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3 A HERBIVORE TAG-AND-TRACE SYSTEM REVEALS CONTACT-  
4 AND DENSITY-DEPENDENT REPELLENCE OF A ROOT TOXIN

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6 ZOE BONT<sup>1</sup>, CARLA ARCE<sup>1</sup>, MERET HUBER<sup>2</sup>, WEI HUANG<sup>1</sup>, ADRIEN MESTROT<sup>3</sup>, CRAIG  
7 J. STURROCK<sup>4</sup> AND MATTHIAS ERB<sup>1\*</sup>

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9 *<sup>1</sup>Institute of Plant Sciences, University of Bern, Bern, Switzerland*

10 *<sup>2</sup>Department of Biochemistry, Max-Planck Institute for Chemical Ecology, Jena, Germany*

11 *<sup>3</sup>Institute of Geography, University of Bern, Bern, Switzerland*

12 *<sup>4</sup>Centre for Plant Integrative Biology, School of Biosciences, University of Nottingham, Sutton  
13 Bonington, Leicestershire, UK*

14 \*Corresponding author: matthias.erb@ips.unibe.ch, +41 31 631 86 68

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27 **Abstract** - Foraging behaviour of root feeding organisms strongly affects plant-environment-  
28 interactions and ecosystem processes. However, the impact of plant chemistry on root  
29 herbivore movement in the soil is poorly understood. Here, we apply a simple technique to trace  
30 the movement of soil-dwelling insects in their habitat without disturbing or restricting their  
31 interaction with host plants: We tagged the root feeding larvae of *Melolontha melolontha* with a  
32 copper ring and repeatedly located their position in relation to their preferred host plant,  
33 *Taraxacum officinale*, using a commercial metal detector. This method was validated and used  
34 to study the influence of the sesquiterpene lactone taraxinic acid  $\beta$ -D-glucopyranosyl ester (TA-  
35 G) on the foraging of *M. melolontha*. TA-G is stored in the latex of *T. officinale* and protects the  
36 roots from herbivory. Using behavioural arenas with TA-G deficient and control plants, we  
37 tested the impact of physical root access and plant distance on the effect of TA-G on *M.*  
38 *melolontha*. *M. melolontha* larvae preferred TA-G deficient plants over control plants, but only  
39 when physical root contact was possible and the plants were grown at a distance of 5 cm. *M.*  
40 *melolontha* showed no preference for TA-G deficient plants when the plants were grown 15 cm  
41 apart, which may indicate a trade-off between the cost of movement and the benefit of  
42 consuming less toxic food. We demonstrate that *M. melolontha* integrates host plant quality  
43 and distance into its foraging patterns and suggest that plant chemistry affects root herbivore  
44 behaviour in a plant-density dependent manner.

45

46 **Key Words** - Root herbivore, Foraging, Tag-and-trace, Imaging, *Melolontha melolontha*,  
47 *Taraxacum officinale*.

48

## INTRODUCTION

49 Root feeding herbivores strongly influence natural and agricultural ecosystems (Hunter, 2001):  
50 They can shape plant fitness and plant community composition (van der Putten 2003; Zvereva  
51 and Kozlov 2012), above ground herbivore damage (Erb et al. 2008; Wäckers and Bezemer  
52 2003) and tritrophic interactions (Rasmann et al. 2005; Soler et al. 2005; van Tol et al. 2001).  
53 In contrast to wingless shoot feeders such as caterpillars, belowground herbivores are less  
54 dependent on the physical structure of their host plants as they can move around freely in the  
55 soil matrix. However, the energetic costs of moving through the soil are presumably higher  
56 (Johnson et al. 2016).

57 In the absence of visual stimuli, belowground herbivores rely on chemical cues to find suitable  
58 hosts. They are for instance able to detect plants from a distance by tracing plant-derived  
59 volatiles and exudates in the rhizosphere (Liu et al. 2016; Nordenhem and Nordlander 1994;  
60 Weissteiner et al. 2012; reviewed by Johnson and Gregory 2006 and by Johnson and Nielsen  
61 2012). After host plant encounter, biting into the root enables root herbivores to assess host  
62 quality (van Dam 2009; Watts et al. 2011). The specialist root feeder *Diabrotica virgifera* for  
63 example is able to use plant volatiles such as (*E*)- $\beta$ -caryophyllene to select maize plants from  
64 a distance, exudates such as benzoxazinoids to locate nutritious maize roots and endogenous  
65 metabolites such as conjugated phenylpropanoids to avoid leaf-infested plants (Robert et al.  
66 2012a, 2012b; Erb et al. 2015).

67 According to optimal foraging theory, animals should forage in a way that maximizes their  
68 fitness (Pyke 1984). In terms of food location and acquisition, this means that animals should  
69 aim at maximizing energy intake while minimizing energy loss through movement (Charnov  
70 1976). Bumblebees for instance integrate information about the quality and distance of different  
71 feeding sites to optimize their foraging patterns (Lihoreau et al. 2011). Because movement in  
72 the soil is costly, the distance between different roots and plants should strongly influence  
73 foraging and host plant preferences of root feeding herbivores (Johnson et al. 2016). To date,  
74 this aspect is not well investigated in the context of root-herbivore interactions (Sonnemann et  
75 al. 2014).

76 One reason why we know so little about root herbivore foraging is related to the fact that

77 observing herbivores in the soil is challenging. So far, most behavioral experiments were  
78 conducted using artificial setups such as slant-boards (Dawson et al. 2002; Gerard et al. 2005),  
79 transparent sandwich systems (Eilers et al. 2016; Reinecke et al. 2008; Schumann et al. 2013)  
80 or petri-dishes (Robert et al. 2012b; Weissteiner et al. 2012). These set-ups allow detailed and  
81 continuous observations, but constrain plant growth and/or insect movement. Other  
82 conventional methods rely on destructive sampling (Murray et al. 2010; Schumann and Vidal  
83 2012) and are therefore not suited to trace movement patterns over time. Non-destructive, non-  
84 restricting techniques to track root herbivore movements include acoustic detection and X-ray  
85 microtomography (reviewed in Johnson et al. 2007). The latter has been used to screen soil  
86 columns and gives interesting insights into herbivore activity, but is restricted to small sample  
87 sizes and requires high-end equipment (Johnson et al. 2004). Acoustic detection on the other  
88 hand is limited in detection range and sensitivity (Mankin et al. 2000). An accurate, inexpensive,  
89 non-invasive tracking system to trace root herbivores would complement the already available  
90 tools and help to better understand patterns of root herbivory.

91 We studied the foraging patterns of the root-feeding larvae of the European cockchafer  
92 (*Melolontha melolontha*; Coleoptera: Scarabaeidae) when feeding on their preferred host plant  
93 (Hauss 1975; Hauss and Schütte 1976), the common dandelion (*Taraxacum officinale* agg.;  
94 Flora Helvetica, 5th edition). *M. melolontha* is capable to detecting a variety of different  
95 chemical stimuli through its frontal sensory organs, including CO<sub>2</sub>, water and various alcohols,  
96 aldehydes, ketones, acids, amines and terpenoids (Eilers et al. 2012). The roots of *T. officinale*  
97 exude high amounts of bitter latex sap upon wounding which can influence the behavior of *M.*  
98 *melolontha* (Huber et al. 2016a). *T. officinale* latex contains three major classes of secondary  
99 metabolites: Phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene  
100 lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) (Huber et al. 2015). We recently  
101 demonstrated that TA-G protects *T. officinale* against herbivory by *M. melolontha* by repelling  
102 the root herbivore (Huber et al. 2016a). In small scale choice experiments *M. melolontha*  
103 preferred to feed on TA-G deficient plants that were silenced in the expression of the  
104 Germacrene A synthase ToGAS1 rather than wild type plants. Based on these findings and the  
105 fact that TA-G is mostly produced and stored in the laticifers of *T. officinale*, we hypothesized  
106 that the compound acts as a negative gustatory stimulus for *M. melolontha*. Furthermore, based

107 on the potentially high energetic cost of movement in the soil, we hypothesized that *M.*  
108 *melolontha* should be more likely to choose between plants of different quality when they are  
109 growing in close proximity.

110 To test these hypotheses, we developed a metal-tag based tag-and-trace system based on an  
111 approach which was developed by Piper and Compton (2002) to track the larvae of a leaf-litter  
112 dwelling chrysomelid beetle. To record the movement patterns of *M. melolontha* we marked the  
113 larvae with copper rings and located their position with a metal detector. We next validated the  
114 system in a series of preference and performance experiments, including X-ray  
115 microtomography. We then used the validated system to elucidate the effect of TA-G on *M.*  
116 *melolontha* behaviour and to test whether *M. melolontha* can integrate host plant distance into  
117 its foraging behaviour.

118

## METHODS AND MATERIALS

119 *Insect Rearing.* Third instar *M. melolontha* larvae were used for all experiments. Larvae were  
120 collected from meadows in Urmein, Bristen and Sion (Switzerland) and reared at 10 °C and  
121 darkness in individual plastic cups filled with a mixture of moist potting soil and grated carrots.  
122 Before experiments, larvae were starved for seven days at room temperature in single cups  
123 filled with moist potting soil.

124 *Plant Cultivation.* Wild type and transgenic *T. officinale* plants in the background A34 were used  
125 in the present study. A34 was originally created by crossing diploid pollen of a triploid apomict  
126 from the Netherlands with a diploid mother from France (Verhoeven et al. 2010). F2 plants of  
127 two transgenic lines (RNAi-1, RNAi-16) which are silenced in the expression of the Germacrene  
128 A synthase ToGAS1 and show a high reduction of TA-G accumulation in the latex were used  
129 together with a transgenic control line (RNAi-15), which was transformed in an identical manner  
130 but does not exhibit ToGAS1 silencing. The transgenic lines were described and characterized  
131 previously (Huber et al. 2015). All seeds were germinated on seedling substrate and  
132 transplanted into individual pots filled with potting soil (5 parts 'Landerde', 4 parts peat and 1  
133 part sand) after 2.5 weeks. Unless specified otherwise, cultivation and experiments took place  
134 in a climate chamber operating under the following conditions: Temperature at day 22 °C and  
135 at night 18 °C; 16 h light and 6 h darkness; 65% humidity.

136 *Development of a Tag-and-trace System for M. melolontha.* To study *M. melolontha* behaviour  
137 in the soil, we developed a tag-and-trace system which consisted of tagging individual grubs  
138 with a copper-ring and detecting their movement and position in the soil using a commercial  
139 metal detector (Bullseye TRX Pinpointer, White's Electronics, USA). To tag the grubs, thin  
140 copper wire (0.5 mm) was coiled around the larval body between the second and third pair of  
141 front legs. The total weight of an individual tag was around 105 mg, which corresponds to an  
142 increase in larval mass of 5%. The wire endings were twisted together to form a small antenna  
143 (Fig. 1a). Preliminary experiments showed that this particular shape of the copper tag optimizes  
144 the detectability of the larvae in the soil as it complements the ring around the body in a way  
145 that maintains electromagnetic inducibility independently of the position of the larva.

146 *Influence of the Copper Tag on M. melolontha Performance.* To investigate whether the metal  
147 tag influences *M. melolontha* development and behaviour, we carried out three experiments.

148 First, we measured the weight gain of third instar larvae with ( $N = 12$ ) and without a copper ring  
149 ( $N = 12$ ) every seven days for nine weeks. Larvae were kept in darkness at 20-22 °C in plastic  
150 cups filled with potting soil and grated carrots. The substrate was changed every week, and  
151 larval weight was determined at the same time.

152 Second, to assess whether the copper from the tag is taken up by the larvae, we determined  
153 copper accumulation in the larvae using inductively coupled plasma mass spectrometry (ICP-  
154 MS). Tagged ( $N = 6$ ) and non-tagged larvae ( $N = 6$ ) were anaesthetized at the end of the  
155 performance assay and haemolymph was collected into ice-cold phosphate buffered saline  
156 (PBS, Green and Sambrook 2012), diluted to a final volume of 1:10 with PBS, centrifuged at  
157 5900 g for 3 min at 4 °C and supernatant was stored at -80 °C (Popham et al. 2004). Fat tissue  
158 and body (without gut) were separated, frozen in liquid nitrogen and stored at -80 °C until  
159 analysis. For sample preparation, body and fat tissue samples were lyophilized overnight and  
160 freeze-dried body samples were ground to fine powder using mortar and pestle. Samples were  
161 then weighed into polyethylene tubes (approximately 100 mg for haemolymph, 50 mg for body  
162 tissue and 20 mg for fat tissue) and accurate weight was noted. For digestion, 4 ml of 69%  
163 ultrapure HNO<sub>3</sub> (Rotipuran, Supra, Carl Roth, Karlsruhe, Germany) was added to each sample  
164 and left for 10 min. Then 1 ml of 30% ultrapure H<sub>2</sub>O<sub>2</sub> (Rotipuran, Supra, Carl Roth, Karlsruhe,  
165 Germany) was added to each tube and samples were digested using a microwave digestion  
166 unit (Ethos cont-FLOW 1600, MLS, Leutkirch, Germany) operating for 5 min at 120 °C and 300  
167 W, then for 15 min at 200 °C and 700 W and for 20 min at 200 °C and 450 W. After cooling  
168 down, samples were diluted with ultrapure water to a final volume of 25 ml. Digestion of each  
169 analytical batch was accompanied by one blank sample and one certified reference material  
170 sample (CRM, ERM-BB422 Fish muscle). Sample solutions were then diluted by a factor of 2  
171 and determination of copper content was performed using ICP-MS (7700x ICP-MS, Agilent  
172 Technologies, Santa Clara, USA). A multi-element standard solution (ICPMS-71A, Inorganic  
173 Ventures, Christiansburg VA, USA) was used to establish an external calibration curve. Copper  
174 concentrations were then compared separately for body, fat tissue and haemolymph between  
175 tagged and non-tagged larvae.

176 Third, we used high resolution X-ray micro Computed Tomography (X-ray  $\mu$ CT) to profile the  
177 foraging behaviour of tagged and non-tagged *M. melolontha* feeding on the roots of *T. officinale*

178 plants. Seven-week old plants in single pots (7.5 cm diameter, 20 cm length) filled with sandy  
179 soil (clay loam with 50% silver sand) which were grown in growth rooms with a 16/8 h light cycle  
180 at a temperature of 23/18 °C were used for the experiment. Half of the pots ( $N = 8$ ) were infested  
181 with one unmarked *M. melolontha* larva, whereas the other half of the plants ( $N = 8$ ) were  
182 infested with one larva carrying a copper ring by carefully inserting the larva into the soil in 3  
183 cm distance to the plant. The device used for imaging was a Phoenix v|tome|x M (GE Sensing  
184 and Inspection Technologies, Wunstorf, Germany) at The Hounsfield Facility (University of  
185 Nottingham, UK) set to 180 kV and 160  $\mu$ A. Per scan, 1199 projections were taken  
186 (average/skip = 1/0) with 250 ms detector timing, resulting in a scan time of 5 min per pot and  
187 a resolution of 105  $\mu$ m microns. All pots were scanned twice a day for three consecutive days.  
188 Data were reconstructed for visualisation using datos|x software (GE Sensing). While moving  
189 through the soil, the larvae left behind air filled tunnels due to soil compression. The tunnels  
190 could be visualized in the scans, allowing the reconstruction of the complete moving pattern of  
191 the larvae. VGStudio MAX V.2.2 (Volume Graphics GmbH, Heidelberg, Germany) software  
192 was used to trace the tunnels and measure their lengths using the polyline tool. The “*Region*  
193 *Growing*” selection tool was used to segment plant root system and to calculate the amount of  
194 main root that was consumed by the larvae. Foraging and moving parameters from  
195 measurements of the first two days were then compared between marked and unmarked  
196 larvae. Values from the third day were excluded as tunnel tracing was inconclusive due to high  
197 movement activity of some larvae.

198 *Development of Arenas for Behavioural Experiments.* To profile the behaviour of tagged *M.*  
199 *melolontha* larvae, we developed an inexpensive arena system using customized PVC cable  
200 canals (15 - 25 cm length, 6 cm width, 4 cm height). The cable canals were closed with root  
201 barrier tissue (Trenn-Vlies, GeoTex Windhager, Switzerland) on both sides (Fig. 1b). The size  
202 of the arenas allowed us to plant up to two plants on either side of the arena while still leaving  
203 enough space for a neutral zone in the middle. Unless specified otherwise, *T. officinale* plants  
204 were transplanted three days before the start of the experiments. In some experiments, we  
205 inserted root barriers (6.5 cm x 4 cm; Trenn-Vlies, GeoTex Windhager, Switzerland) to restrict  
206 root growth while allowing root exudates and volatiles to diffuse into the centre of the arenas.  
207 The arenas were filled with potting soil (5 parts ‘Landerde’, 4 parts peat and 1 part sand). Plants



208 were watered every 1-2 days. Individual tagged *M. melolontha* larvae were then carefully  
209 inserted in the middle of the arenas. Using the metal detector, we recorded the positions of the  
210 larvae at different time intervals. At the end of the experiments, the larvae were recaptured, the  
211 copper rings removed and the plants harvested for chemical analysis. To validate the setup,  
212 we conducted a first behavioural experiment in which we offered one seven-week old *T.*  
213 *officinale* control plant (RNAi-15) on one side and soil without plant on the other side of the  
214 arena to *M. melolontha* larvae ( $N = 20$ ). Root barriers prevented direct contact of the larvae  
215 with the plant or the empty control side. Three days after transplanting, the tagged larvae were  
216 put into the arenas and their positions were recorded six times over 48 h as described above.  
217 Larval position was recorded by dividing the arena into two segments of equal size containing  
218 test plants and one central part (2 cm length) in between. To analyse larval foraging locomotion  
219 patterns in more detail, we conducted an additional experiment in which we exposed tagged  
220 larvae to three different choice situations: i) No plant on both sides of the arena, ii) no plant vs.  
221 *T. officinale* on one side and iii) *T. officinale* on both sides. Seven-week old A34 plants were  
222 transplanted into the arenas as described above. One day after transplanting, one tagged larva  
223 was put into each arena ( $N = 18-20$  for each choice situation) and the position of the larvae  
224 was recorded on a centimetre scale every 10 min for 6 h. The central part of 2 cm length was  
225 defined as neutral area. For each larva we determined the overall preference behaviour.  
226 Furthermore, we calculated the mean moving speed of the larvae per hour.

227 *Influence of Plant Toxins on M. melolontha Foraging.* In a next step, we used the validated tag-  
228 and-trace system together with the behavioural arenas to analyse the impact of root secondary  
229 metabolites on *M. melolontha* foraging. We focused our experiments on taraxinic acid  $\beta$ -D-  
230 glucopyranosyl ester (TA-G), a sesquiterpene lactone that is produced in high quantities in the  
231 latex of *T. officinale* and has been shown to reduce *M. melolontha* damage and food  
232 consumption (Huber et al. 2016a, 2016b).

233 In a first experiment, we offered seven-week old TA-G deficient (RNAi-1, RNAi-16) and TA-G  
234 producing (RNAi-15) *T. officinale* plants to *M. melolontha* using 25 cm long arenas. One TA-G  
235 deficient (RNAi-1 or RNAi-16) and one control (RNAi-15) plant were transplanted at a distance  
236 of 5 cm or 15 cm ( $N = 16$  for each distance and genotype combination). Root barriers were  
237 used to restrict *T. officinale* root growth during the acclimatization phase and removed before

238 the start of the behavioural experiments to give *M. melolontha* direct access to the roots. This  
239 setup allowed us to test whether *M. melolontha* behaviour is influenced by TA-G production  
240 and whether this effect depends on the distance between the different host plants. Based on  
241 the results of our first experiments, the position of the larvae was recorded 6 times over 48 h.  
242 In a second experiment, we tested whether *M. melolontha* needs direct root contact to choose  
243 between TA-G deficient and control plants. To answer this question, we used 15 cm long arenas  
244 and one seven-week old TA-G deficient (RNAi-1 or RNAi-16) and one control (RNAi-15) plant  
245 were transplanted at either end of the arenas with root barriers restricting their growth. After  
246 three days, root barriers were removed for half of the arenas to enable direct root contact. For  
247 the other half of the arenas the root barriers were left in the arenas ( $N = 20$  for each treatment  
248 and genotype combination). Larval behaviour was determined as described above.

249 *Phenotyping of TA-G Deficient and TA-G Producing Plants.* To validate the phenotypes of the  
250 transgenic plants, we compared biomass and latex profiles of TA-G deficient and TA-G  
251 producing plants. For biomass determination, we used six replicates of eight-week old TA-G  
252 deficient (RNAi-1, RNAi-16) and TA-G producing (RNAi-15) plants. The root systems were  
253 washed under tap water, the plants were separated into above and belowground parts and the  
254 dry mass was determined after drying the plant parts in an oven at 80 °C until constant weight  
255 was reached. Dry mass was analysed between genotypes for leaves and roots separately.

256 To confirm reduced TA-G production in ToGAS1 silenced plants and assess them for potential  
257 pleiotropic effects, we conducted chemical analyses of latex of seven-week old plants (RNAi-  
258 1, RNAi-15, RNAi-16;  $n=10$  per genotype). To collect latex, main roots were cut 0.5 cm below  
259 the tiller and 2  $\mu$ l of exuding latex was pipetted immediately into Eppendorf tubes containing  
260 200  $\mu$ l methanol. The tubes were vortexed for 10 min, put into ultrasonic bath for 10 min,  
261 centrifuged at 14'000  $g$  for 20 min and supernatants were stored at -20 °C. The methanol  
262 extracts were injected into an Acquity-TQD UPLC-PDA-MS (Waters) with electrospray  
263 ionization in positive mode, consisting of an ultra-performance liquid chromatograph (UPLC)  
264 coupled to a photodiode array detector (PDA) and a single quadrupole mass specific detector  
265 (QDa). Metabolites were separated on an Acquity BEH-C18 column (2.1 mm x 100 mm, particle  
266 size 1.7  $\mu$ m, Waters). The flowrate was set to 0.4 ml min<sup>-1</sup>, column temperature was 55 °C and  
267 injection volume 2.5  $\mu$ l. The mobile phase A consisted of H<sub>2</sub>O and formic acid (99.9:0.1), and

268 the mobile phase B of ACN and formic acid (99.9:0.1). The following gradient was used: 0-1.5  
269 min: 5% B; 2.5 min 20% B; 3 min 40% B; 5 min 95% B; 6 min 5% B. The QDa was operated in  
270 ESI- using a cone voltage of 10 V and a scan range of 150-650 *m/z*. The PDA scan range was  
271 200-600 nm. TA-G was identified based on its absorption and mass spectrum, and its identity  
272 was confirmed by co-injection of purified TA-G. For quantification of TA-G, peak areas at 245  
273 nm were integrated and concentrations were calculated using a standard curve with loganin as  
274 external standard and the corresponding response factor to pure TA-G. To assess potential  
275 pleiotropic effects due to silencing ToGAS1, we further analyzed another major class of latex  
276 metabolites, di- and tri-4-hydroxyphenylacetate inositol esters (PIEs, Huber et al. 2015) in the  
277 latex methanol extracts. UPLC-PDA-MS analysis was performed as described, and PIEs were  
278 quantified by integrating peak areas at 275 nm. Relative concentrations were calculated for di-  
279 and tri-PIEs separately.

280 *Data Analysis.* To test whether metal-marking had an effect on larval performance, we analysed  
281 weight gain of tagged and non-tagged larvae with a generalized linear model (GLM, function  
282 'glm') which takes into account the repeated measurements of the same individuals, followed  
283 by Wald tests (package 'car' (Fox and Weisberg 2011)). To analyse copper contents of the  
284 larvae, we compared individual tissues using Student's T-tests. Furthermore, we employed a  
285 linear mixed effect model (LMM, function 'lmer' of package 'lme4' (Bates et al. 2015)) to analyse  
286 the effect of the copper tag across tissues. 'Tissue' and 'Treatment' were used as fixed factors,  
287 and 'Larval identity' was included as a random factor. To analyse larval movement patterns in  
288 the high-resolution behaviour assay, we used a linear model with 'position' as response  
289 variable, 'Time' and 'Treatment' as fixed factors and '(1|Arena)' as random factor. To compare  
290 moving speed in each hour of measurement between the choice situations, we used a linear  
291 model with 'Moving speed' as response variable and 'Time' and 'Treatment' as fixed factors.  
292 To analyse larval behaviour in the preference assays, we calculated the percentage of  
293 detections in each side of the arena for each larva. The percentage of detections in the central  
294 area did not differ between choice situations and was therefore excluded from the analysis. To  
295 test whether the larvae prefer a specific host plant, linear mixed models were established for  
296 each choice situation separately, using 'Percentage of detection' as response variable, 'Side'  
297 and 'Genotype' (TA-G deficient line RNAi-1 or -16) as fixed factors and '(1|Arena)' as random

298 factor. To test if the distance or accessibility of the roots has an influence on the percentage of  
299 detection in each side of the arena, we established linear models with 'Percentage of detection'  
300 as response variable and 'Distance' respectively 'Root accessibility' and 'Genotype' (TA-G  
301 deficient line RNAi-1 or -16) as explanatory variables separately for each test plant side. All  
302 models were tested using Wald tests and pairwise comparisons of Least Squares Means of  
303 significant terms were performed using the function 'lsmeans' (package 'lsmeans' (Lenth  
304 2016)). The results were plotted using the package 'ggplot2' (Wickham 2009). All statistical  
305 analyses were performed in R 3.3.0 (R Core Team 2016).

306

## RESULTS

307 *Influence of Copper Tagging on M. melolontha Performance and Host Location.* The copper  
308 tag did not impair the growth of third instar *M. melolontha* larvae over 9 weeks (GLM:  $P = 0.146$ ;  
309 Fig. 2a). Furthermore, copper concentrations of fat tissue ( $t$ -test:  $P = 0.280$ ; Fig. 2b),  
310 haemolymph ( $t$ -test:  $P = 0.576$ ; Fig. 2b) and the remaining body ( $t$ -test:  $P = 0.180$ ; Fig. 2b) did  
311 not significantly increase in metal tagged grubs compared to non-tagged individuals after 9  
312 weeks. Across all measured tissues, the copper tag tended to increase copper concentrations  
313 (LMM:  $P = 0.059$ ; Fig 2b). The profiling of *M. melolontha* behaviour in the rhizosphere of *T.*  
314 *officinale* using X-ray  $\mu$ CT revealed that in general, larvae first moved downwards into the  
315 bottom half of the pot, then turned towards the plant and moved upwards again while  
316 consuming the tap root. The scanning further visualized small air-filled caves around the larvae  
317 (Fig. 3a). The activity of *M. melolontha* varied greatly between individuals, ranging from no  
318 movement up to 115 cm moving distance in 72 h (Fig. 3b). No differences in consumed root  
319 volume ( $t$ -test:  $P = 0.222$ ; Fig. 3d) or distance covered until first tap root contact ( $t$ -test:  $P =$   
320  $0.125$ ; Fig. 3e) between grubs with and without copper ring were found. However, metal tagged  
321 larvae covered less distance in the same time than non-tagged individuals ( $t$ -test:  $P = 0.008$ ;  
322 Fig. 3c).

323 *Profiling of Root Herbivore Behaviour in the Soil.* Metal tagged larvae moved slower through  
324 the soil than non-tagged grubs, but showed no differences in other foraging parameters (Fig.  
325 2). We therefore concluded that small-scale arenas (Fig. 1b) can be used to profile the foraging  
326 behaviour of the tagged larvae. Indeed, when presented with a single *T. officinale* plant, *M.*  
327 *melolontha* larvae could find their host plant reliably, even in the absence of direct root contact  
328 (LMM:  $P < 0.001$ ; Fig. 1c).

329 *Locomotion Patterns of M. melolontha.* The position of the larvae significantly changed over  
330 time and depended on the number of available plants in the arena (Fig. 4a). *M. melolontha*  
331 preferred the side of the plant when they had the choice between *T. officinale* and only soil (Fig.  
332 4a). By contrast, the larvae moved back and forth between plants when two genetically identical  
333 *T. officinale* plants were grown in the arena. Without host plants, the larvae moved around  
334 randomly in the choice arena (Fig. 4a). During the six hours of measurement the larvae covered  
335 an average distance of  $35.0 \pm 3.3$  cm (no plant in the arena),  $29.1 \pm 2.2$  cm (one plant in the

336 arena) and  $37.7 \pm 3.6$  cm (two plants in the arena; one-way ANOVA:  $P=0.147$ ). On a per hour  
337 basis, *M. melolontha* moving speed was significantly lower when one plant was available  
338 compared to when two plants were present (LM:  $P=0.010$ ; Fig. 4b). On average, *M. melolontha*  
339 clearly preferred the side of the arena containing *T. officinale* when only one plant was present  
340 (LMM:  $P=0.011$ ; Fig. 4c), but showed no preference for neither side of the arena when no or  
341 two plants were available.

342 *M. melolontha* Prefers TA-G Deficient Genotypes over Short Distances. When TA-G deficient  
343 plants and control plants were growing at a distance of 5 cm, *M. melolontha* showed a clear  
344 preference for TA-G deficient plants (LMM:  $P=0.010$ ; Fig. 5a). However, when the genotypes  
345 were growing at a distance of 15 cm, the grubs showed no preference any more (LMM:  $P=$   
346  $0.464$ ; Fig. 5a). When the genotypes were grown in a distance of 5 cm, the larvae were detected  
347 significantly less often close to the TA-G producing plant (LM:  $P=0.048$ ; Fig. 5a) than when  
348 the plants were at 15 cm distance. Choice patterns were similar for both TA-G deficient  
349 transgenic lines in both choice setups (LMM:  $P>0.479$ ).

350 *Direct Root Contact Is Required for the Repellent Effect of TA-G.* We found that the preference  
351 of *M. melolontha* for TA-G deficient plants was directly dependent on direct root contact (LMM:  
352  $P=0.015$ ; Fig. 5b): As soon as root barriers were used to restrict the grubs from biting into the  
353 roots, *M. melolontha* did not distinguish between TA-G deficient and control plants any more  
354 (LMM:  $P=0.271$ ; Fig. 5b). The root accessibility had no influence on the percentage of  
355 detections close to the TA-G deficient plant (LM:  $P=0.167$ ; Fig. 5b), but if root contact was  
356 possible, the larvae were detected less often close to the TA-G producing plant (LM:  $P=0.024$ ;  
357 Fig. 5b). Choice patterns were similar for both TA-G deficient transgenic lines in both choice  
358 setups (LMM:  $P>0.297$ ).

359 *Effect of Silencing ToGAS1 on Biomass and Latex Metabolites.* Biomass of neither roots nor  
360 shoots differed between genotypes (one-way ANOVA:  $P=0.273$  reps.  $P=0.277$ ; Fig. 6a).  
361 Chemical analysis confirmed that concentration of TA-G in line RNAi-1 and RNAi-16 was  
362 significantly reduced compared to the TA-G concentration in control plants RNAi-15 (one-way  
363 ANOVA:  $P<0.001$ ; Fig. 6b). The TA-G deficient line RNAi-16 produced higher amounts of di-  
364 PIs than the TA-G deficient line RNAi-1 (one-way ANOVA:  $P=0.001$ ; Fig. 6c) and higher  
365 amounts of tri-PIs than RNAi-1 (one-way ANOVA:  $P<0.001$ ; Fig. 6d) and RNAi-15 (one-way

366 ANOVA:  $P = 0.005$ ; Fig. 6d). By contrast, RNAi-1 and the control line RNAi-15 did not differ in  
367 di- or tri-PIE levels.

## DISCUSSION

368

369 Our study introduces a non-invasive system to observe foraging of belowground herbivores in  
370 their natural environment and employs the system to test the impact of a plant toxin on a root  
371 feeder. Through this approach, we demonstrate that the deterrent effect of the plant toxin  
372 depends on direct physical contact and the presence of an alternative host in close proximity.  
373 Several techniques have been developed to document root-herbivore behaviour in the soil, but  
374 their applicability to track root feeders has remained limited (Johnson et al. 2007). Metal  
375 detection has not been used to trace root herbivores so far, but has been successfully applied  
376 to track leaf-litter dwelling beetles (Piper and Compton 2002; Piper et al. 2014) and to relocate  
377 underwater organisms like sea urchins (Duggan and Miller 2001). By gluing small strips of  
378 stainless steel imprinted with unique codes on the dung cases of *Cryptocephalus coryli* larvae,  
379 Piper and Compton (2002) were able to relocate the tagged larvae in the leaf-litter with a  
380 commercial metal detector. In our experiments, we used copper rings to mark the larvae.  
381 Copper is a good electrical conductor and therefore elicits a strong signal for detection, which  
382 enabled us to detect tagged organisms in more than 10 cm soil depth. By fixing the copper wire  
383 as a ring around the larval body we were able to tag the highly mobile and soft-skinned *M.*  
384 *melolontha* larvae without the need for glue or a hard surface. Our study shows that carrying a  
385 copper ring for nine weeks has no negative impact on the weight gain of third instar *M.*  
386 *melolontha*. Copper concentrations in tagged larvae tended to increase after carrying the tag  
387 for 9 weeks. As the larvae are typically only marked for a few days for behavioural screens,  
388 side-effects of copper accumulation were likely negligible in our experiments. However, long-  
389 term experiments need to take potential side effects of increased copper exposure into account.  
390 A crucial part in the development of the tag-and-trace system was the cross validation using X-  
391 ray  $\mu$ CT, which enabled us to assess the influence of the metal-tag on larval behaviour. Tagged  
392 larvae covered less distance than non-tagged larvae during the time of observation, which  
393 indicates that the copper ring may physically constrain larval movements in compact soil  
394 matrices. However, the larvae were still able to move within the soil, and root-foraging  
395 behaviour was not altered by the metal tag. From these observations, we conclude that the  
396 system is an inexpensive and valid method to track medium-sized belowground organisms over  
397 short distances.



398 Root herbivores can use a variety of cues to assess host plant suitability. In a first step, root  
399 herbivores can use cues which are released from the roots, including volatiles and exudates  
400 (Erb et al. 2013; van Dam and Bouwmeester 2016). After biting into the root, various  
401 endogenous metabolites like sugars (Bernklau and Bjostad 2008; Mochizuki et al. 1985;  
402 Sutherland and Hillier 1976) amino acids (Sutherland and Hillier 1974) and secondary  
403 metabolites (Schmelz et al. 2002; Sutherland et al. 1980; Robert et al. 2012b) can play a role  
404 as feeding stimulants or deterrents for the herbivore. Our experiments show that *M. melolontha*  
405 favours *T. officinale* plants with reduced amount of the secondary metabolite TA-G in the latex  
406 over plants with normal TA-G quantities and thus confirm the results of Huber et al. (2016a).  
407 Notably, the preference was dependent on the accessibility of the roots: When physical contact  
408 of larvae and roots was prevented through a fine mesh, *M. melolontha* did not distinguish  
409 between the different genotypes any more. This result is in accordance with the fact that TA-G  
410 is mostly stored in the laticifers, although small amounts are also found in root cortex cells  
411 (Huber et al. 2016b). More than 10% of all vascular land plants possess laticifers, which are  
412 specialised cells with a distinct cytoplasm known as latex (Farrell et al. 1991; Metcalf 1967;  
413 reviewed in Agrawal and Konno 2009). Latex exudation only occurs after tissue disruption, and  
414 to assess the chemical composition of the latex, herbivores therefore need to mechanically  
415 damage the plant e.g. by chewing through the root epidermis. From our experiment we suggest  
416 that TA-G acts as a contact dependent feeding deterrent for *M. melolontha*. *M. melolontha*  
417 larvae are equipped with a sophisticated set of chemoreceptors (Eilers et al. 2012), which  
418 enables them to perceive and distinguish highly diverse olfactory and tactile stimuli, including  
419 TA-G, as shown here.

420 The movement of an organism depends on various factors, including the internal state (why  
421 move?) as well as the capacity of motion (how to move?) and navigation (when and where to  
422 move? Nathan et al. 2008). Subterranean locomotion is energetically expensive, as the soil  
423 matrix is much denser than the phyllosphere (Barnett and Johnson 2013; Luna and Antinuchi  
424 2006; Perissinotti et al. 2009). We therefore hypothesized that the distance between different  
425 food sources may influence the choice behaviour of *M. melolontha*. Sonnemann et al. (2014)  
426 showed that the migration of root-feeding click beetle larvae was food-density dependent. In  
427 our study, we show that the distance between plants has a direct impact on larval preference:

428 With unrestricted root access, *M. melolontha* favours TA-G deficient *T. officinale* when the plant  
429 is growing in 5 cm distance to a TA-G producing plant, but shows no preference between  
430 genotypes when the distance is increased to 15 cm. Although the copper tag reduces  
431 movement speeds, the tagged larvae moved around 6 cm per hour in the choice arenas; their  
432 capacity to move was therefore sufficient to taste and choose between host plants at both 5  
433 and 15 cm distance and is unlikely to have influenced the observed choice patterns. A biological  
434 hypothesis for the observed behaviour is that the cost of moving another 15 cm outweighs the  
435 cost of high TA-G levels and prompts *M. melolontha* to accept an inferior host plant in  
436 environments with low plant densities. To confirm this hypothesis, measuring energy costs of  
437 TA-G tolerance and movement in the soil would need to be determined along with additional  
438 experiments to understand the relationship between host plant distance and food quality.

439

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584

## FIGURE LEGENDS

585 **Fig. 1** Tag-and-trace system to track root herbivores in the soil. (a) Third instar *M. melolontha*  
586 larvae tagged with a copper ring. (b) Preference assay arena in which the larvae were released.  
587 Orange shapes indicate root barriers to avoid direct contact. (c). Choice of *M. melolontha* larvae  
588 between a *T. officinale* host plant and an empty compartment. Mean values ( $N = 20$ ) and  
589 standard errors ( $\pm$  SE) are shown. '\*\*\*\*' indicates a significant difference in larval preference  
590 ( $P < 0.001$ )

591 **Fig. 2** The copper tag does not influence *M. melolontha* performance and Cu content. (a)  
592 Performance of tagged and non-tagged *M. melolontha* larvae. Mean values ( $N = 12$ ) and  
593 standard errors ( $\pm$  SE) are shown. (b) Copper concentration in body, fat tissue and haemolymph  
594 of tagged and non-tagged *M. melolontha* larvae. Mean values ( $N = 4-6$ ) and standard errors  
595 ( $\pm$  SE) are shown. 'n.s.' indicates no significant difference between groups ( $P \geq 0.05$ )

596 **Fig. 3** The copper tag does not influence host location and foraging of *M. melolontha*, but  
597 reduces movement speed. (a) Copper tagged larva foraging on *T. officinale* visualized by X-  
598 ray micro computer tomography ( $\mu$ CT). (b) Example of the movement path of a larva over 72h  
599 measured by X-ray  $\mu$ CT. The green line shows the movement of the larvae over this period. (c)  
600 Speed of movement of tagged and untagged *M. melolontha* larvae. (d) Tap root consumption.  
601 (e) Host location efficiency. Mean values ( $N = 6-8$ ) and standard errors ( $\pm$  SE) are shown. '\*\*\*'  
602 ( $P < 0.01$ ) indicates a significant difference between groups. 'n.s.' indicates no significant  
603 difference ( $P \geq 0.05$ )

604 **Fig. 4** High resolution tracking of *M. melolontha* larvae in the presence of host plants. (a) Colour  
605 maps of *M. melolontha* larval position over time in arenas with 0, 1 or 2 host plants. Darker  
606 colours indicate higher frequencies of detection at a given time point. (b) Horizontal movement  
607 speed of *M. melolontha* averaged over 1 h. (c) Overall preference of *M. melolontha* for the sides  
608 of the arenas. Mean values ( $N = 18-20$ ) and standard errors ( $\pm$  SE) are shown. '\*\*\*' indicates  
609 significant differences between groups ( $P < 0.01$ ). 'n.s.' indicates no significant difference  
610 ( $P \geq 0.05$ )

611 **Fig. 5** *M. melolontha* prefers TA-G deficient *T. officinale* plants only when growing in close  
612 proximity with wild types and when physical root contact is possible. (a) Larval preference of  
613 *M. melolontha* larvae choosing between control and TA-G deficient *T. officinale* plants growing



614 at a distance of 5 cm (top) or 15 cm (bottom). Root access was possible for the larvae in both  
615 cases. Mean values ( $N = 32$ ) and standard errors ( $\pm$  SE) are shown. (b) Larval preference of  
616 *M. melolontha* larvae choosing between control and TA-G-deficient *T. officinale* plants in the  
617 presence of a fine mesh, which allows passage of plant metabolites, but prevents direct  
618 physical contact (top) or without mesh, which allows physical contact (bottom). Mean values ( $N$   
619 = 40) and standard errors ( $\pm$  SE) are shown. ‘\*’ indicates significant differences within and  
620 between choice situations ( $P < 0.05$ ). TA-G: taraxinic acid  $\beta$ -D-glucopyranosyl ester  
621 **Fig. 6** Biomass and latex profiles of TA-G-deficient *T. officinale* plants. Root and leaf dry mass  
622 (a), TA-G (b), di-PIE (c) and tri-PIE (d) concentrations of the different transgenic lines used in  
623 this study. RNAi-15 is a transformed control line that does not exhibit target gene silencing.  
624 Mean values ( $N = 10$ ) and standard errors ( $\pm$  SE) are shown. Different letters indicate significant  
625 differences between genotypes ( $P < 0.05$ ). ‘n.s.’ indicates no significant difference between  
626 groups ( $P \geq 0.05$ ). TA-G: taraxinic acid  $\beta$ -D-glucopyranosyl ester. PIE: Phenolic inositol ester