranosyl ester (TA-G) (Huber et al., 2015). High TA-G levels are  
82 associated with reduced *M. melolontha* damage, and silencing TA-G production makes *T.*  
83 *officinale* more attractive to *M. melolontha*, suggesting that it acts as a direct defense that deters  
84 *M. melolontha* (Bont et al., 2017; Huber et al., 2016). However, even genotypes producing high  
85 levels of TA-G are regularly attacked by *M. melolontha*, suggesting overall low resistance  
86 potential against this herbivore. Recent evidence showed that prolonged herbivory by *M.*  
87 *melolontha* larvae increases seed dispersal of *T. officinale*, which suggests that escaping  
88 herbivory is also part of the defense strategy of this plant species (Bont et al., 2019).

89 Our approach involved a set of manipulative experiments to estimate root damage and  
90 consumption by *M. melolontha* attacking the different species, root regrowth and shoot growth

91 as tolerance mechanisms and volatile- and non-volatile attractiveness of the roots as direct  
92 resistance mechanisms. We also assessed primary metabolite levels, as well as chemical and  
93 structural defense mechanisms in the different species to determine whether low food quality  
94 may be responsible for the observed differences in resistance. By combining these  
95 measurements, we demonstrate that the three different species employ different sets of defense  
96 mechanisms to reduce or tolerate *M. melolontha* damage.

97

## 98 **Materials and Methods**

### 99 *Plants and experimental conditions*

100 Seeds of *C. stoebe* and *T. repens* were purchased from UFA-SAMEN (Bern, Switzerland) and  
101 Samen & Saatgut Shop (Zurich, Switzerland), respectively. For *T. officinale*, the genotype A34  
102 was propagated in the laboratory and used for experiments. All seeds were germinated on  
103 seedling substrate and transplanted into 9 x 9 x 10 cm (L x l x H) pots filled with a mixed  
104 potting soil ('Landerde':peat:sand 5:4:1) after 2.5 weeks. Seedlings were transplanted  
105 individually except for *T. repens* where two seedlings were transplanted per pot to provide a  
106 sufficient amount of root material for *M. melolontha* larvae (hereafter, each pot is treated as a  
107 single replicate). Plants were used for experiments at 10 weeks after sowing. Cultivation and  
108 experiments took place in the same controlled conditions in climatic chambers: photoperiod  
109 16:8 (light:dark), light intensity approx. 350  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (supplied by Radium Bonalux  
110 NL39W 830/840 lamps), temperature 22:18 °C (day:night) and humidity 65%.

111

### 112 *Insects*

113 *M. melolontha* larvae were collected from meadows in different areas of Switzerland (Table 1).  
114 Larvae were reared in controlled conditions (10 °C, darkness) in individual soil-filled plastic  
115 cups with carrot slices as food source. Second-instar (L2) and third-instar (L3) larvae were  
116 starved for five and seven days before experiments, respectively.

117

118 Table 1 – Populations of *Melolontha melolontha* larvae used in this study. L2: second instar,  
119 L3: third instar.

120

Location	Coordinates	Date of collection	Instar at collection	Instar at experiment
Erstfeld	46.82°N, 8.64°E	September 2015	L2	L2
Kesswil	47.60°N, 9.30°E	September 2015	L2	L2
Bristen	46.77°N, 8.69°E	May 2016	L2	L2
Urmein 1	46.69°N, 9.41°E	May 2015	L2	L3
Urmein 2	46.69°N, 9.41°E	September 2015	L3	L3
Valzeina	46.96°N, 9.61°E	September 2015	L3	L3

121

122 *Host suitability and estimation of root consumption*

123 To establish the pattern of host suitability, pre-weighed *M. melolontha* larvae were individually  
124 placed with one plant for a fixed number of days. Larvae were added to plant pots into a 1cm  
125 hole near the center of the pot, and then covered with soil. At the end of the experiment, larvae  
126 were sampled back from the pots and weighed. Host suitability was assessed through larval  
127 performance, which was defined as a relative weight gain: (weight post-experiment – weight  
128 pre-experiment) / weight pre-experiment. To test for the robustness of the pattern, the  
129 experiment was conducted with two populations of L2 larvae (Erstfeld and Kesswil) and two  
130 populations of L3 larvae (Urmein 2 and Valzeina). Experiment duration was 14 days for L2  
131 larvae, 10 days for L3 larvae. Eleven to twelve replicates were performed per population, except  
132 for Erstfeld where five to six replicates were performed due to a lower number of available  
133 larvae. To estimate root consumption, the whole root system of each plant was harvested at the  
134 end of the experiment. Soil was removed by gentle washing with tap water. Roots were then  
135 dried for 5 days at 65 °C and weighed. As a control, twelve other plants of each species were  
136 included in the experimental design. These plants were grown and harvested in the exact same  
137 conditions as the first ones but no larva was added.

138

139 *Estimation of root consumption and capacity for compensatory growth*

140 Since root consumption estimation from the first experiment could be biased by compensatory  
141 regrowth a second experiment was conducted. Plants were grown in two stacked pots filled  
142 with the same soil. The bottom of the upper pot ('systemic compartment') was replaced with a  
143 fine mesh (Windhager, Switzerland). The mesh allowed roots to grow through, but restricted  
144 the herbivore larvae to the lower pot ('attacked compartment'). Three treatments were  
145 conducted for each plant species: 'control', 'larva' (one L3 larva of population Urmein 1 placed  
146 in the attacked belowground compartment) and 'root removal' (mechanical removal of all roots  
147 of the attacked belowground compartment by cutting them with scissors just below the mesh,  
148 one day after the beginning of the experiment). The 'root removal' treatment was included to  
149 test whether plants are able to compensate for root loss without the confounding factor of  
150 different larval feeding patterns across the three species. Ten days after the beginning of the  
151 experiment, roots of each belowground compartment as well as aboveground organs were  
152 harvested separately, dried as explained above and weighed. No root could be harvested from  
153 the attacked belowground compartment of the 'root removal' treatment. Before harvesting of  
154 the attacked belowground compartment of the 'larva' treatment, damage to roots was visually  
155 assessed using a three-level damage scale: no damage except for a small spherical area around  
156 the larva ('+'), one or several tunnels but  $\leq 50\%$  of roots removed ('++') or  $> 50\%$  of roots  
157 removed ('+++'). Six to seven replicates were performed per species and treatment.

158

159 *Contribution of distance and contact cues to plant resistance*

160 Two experiments were conducted to assess whether the capacity of *C. stoebe* and *T. repens* to  
161 inhibit *M. melolontha* feeding was due to the release of repellent volatiles and/or exudates or  
162 due to contact-dependent defenses. At the beginning of the first experiment, the bottom of the

163 pots were removed and replaced with a fine mesh (Windhager, Switzerland), then the pots were  
164 placed in a second pot filled with the same soil. The mesh was used to prevent roots from  
165 growing through and larvae from attaining the plants, while allowing exudates and volatiles to  
166 pass into the lower pot. A round piece of artificial diet (4 cm diameter, 1 cm height, 12 g,  
167 composition modified from Allsopp (1994)) was added to the lower belowground compartment,  
168 just below the mesh, and one L2 larva was placed at the bottom of the lower belowground  
169 compartment (Figure S1). After 14 days, the piece of artificial diet was recovered from the soil  
170 and damage was visually assessed using a five-level damage scale: no consumption ('0'), 1-  
171 30% piece consumed ('+'), 31-60% piece consumed ('++'), 61-90% piece consumed ('+++'),  
172 91-100% piece consumed ('++++'). Twelve replicates were performed per plant species (half  
173 with larvae from population Kesswil and half with larvae from population Erstfeld).

174 At the beginning of the second experiment, the bottom of the pots were removed and replaced  
175 with a fine mesh as in the first experiment. Root chemicals were allowed to diffuse into the  
176 lower pot over four days. At this time, one side of the lower pot was opened and this pot was  
177 fixed to another pot containing fresh soil of the same composition and moisture. A pot filled  
178 with soil was placed on the top of this second lower pot to equalize pressure in the two lower  
179 pots. At the same time, one L2 larva (population Bristen) was placed at the bottom of the pot  
180 below the plant (Figure S1). Twenty-four hours later, larvae were sampled back to assess  
181 whether they escaped from the pot containing root chemicals to the pot with fresh soil. Nineteen  
182 to twenty replicates were performed per plant species.

183

#### 184 *Importance of root exudates for C. stoebe resistance*

185 Since previous experiments showed chemicals released by *C. stoebe* reduce *M. melolontha* diet  
186 consumption, an additional experiment was performed to test whether this effect could be  
187 reproduced by using soluble root exudates. Exudates of *C. stoebe* and *T. officinale* were



188 collected by placing the root system of a single intact plant (which was previously shaken gently  
189 to remove most of the surrounding soil) into 50 ml of deionized water for 3 h. The water was  
190 then centrifuged for 10 min at 3500 rpm at room temperature and the supernatant collected and  
191 freeze-dried. Four plants were used per species, which exudates were mixed after freeze-drying  
192 and re-diluted into 70 ml of deionized water. This solution was used to prepare diet pieces by  
193 mixing it with agar (size, weight and proportion of agar similar to artificial diet pieces). Pieces  
194 were then offered to single L2 larva (population Bristen) in pots filled with the same soil as in  
195 the other experiments. After seven days, the pieces were recovered from the soil and damage  
196 was visually assessed using the five-level damage scale explained above. Eight replicates were  
197 performed per species.

198

199 *Contribution of structural factors and exuded or non-exuded deterrent compounds to T. repens*  
200 *resistance*

201 Since previous experiments showed that *T. repens* had a negative effect on *M. melolontha* larvae  
202 upon direct contact, but that this effect was not associated with a repellent effect of released  
203 chemicals, a series of experiments were performed on *T. repens* and *T. officinale* to test whether  
204 this effect was due to structural factors, exuded deterrent chemicals or non-exuded deterrent  
205 chemicals.

206 *Structural factors* – The effect of structural factors was tested with a setup based on feeding  
207 piece. Agar pieces were spiked with either 100 mg of fresh root pieces (~2 cm long) or 100 mg  
208 of fresh root powder obtained after grinding roots in liquid nitrogen. We hypothesized that  
209 grinding the roots would destroy plant structural features, including lignified cell walls, and  
210 would thus result in a food matrix in which root toughness could no longer be assessed by the  
211 larvae and thus influence their feeding behavior. Seven to twelve replicates per experiment and  
212 plant species were carried out, all of them with L2 larvae from population Bristen. To obtain a

213 complementary chemical measure of root toughness, total lignin was quantified in roots of *T.*  
214 *officinale* and *T. repens*. Measurements were performed on six randomly chosen control plants  
215 per species. Lignin was extracted and quantified as described in Maia et al. (2012) based on 20  
216 mg of dried powder.

217 *Soluble exuded chemicals* – Soluble exuded compounds were tested as described in the  
218 experiment comparing *T. officinale* and *C. stoebe* root exudates. The same *T. officinale* feeding  
219 pieces were used for comparison with *C. stoebe* and *T. repens*, all three plants having been  
220 tested simultaneously.

221 *Soluble non-exuded chemicals* – The potential of internal root-derived soluble chemicals to  
222 reduce *M. melolontha* feeding on *T. repens* was further tested by spiking agar pieces with root  
223 extracts from *T. officinale* or *T. repens*. Three kinds of extracts were prepared to test for a broad  
224 range of compound polarity: water, methanol and hexane. The water extract was prepared by  
225 continuous shaking of 1200 mg of fresh root powder (quantity equivalent to 100 mg per final  
226 feeding piece) into 40 ml of deionized water for 1 h. The extract was then centrifuged for 10  
227 min at 3500 rpm at room temperature and the supernatant collected, then the volume completed  
228 to 70 ml using deionized water. The methanol extract was prepared by continuous shaking of  
229 1200 mg of fresh root powder into 40 ml of methanol for 1 h. The extract was then centrifuged  
230 as above and the supernatant collected, then evaporated in a rotary vacuum evaporator at 45 °C  
231 until a volume of 5 ml was obtained. This was added to 65 ml of deionized water prior to the  
232 preparation of feeding pieces. Finally, the hexane extract was prepared by continuous shaking  
233 of 1200 mg of fresh root powder into 40 ml of hexane for 1 h. The extract was then centrifuged  
234 as above and the supernatant collected, then completely evaporated in a rotary vacuum  
235 evaporator at 45 °C. The dry residue was diluted into 5 ml of hexane:isopropanol 50:50 to  
236 improve mixing with 65 ml of deionized water during feeding piece preparation.

237

238 *Profiling of root primary metabolites*

239 Metabolic profiling of root primary metabolites and elements was performed (i) to assess the  
240 relative nutritional quality of the different plant species, and (ii) to test whether infestation by  
241 *M. melolontha* reconfigures primary metabolism, potentially as a part of induced tolerance  
242 through resource reallocation. We assessed concentrations of essential amino acids (arginine,  
243 histidine, isoleucine, leucine, lysine, phenylalanine, threonine, valine), major simple sugars  
244 (fructose, glucose, sucrose), phytosterols (campesterol, stigmasterol,  $\beta$ -sitosterol) and elements  
245 (Ca, K, Mg, Na, P). Dried roots from plants of the experiment on host suitability were used as  
246 material. Measurements were performed on the same six control plants per species that were  
247 used for lignin quantification and on the twelve plants per species placed with L3 larvae from  
248 population Valzeina. Extraction and quantification of amino acids, sugars and elements was  
249 performed as described in Hervé, Delourme, Leclair, Marnet, & Cortesero (2014), Machado et  
250 al. (2013) and Neba, Newbery, & Chuyong (2016), respectively (based on 10, 10 and 30 mg of  
251 dried powder, respectively). Phytosterols were extracted according to Feng, Liu, Luo, & Tang  
252 (2015) based on 10 mg of dried powder and quantified by ultraperformance convergence  
253 chromatography – mass spectrometry. Chromatography was performed on a Waters Acquity  
254 UPC<sup>2</sup> with a BEH 100 mm x 3.0 mm x 1.7  $\mu$ m column, with the following parameters: column  
255 temperature 40 °C, solvent A supercritical CO<sub>2</sub>, solvent B methanol, column flow 2 ml.min<sup>-1</sup>,  
256 make-up solvent methanol, make-up flow 0.2 ml.min<sup>-1</sup>, CO<sub>2</sub> back-pressure 2000 psi. The  
257 gradient of solvents was 0-1 min 98% A, 1-2 min linear decrease to 65% A, 2-2.5 min 65% A,  
258 2.5-2.6 min linear increase to 98% A, 2.6-3 min equilibration at 98% A. Compounds were  
259 quantified on a Xevo G2-XS QToF high-resolution mass spectrometer with the following  
260 parameters: positive-mode ESCi multi-mode ionization (high-speed switching between  
261 electrospray ionization and atmospheric pressure chemical ionization), source temperature 120  
262 °C, capillary voltage 3 kV, corona current 15  $\mu$ A, dry gas (nitrogen) temperature 400 °C.

263 Compounds were identified and quantified based on the following  $[M+H]^+$  fragments (amu):  
264 campesterol 383.3677,  $\beta$ -sitosterol 397.3833 and stigmasterol 395.3673. All compounds were  
265 quantified using calibration curves from pure standards.

266

#### 267 *Data analysis*

268 All statistical analyses were performed with the R software v. 3.4.0 (R Core Team, 2017).  
269 Pairwise comparisons of Estimated Marginal Means (EMMeans) were systematically  
270 performed if not otherwise stated, using the ‘emmeans’ package (Lenth, 2018). *P*-values of  
271 pairwise comparisons were always adjusted using the False Discovery Rate correction. The  
272 performance of larvae was analyzed using an ANOVA (one model per larval instar) taking into  
273 account the plant species, the larval population and the interaction between these two factors.  
274 Root consumption data were analyzed separately for each plant species using ANOVAs, which  
275 were performed separately for each larval instar in the first experiment and for each  
276 compartment (aboveground, upper belowground, lower belowground) in the second  
277 experiment. The proportion of larvae that escaped in the ‘escape experiment’ was compared  
278 between the three plant species using a likelihood ratio test applied on a generalized linear  
279 model (family: binomial, link: logit). Damage data obtained on feeding pieces or artificial diet  
280 pieces were analyzed using likelihood ratio tests applied on Cumulative Link Models (CLM),  
281 which were built using the ‘ordinal’ package (Christensen, 2018). Due to impossibility to adjust  
282 a proper CLM, root damage data were analyzed using a Kruskal-Wallis test followed by  
283 pairwise Mann-Whitney-Wilcoxon tests. Since CLMs work on latent variables which values do  
284 not make direct biological sense, medians and associated 95 % confidence intervals are  
285 systematically used for graphical representation of damage data. Primary metabolites and  
286 elements were compared between plant species using both a multivariate approach (redundancy  
287 analysis (RDA) on centered and scaled data, and associated permutation test with 9999

288 permutations, ‘vegan’ package (Oksanen et al., 2018)) and a univariate approach (Welch *t*-test  
289 for each compound, all *p*-values being further adjusted using a FDR correction). The same  
290 process was used to compare control and infested plants, separately for each species. Lignin  
291 content was also compared between plant species using a Welch *t*-test.

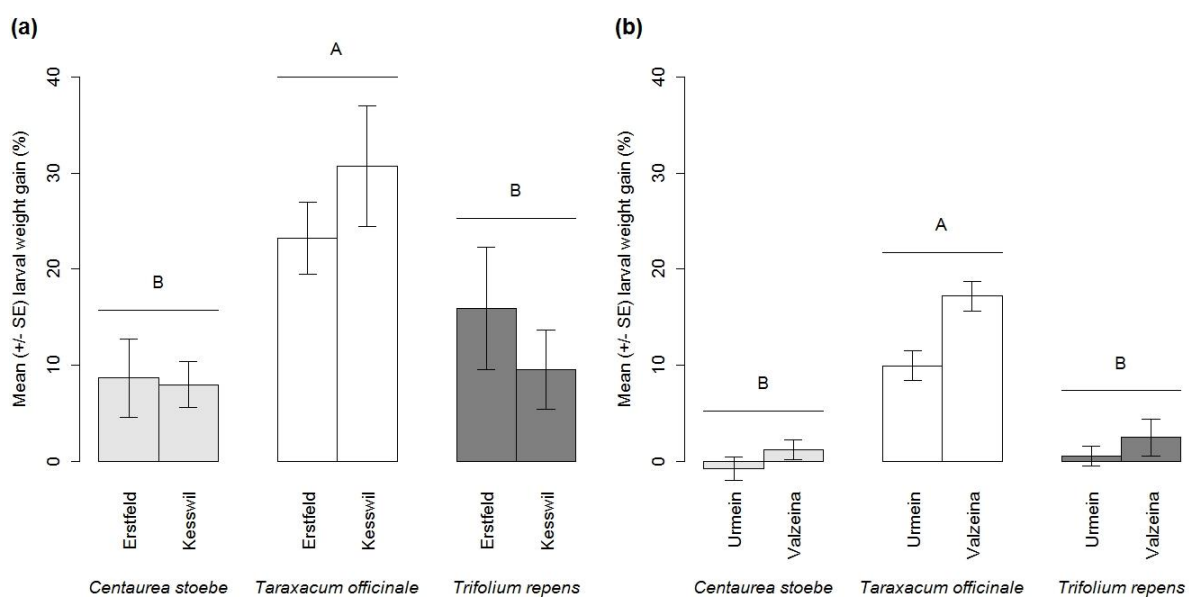
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## 293 Results

294 *M. melolontha* larvae perform better on *T. officinale* than on *C. stoebe* and *T. repens*

295 Larval performance differed significantly between the three plant species for both L2 larvae  
296 ( $F_{2,46} = 9.135, p < 0.001$ ) and L3 larvae ( $F_{2,66} = 55.542, p < 0.001$ ). Overall, the L3 population  
297 Valzeina performed systematically better than the L3 population Urmein ( $F_{1,66} = 10.563, p =$   
298  $0.002$ ). No differences between the two L2 populations were observed ( $F_{1,46} = 0.002, p = 0.969$ ).  
299 The population origin had no effect on performance patterns (L2:  $F_{2,46} = 0.889, p = 0.418$ , L3:  
300  $F_{2,66} = 2.409, p = 0.098$ ). In all cases, larval performance was better on *T. officinale* than on the  
301 two other plant species (Figure 1). Strikingly, L3 larvae did not gain any weight when feeding  
302 on *T. repens* or *C. stoebe*, suggesting the presence of strong resistance traits in these species.

303

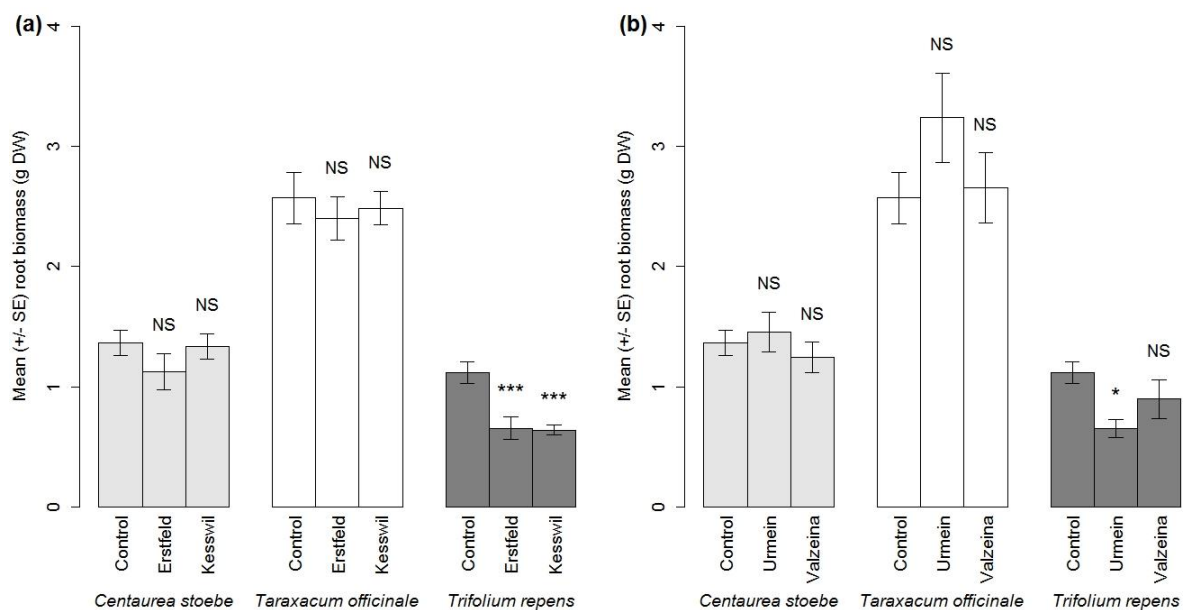


304

305 **Figure 1.** Root herbivore performance on different plant species. Performance of *Melolontha*  
 306 *melolontha* larvae from different populations on *Centaurea stoebe*, *Taraxacum officinale* and  
 307 *Trifolium repens*. (a) Growth of second-instar larvae, (b) growth of third-instar larvae.  
 308 Different letters indicate significant differences between plant species ( $p < 0.05$ ).  
 309

310 *T. officinale* specifically compensates for high root consumption through regrowth

311 No difference in *T. officinale* and *C. stoebe* root biomass was observed between control plants  
 312 and plants that were infested with *M. melolontha* (*T. officinale*: L2:  $F_{2,27} = 0.166$ ,  $p = 0.848$ ,  
 313 L3:  $F_{2,33} = 1.471$ ,  $p = 0.244$ ; *C. stoebe*: L2:  $F_{2,25} = 0.869$ ,  $p = 0.432$ , L3:  $F_{2,33} = 0.615$ ,  $p = 0.547$ )  
 314 (Figure 2). By contrast, *T. repens* root dry mass was reduced significantly upon infestation by  
 315 *M. melolontha* (L2:  $F_{2,27} = 13.494$ ,  $p < 0.001$ ; L3:  $F_{2,33} = 4.085$ ,  $p = 0.026$ ) (Figure 2).  
 316



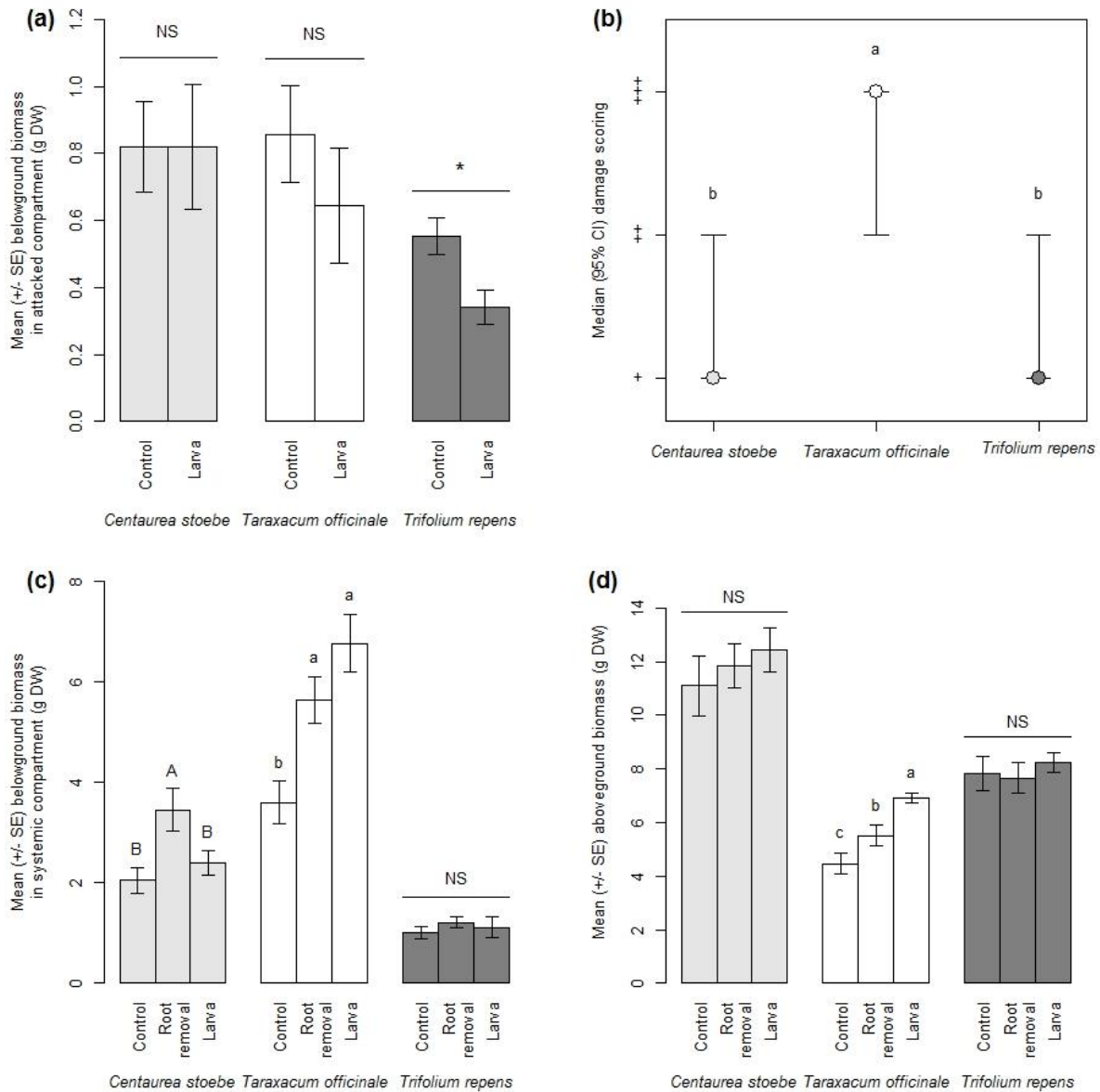
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318 **Figure 2.** Changes in root biomass following root herbivore infestation. Root biomass of  
 319 *Centaurea stoebe*, *Taraxacum officinale* and *Trifolium repens* plants that were infested with  
 320 *Melolontha melolontha* larvae from different populations (Erstfeld, Kesswil, Urmein, Valzeina)  
 321 or left uninfested (Control). (a) Second-instar larvae, (b) third-instar larvae. Asterisks indicate  
 322 significant differences between control and infested plants (\*  $p < 0.05$ , \*\*\*  $p < 0.001$ ). NS: not  
 323 significant.  
 324

325 The same pattern was observed when larvae were restricted to the lower parts of the root  
 326 systems of the different species. Root biomass of the attacked compartment was not different

327 between control and infested plants for *T. officinale* ( $F_{1,12} = 0.887$ ,  $p = 0.365$ ) and *C. stoebe*  
328 ( $F_{1,11} = 0.000$ ,  $p = 1.000$ ), whereas root biomass of *T. repens* plants was significantly reduced  
329 by *M. melolontha* attack ( $F_{1,12} = 8.072$ ,  $p = 0.015$ ) (Figure 3a). Root damage scores differed  
330 between species ( $\chi^2 = 13.475$ ,  $df = 2$ ,  $p = 0.001$ ), with *T. officinale* roots showing significantly  
331 more damage than the other two species (Figure 3b). Thus, root herbivore performance on the  
332 different species can be explained by the extent of root damage, and hence herbivore feeding,  
333 but these parameters are not reflected in final root biomass. A possible explanation for this  
334 apparent contradiction was uncovered when assessing the growth responses of the different  
335 plants upon herbivore attack and mechanical root damage. While the biomass of the shoots and  
336 the systemic roots did not change in *T. repens* in response to *M. melolontha* attack and  
337 mechanical root damage, both treatments significantly increased shoot and root biomass in *T.*  
338 *officinale* while in *C. stoebe* only mechanical damage increase root, but not shoot, biomass.  
339 (Figure 3c,d). Thus, *T. officinale* is most damaged and readily consumed by *M. melolontha*, but  
340 shows the strongest capacity for compensatory growth, and thus does not suffer from a  
341 reduction in vegetative growth under the given conditions. *Centaurea stoebe* on the other hand  
342 does not seem to be consumed by *M. melolontha* at all, which is reflected in the absence of root  
343 biomass increase despite capacity for compensatory growth. This plant is thus highly resistant  
344 to *M. melolontha*. Finally, *Trifolium repens* is fed upon by *M. melolontha*, as it suffers from a  
345 reduction in root biomass upon infestation, but damage remains low, suggesting that root  
346 consumption is limited. This suggests that this species is at least partially resistant to the root  
347 herbivore.





348

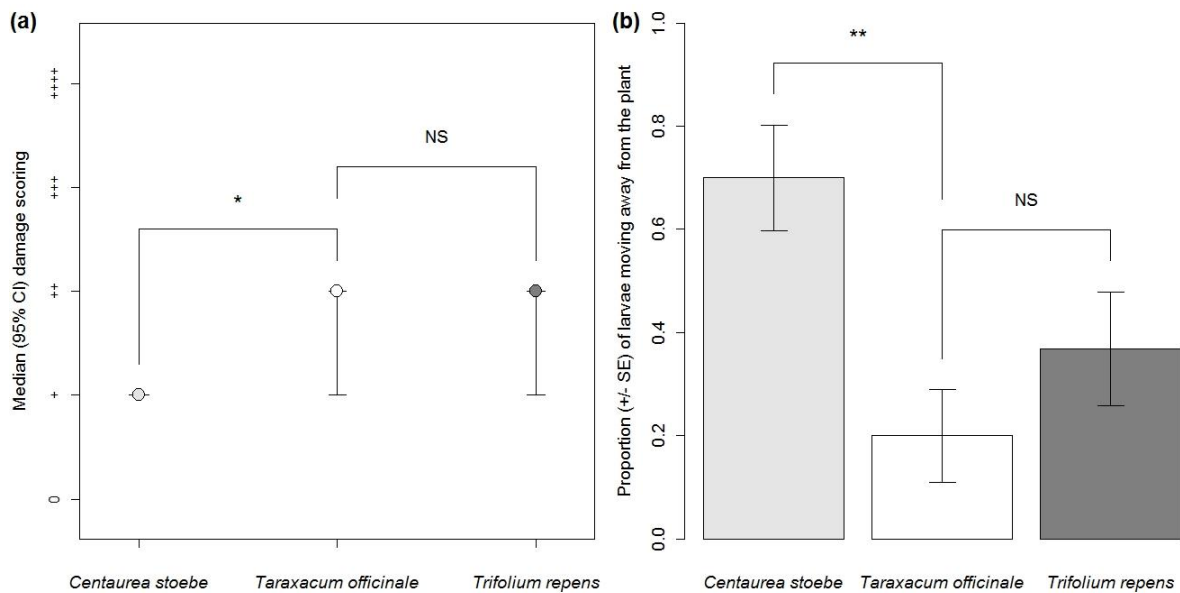
349 **Figure 3.** Root damage and regrowth patterns of different plant species in a split-root system.  
 350 (a) Root biomass in the attacked belowground compartment in control and *Melolontha*  
 351 *melolontha* infested plants (“Larva”). (b) Visual assessment of damage of roots within the  
 352 attacked belowground compartment. Scores were ‘+’: no damage except for a small spherical  
 353 area around the larva; ‘++’: one or several tunnels, but ≤ 50% of roots removed; and ‘+++’: >  
 354 50% of roots removed. (c) Root biomass in the systemic belowground compartments that were  
 355 not directly attacked by *M. melolontha*. (d) Aboveground biomass. Different letters indicate  
 356 significant differences between treatments or species ( $p < 0.05$ ). Asterisks indicate significant  
 357 differences between species (\*  $p < 0.05$ ).

358

359



360 *C. stoebe* reduces *M. melolontha* feeding by releasing chemicals in the rhizosphere  
361 Compared to *T. officinale*, exposure to *C. stoebe* at a distance reduced *M. melolontha* feeding  
362 on artificial diet (Figure 4a) and prompted the majority of the larvae to move away from the  
363 plant into a pot containing soil only (Figure 4b). No difference was shown between *T. officinale*  
364 and *T. repens*, either for damage (Figure 4a) or for the proportion of larvae moving away from  
365 the plant (Figure 4b). Therefore, *C. stoebe* has the capacity to repel *M. melolontha* without  
366 direct physical contact, which may contribute to its strong resistance phenotype.  
367



368  
369 **Figure 4.** Influence of released chemicals on root-herbivore feeding behavior. (a) Feeding  
370 activity of *Melolontha melolontha* larvae on pieces of artificial diet in the vicinity of roots of  
371 the different plant species. '0': no consumption; '+': 1-30% piece consumed; '++': 31-60%  
372 piece consumed; '+++': 61-90% piece consumed; '++++': 91-100% piece consumed. (b)  
373 Proportion of larvae moving away from the vicinity of the roots of the different species into a  
374 soil-filled pot without plant. Stars indicate significant differences between species (\*  $p < 0.05$ ,  
375 \*\*  $p < 0.01$ ).  
376

377 *The negative effect of C. stoebe is most likely not due to soluble root exudates*  
378 No difference was observed in damage scoring of feeding pieces containing root exudates of *C.*  
379 *stoebe* compared to *T. officinale* ( $\chi^2 = 2.044$ ,  $df = 1$ ,  $p = 0.153$ ). The median [95 % CI] damage

380 scoring on *C. stoebe* was ‘+++’ [‘0’ – ‘++++’] whereas on *T. officinale* it was ‘++++’ [‘+++’ –  
381 ‘++++’].

382

383 *Structural integrity of T. repens roots is associated with lower M. melolontha root consumption*

384 Experiments on feeding pieces showed that those containing root pieces of *T. repens* were

385 significantly less damaged than those containing root pieces of *T. officinale*. This difference

386 was lost when roots were ground into powder (Figure 5). Lignin content was significantly

387 higher in roots of *T. repens* (mean  $\pm$  SE  $24.33 \pm 1.02 \mu\text{g}\cdot\text{mg}^{-1}$ ) than in *T. officinale* ( $18.69 \pm$

388  $1.50 \mu\text{g}\cdot\text{mg}^{-1}$ ) ( $t_{8.814} = -3.064, p = 0.014$ ). No difference in damage was observed neither in the

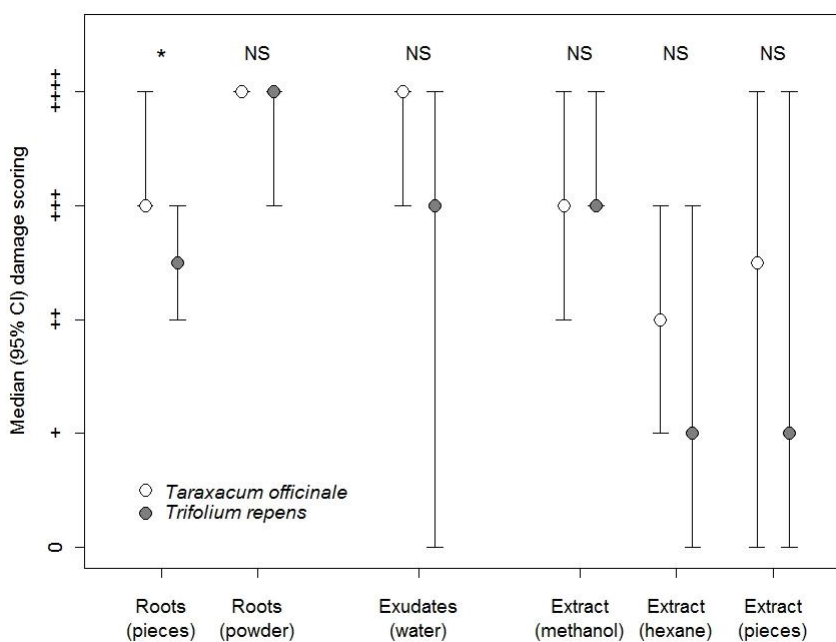
389 experiment with feeding pieces containing root exudates nor in the three experiments with

390 feeding pieces containing root extracts (Figure 5). Thus, the higher resistance of *T. repens* is

391 most closely associated with root structural features such as lignin-mediated toughness. Labile

392 chemical defenses that are destroyed during root grinding and extraction may also contribute to

393 the observed pattern.

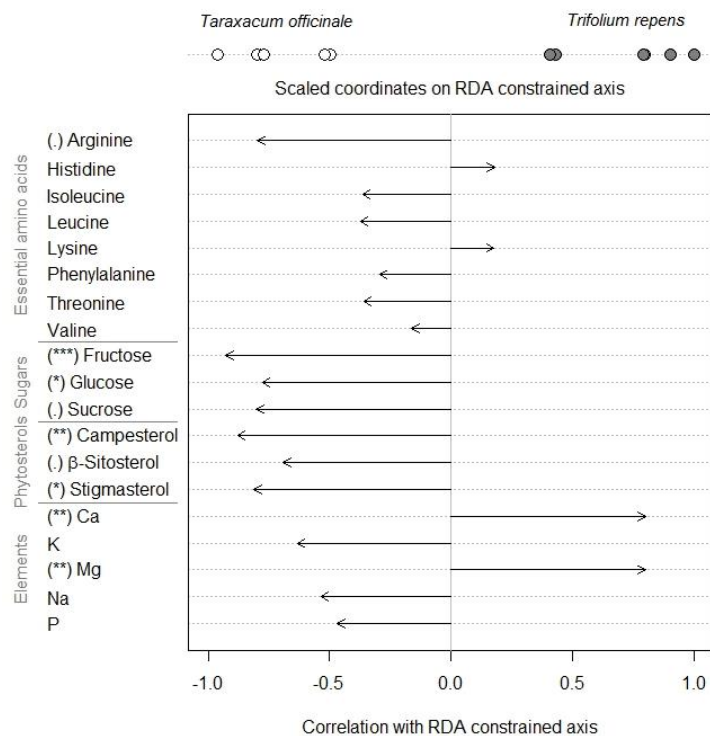


394

395 **Figure 5.** Influence of different root traits on *Melolontha melolontha* feeding. Median damage  
 396 scoring of feeding pieces in a series of experiments aiming at deciphering the contribution of  
 397 structural factors and phagodeterrent compounds in the negative effect of *Trifolium repens* on  
 398 *Melolontha melolontha* larvae. \*  $p < 0.05$ .  
 399

400 *T. repens* roots are less nutritious than *T. officinale* roots

401 The RDA showed that root nutrient contents differed between *T. officinale* and *T. repens*  
 402 (34.2% of constrained variance,  $F = 5.201$ ,  $p = 0.006$ ). Both multivariate and univariate  
 403 approaches revealed that *T. officinale* roots contained more nutrients than *T. repens* roots  
 404 (Figure 6, Table S1). The strongest differences were found for glucose (x10.9 in *Taraxacum*),  
 405 fructose (x4.4), stigmatersol (x3.4) and campesterol (x2.1). There was no difference in nutrients  
 406 between *T. officinale* roots and *C. stoebe* roots, both multivariately (14.4% of constrained  
 407 variance,  $F = 1.678$ ,  $p = 0.156$ ) and univariately (all  $p \geq 0.450$ , Table S2). Thus, the three species  
 408 vary substantially in their nutrient content, with *T. officinale* roots being richer than *T. repens*  
 409 roots in essential nutrients such as sugars and sterols but not different from *C. stoebe* roots.  
 410



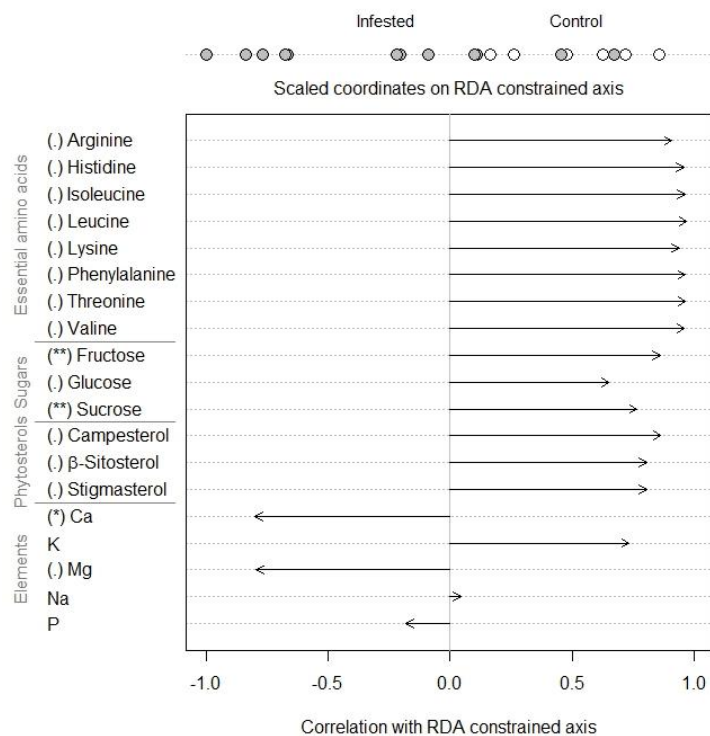
411 **Figure 6.** *Taraxacum officinale* roots are richer in sugars and sterols than roots of *Trifolium*  
 412 *repens*. Redundancy analysis (RDA) performed on nutrient content of control *Taraxacum*  
 413

414 *officinale* and *Trifolium repens*. Sample coordinates on the RDA constrained axis scaled to [-  
 415 1;1] and species names placed at the mean of the corresponding samples. Arrows show  
 416 correlations between nutrient concentrations and the RDA constrained axis. Symbols in  
 417 brackets show results of univariate tests: .  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . For  
 418 absolute levels of nutrients, refer to Supplementary Information Table 1.  
 419

420 *M. melolontha* attack reconfigures *T. officinale* primary metabolism

421 The RDA showed that herbivory by *M. melolontha* larvae induces significant changes in the  
 422 roots' primary metabolism of *T. officinale* (24.9% of constrained variance,  $F = 5.307$ ,  $p =$   
 423  $0.011$ ). The concentration of the vast majority of nutrients was lower in roots of infested plants  
 424 compared to control plants (Figure 7, Table S3). The most important decrease was for simple  
 425 sugars (-55.3 to -68.9%) and phytosterols (-33.4 to -46.3%). On the other hand, both  
 426 multivariate and univariate approaches showed no significant change with infestation in roots  
 427 of *C. stoebe* (RDA: 9.2% of constrained variance,  $F = 1.611$ ,  $p = 0.142$ ;  $t$ -tests: all  $p \geq 0.165$ ,  
 428 Table S4) and *T. repens* (RDA: 1.5% of constrained variance,  $F = 0.241$ ,  $p = 0.952$ ;  $t$ -tests: all  
 429  $p = 0.989$ , Table S5).

430



431

432 **Figure 7.** *Taraxacum officinale* roots are depleted in primary metabolites upon root herbivore  
433 attack. Redundancy analysis (RDA) performed on nutrient content of control and infested  
434 *Taraxacum officinale* plants. Sample coordinates on the RDA constrained axis scaled to [-1;1]  
435 and treatment names placed at the mean of the corresponding samples. Arrows show  
436 correlations between nutrient concentrations and the RDA constrained axis, symbols in brackets  
437 show results of univariate tests: .  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ . For absolute levels of nutrients,  
438 refer to Supplementary Information Table 3.  
439

## 440 Discussion

441 Plants directly defend themselves against root-feeding insects through a variety of strategies,  
442 including the storage and release of repellent chemicals, the construction of mechanical barriers  
443 and the reallocation of resources for future regrowth (Johnson, Benerfer, et al., 2016; Johnson,  
444 Erb, et al., 2016). These strategies have so far mostly been investigated in isolation in individual  
445 plant species. Here, we demonstrate that three co-occurring grassland species that are threatened  
446 by the same generalist root herbivore have evolved widely different defense strategies. Below,  
447 we discuss these strategies from mechanistic and ecological points of view.

448 The release of repellent chemicals can be an effective strategy to avoid herbivore attack  
449 (Unsicker, Kunert, & Gershenson, 2009). We found that, although *C. stoebe* contains high  
450 levels of nutrients similar to *T. officinale*, it does not support *M. melolontha* growth, an effect  
451 that is associated with low damage and root removal. Thus, we hypothesized that *C. stoebe*  
452 exhibits strong, almost qualitative resistance against *M. melolontha*. Indeed, *M. melolontha*  
453 feeding is inhibited even in the absence of direct root contact, and the larvae actively try to  
454 move away from *C. stoebe*. This is one of a very few examples of repellent compounds acting  
455 at distance belowground (Johnson & Nielsen, 2012). Semi-artificial diets incorporating root  
456 exudates showed no adverse effect on *M. melolontha*, suggesting that repellent volatiles may  
457 be involved. *Melolontha melolontha* possess numerous olfactory receptors and is able to detect  
458 a diversity of volatile compounds (Eilers, Talarico, Hansson, Hilker, & Reinecke, 2012).  
459 Moreover, volatile-oriented behavior has been proven in two close relative species, *M.*  
460 *hippocastani* (Weissteiner et al., 2012) and *Costelytra zealandica* (Rostás, Cripps, & Silcock,

461 2015). The repellent volatiles of *C. stoebe* are not identified yet. However, it is known that  
462 volatile bouquets emitted by roots of *C. stoebe* are dominated by high amounts of  
463 sesquiterpenes, among a diversity of other compounds (Gfeller et al., 2019). These terpenes  
464 have so far been associated with an increase rather than a decrease of *M. melolontha* growth on  
465 neighboring plants (Huang, Gfeller, & Erb, 2019). Whether the reduction in feeding observed  
466 here is dose-dependent or due to other volatile chemical cues, and whether labile soluble  
467 exudates may play a role remains to be determined. Taken together, our profiling suggests that  
468 *C. stoebe* is protected against *M. melolontha* through the release of repellent chemicals rather  
469 than strong regrowth capacity or poor nutritional value.

470 Apart from the release of chemicals, plants can protect their tissues through internal structural  
471 and chemical resistance traits. We found that *T. repens* is resistant to *M. melolontha* as *C.*  
472 *stoebe*, but that this trait is not associated with repellency from a distance. The semi-artificial  
473 diet further showed that neither root exudates, nor soluble internal chemicals can explain this  
474 resistance. Instead, intact root pieces seem to be disliked by *M. melolontha*, a pattern that is  
475 associated with high levels of root lignin in *T. repens*. As lignin directly contributes to tissue  
476 toughness, it is conceivable that higher lignification may stop *M. melolontha* from feeding on  
477 *T. repens* (Johnson, Benefer, et al., 2016). Lignin content was documented to increase root  
478 toughness and *Agriotes* spp. resistance in tobacco (Johnson et al., 2010). Additionally, our  
479 metabolic profiling showed that the nutritional quality of *T. repens* is substantially lower than  
480 that of *T. officinale*. Thus, apart from structural defenses, low nutrient levels may contribute to  
481 the low performance of *M. melolontha* on *T. repens*. Together, these results suggest that *T.*  
482 *repens* becomes resistant to *M. melolontha* because of low digestibility associated with high  
483 lignin and low nutrient contents.

484 The performance of the herbivore was the best on *T. officinale*, confirming that this species is  
485 a good host for *M. melolontha* larvae (Hauss, 1975; Hauss & Schütte, 1976). This is in line with

486 the fact that *T. officinale* roots are nutrient rich. In an interspecific study, Sukovata et al. (2015)  
487 showed that *M. melolontha* larvae grow better on plants that are more sugar-rich. Although  
488 latex defenses protect *T. officinale* to a certain degree by prompting larvae to move to congeners  
489 with lower latex defense levels (Bont et al., 2017; Huber et al., 2016), this form of resistance is  
490 not sufficient for *T. officinale* to avoid attack by *M. melolontha* in the field. Instead, as shown  
491 here, *T. officinale* has a high capacity to compensate for root loss by increasing root growth in  
492 undamaged parts of the root system as well as shoot growth. This response is associated with a  
493 substantial reduction of primary metabolites in the attacked roots, which could have been  
494 selected as a reallocation to aboveground organs favoring tolerance, a sequestration strategy to  
495 protect nutrients away from the tissues under attack and/or a direct defense strategy decreasing  
496 nutritional quality for the herbivore, as hypothesized in cases of generalist herbivores with low  
497 mobility (Berenbaum, 1995; Johnson, Erb, et al., 2016). Taken together, *T. officinale* seems to  
498 be highly nutritious and little defended towards *M. melolontha*, but seems to be able to tolerate  
499 attack through compensatory growth.

500 Of note, the defense strategies of the plant species tested in this study closely match the defense  
501 syndromes described for aboveground traits of milkweeds by Agrawal & Fishbein (2006).  
502 *Centaurea stoebe* seems to follow ‘Nutrition and defense’, with good nutritional quality but  
503 strong resistance traits repelling *M. melolontha* larvae. *Trifolium repens* would fit into the  
504 category ‘Low nutritional quality’, with structural defenses combined with low nutritional  
505 quality. *Taraxacum officinale* seems to follow a ‘Tolerance/escape’ strategy, with important  
506 abilities to compensate for root loss and, as shown by Bont et al. (2019), increased seed  
507 dispersal. The fact that tolerance is expected to exert no selection pressure on herbivores (Weis  
508 & Franks, 2006) may explain why *T. officinale* is the preferred host plant of *M. melolontha* and  
509 why there is a positive historical relationship between *M. melolontha* and *T. officinale*  
510 abundance in European grasslands (Schütte, 1996). Interestingly, *T. officinale* is also one of the



511 preferred host plants of wireworms, that co-occur with *M. melolontha* in European grasslands  
512 (Wallinger et al., 2014). This suggests that the defense strategy of *T. officinale* against generalist  
513 root herbivores might be independent of the herbivore species. From the perspective of the  
514 herbivore, our work raises questions regarding the evolution of host preference in generalist  
515 root herbivores. Could it be that host preferences in these insect species are driven by intrinsic  
516 defense strategies of their hosts, resulting in preferences for tolerant over resistant plants over  
517 evolutionary time? If this were the case, we would expected generalist root herbivores to  
518 accumulate on tolerant plants in the field. The hypothesis that accumulation of generalists  
519 predicts the defense syndrome of plants within natural communities remains to be tested.

520

#### 521 **Authors' Contributions**

522 MRH and ME conceived the ideas and designed methodology; MRH collected the data; MRH  
523 analyzed the data; MRH and ME interpreted the data and wrote the manuscript.

524

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531

#### 532 **Data Accessibility**

533 The data of this manuscript has been deposited on Dryad [to be inserted at a later date].

534

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