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

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

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

INTRODUCTION

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

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

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

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

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

We studied the foraging patterns of the root-feeding larvae of the European cockchafer (*Melolontha melolontha;* Coleoptera: Scarabaeidae) when feeding on their preferred host plant (Hauss 1975; Hauss and Schütte 1976), the common dandelion (*Taraxacum officinale* agg.; Flora Helvetica, 5th edition). *M. melolontha* is capable to detecting a variety of different chemical stimuli through its frontal sensory organs, including CO_2, water and various alcohols, aldehydes, ketones, acids, amines and terpenoids (Eilers et al. 2012). The roots of *T. officinale* exude high amounts of bitter latex sap upon wounding which can influence the behavior of *M. melolontha* (Huber et al. 2016a). *T. officinale* latex contains three major classes of secondary metabolites: Phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) (Huber et al. 2015). We recently demonstrated that TA-G protects *T. officinale* against herbivory by *M. melolontha* by repelling the root herbivore (Huber et al. 2016a). In small scale choice experiments *M. melolontha* preferred to feed on TA-G deficient plants that were silenced in the expression of the Germacrene A synthase ToGAS1 rather than wild type plants. Based on these findings and the fact that TA-G is mostly produced and stored in the laticifers of *T. officinale*, we hypothesized 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.

METHODS AND MATERIALS

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

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

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

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

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

Second, to assess whether the copper from the tag is taken up by the larvae, we determined copper accumulation in the larvae using inductively coupled plasma mass spectrometry (ICP-MS). Tagged (*N* = 6) and non-tagged larvae (*N* = 6) were anaesthetized at the end of the performance assay and haemolymph was collected into ice-cold phosphate buffered saline (PBS, Green and Sambrook 2012), diluted to a final volume of 1:10 with PBS, centrifuged at 5900 *g* for 3 min at 4 °C and supernatant was stored at -80 °C (Popham et al. 2004). Fat tissue and body (without gut) were separated, frozen in liquid nitrogen and stored at -80 °C until analysis. For sample preparation, body and fat tissue samples were lyophilized overnight and freeze-dried body samples were ground to fine powder using mortar and pestle. Samples were then weighed into polyethylene tubes (approximately 100 mg for haemolymph, 50 mg for body tissue and 20 mg for fat tissue) and accurate weight was noted. For digestion, 4 ml of 69% ultrapure HNO_3 (Rotipuran, Supra, Carl Roth, Karlsruhe, Germany) was added to each sample and left for 10 min. Then 1 ml of 30% ultrapure H_2O_2 (Rotipuran, Supra, Carl Roth, Karlsruhe, Germany) was added to each tube and samples were digested using a microwave digestion unit (Ethos cont-FLOW 1600, MLS, Leutkirch, Germany) operating for 5 min at 120 °C and 300 W, then for 15 min at 200 °C and 700 W and for 20 min at 200 °C and 450 W. After cooling down, samples were diluted with ultrapure water to a final volume of 25 ml. Digestion of each analytical batch was accompanied by one blank sample and one certified reference material sample (CRM, ERM-BB422 Fish muscle). Sample solutions were then diluted by a factor of 2 and determination of copper content was performed using ICP-MS (7700x ICP-MS, Agilent Technologies, Santa Clara, USA)**.** A multi-element standard solution (ICPMS-71A, Inorganic Ventures, Christiansburg VA, USA) was used to establish an external calibration curve. Copper concentrations were then compared separately for body, fat tissue and haemolymph between tagged and non-tagged larvae.

Third, we used high resolution X-ray micro Computed Tomography (X-ray µCT) to profile the 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 µA. 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 µm 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

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

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

In a first experiment, we offered seven-week old TA-G deficient (RNAi-1, RNAi-16) and TA-G producing (RNAi-15) *T. officinale* plants to *M. melolontha* using 25 cm long arenas. One TA-G deficient (RNAi-1 or RNAi-16) and one control (RNAi-15) plant were transplanted at a distance of 5 cm or 15 cm (*N* = 16 for each distance and genotype combination). Root barriers were 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 µl of exuding latex was pipetted immediately into Eppendorf tubes containing
260 200 µl 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 µm, Waters). The flowrate was set to 0.4 ml min^{-1}, column temperature was 55 °C and
267 injection volume 2.5 µl. The mobile phase A consisted of H_2O and formic acid (99.9:0.1), and

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

Data Analysis. To test whether metal-marking had an effect on larval performance, we analysed weight gain of tagged and non-tagged larvae with a generalized linear model (GLM, function 'glm') which takes into account the repeated measurements of the same individuals, followed by Wald tests (package 'car' (Fox and Weisberg 2011)). To analyse copper contents of the larvae, we compared individual tissues using Student's T-tests. Furthermore, we employed a linear mixed effect model (LMM, function 'lmer' of package 'lme4' (Bates et al. 2015)) to analyse the effect of the copper tag across tissues. 'Tissue' and 'Treatment' were used as fixed factors, and 'Larval identity' was included as a random factor. To analyse larval movement patterns in the high-resolution behaviour assay, we used a linear model with 'position' as response variable, 'Time' and 'Treatment' as fixed factors and '(1|Arena)' as random factor. To compare moving speed in each hour of measurement between the choice situations, we used a linear model with 'Moving speed' as response variable and 'Time' and 'Treatment' as fixed factors. To analyse larval behaviour in the preference assays, we calculated the percentage of detections in each side of the arena for each larva. The percentage of detections in the central area did not differ between choice situations and was therefore excluded from the analysis. To test whether the larvae prefer a specific host plant, linear mixed models were established for each choice situation separately, using 'Percentage of detection' as response variable, 'Side' 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 µCT 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 ± 3.3 cm (no plant in the arena), 29.1 ± 2.2 cm (one plant in the

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

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

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

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

Our study introduces a non-invasive system to observe foraging of belowground herbivores in their natural environment and employs the system to test the impact of a plant toxin on a root feeder. Through this approach, we demonstrate that the deterrent effect of the plant toxin depends on direct physical contact and the presence of an alternative host in close proximity.

Several techniques have been developed to document root-herbivore behaviour in the soil, but their applicability to track root feeders has remained limited (Johnson et al. 2007). Metal detection has not been used to trace root herbivores so far, but has been successfully applied to track leaf-litter dwelling beetles (Piper and Compton 2002; Piper et al. 2014) and to relocate underwater organisms like sea urchins (Duggan and Miller 2001). By gluing small strips of stainless steel imprinted with unique codes on the dung cases of *Cryptocephalus coryli* larvae, Piper and Compton (2002) were able to relocate the tagged larvae in the leaf-litter with a commercial metal detector. In our experiments, we used copper rings to mark the larvae. Copper is a good electrical conductor and therefore elicits a strong signal for detection, which enabled us to detect tagged organisms in more than 10 cm soil depth. By fixing the copper wire as a ring around the larval body we were able to tag the highly mobile and soft-skinned *M. melolontha* larvae without the need for glue or a hard surface. Our study shows that carrying a copper ring for nine weeks has no negative impact on the weight gain of third instar *M. melolontha*. Copper concentrations in tagged larvae tended to increase after carrying the tag for 9 weeks. As the larvae are typically only marked for a few days for behavioural screens, side-effects of copper accumulation were likely negligible in our experiments. However, long-term experiments need to take potential side effects of increased copper exposure into account.

A crucial part in the development of the tag-and-trace system was the cross validation using X-ray µCT, which enabled us to assess the influence of the metal-tag on larval behaviour. Tagged larvae covered less distance than non-tagged larvae during the time of observation, which indicates that the copper ring may physically constrain larval movements in compact soil matrices. However, the larvae were still able to move within the soil, and root-foraging behaviour was not altered by the metal tag. From these observations, we conclude that the system is an inexpensive and valid method to track medium-sized belowground organisms over short distances.

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

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

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

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583

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 (± SE) are shown. '***' indicates a significant difference in larval preference
590 ($P < 0{\cdot}001$)

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 (± 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 (± SE) are shown. 'n.s.' indicates no significant difference between groups ($P \geq 0{\cdot}05$)

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 (µCT). (b) Example of the movement path of a larva over 72h
599 measured by X-ray µCT. 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 (± SE) are shown. '**'
602 ($P < 0{\cdot}01$) indicates a significant difference between groups. 'n.s.' indicates no significant
603 difference ($P \geq 0{\cdot}05$)

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 (± SE) are shown. '**' indicates
609 significant differences between groups ($P < 0{\cdot}01$). 'n.s.' indicates no significant difference
610 ($P \geq 0{\cdot}05$)

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 (± 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 (± SE) are shown. '*' indicates significant differences within and
620 between choice situations ($P < 0{\cdot}05$). TA-G: taraxinic acid β-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 (± SE) are shown. Different letters indicate significant
625 differences between genotypes ($P < 0{\cdot}05$). 'n.s.' indicates no significant difference between
626 groups ($P \geq 0{\cdot}05$). TA-G: taraxinic acid β-D-glucopyranosyl ester. PIE: Phenolic inositol ester