

SPECIAL ISSUE: MOLECULAR DETECTION OF TROPHIC INTERACTIONS

How generalist herbivores exploit belowground plant diversity in temperate grasslands

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Abstract

Belowground herbivores impact plant performance, thereby inducing changes in plant community composition, which potentially leads to cascading effects onto higher trophic levels and ecosystem processes and productivity. Among soil-living insects, external root-chewing generalist herbivores have the strongest impact on plants. However, the lack of knowledge on their feeding behaviour under field conditions considerably hampers achieving a comprehensive understanding of how they affect plant communities. Here, we address this gap of knowledge by investigating the feeding behaviour of *Agriotes* click beetle larvae, which are common generalist external root-chewers in temperate grassland soils. Utilizing diagnostic multiplex PCR to assess the larval diet, we examined the seasonal patterns in feeding activity, putative preferences for specific plant taxa, and whether species identity and larval instar affect food choices of the herbivores. Contrary to our hypothesis, most of the larvae were feeding-active throughout the entire vegetation period, indicating that the grassland plants are subjected to constant belowground feeding pressure. Feeding was selective, with members of Plantaginaceae and Asteraceae being preferred; Apiaceae were avoided. Poaceae, although assumed to be most preferred, had an intermediate position. The food preferences exhibited seasonal changes, indicating a fluctuation in plant traits important for wireworm feeding choice. Species- and instar-specific differences in dietary choice of the *Agriotes* larvae were small, suggesting that species and larval instars occupy the same trophic niche. According to the current findings, the food choice of these larvae is primarily driven by plant identity, exhibiting seasonal changes. This needs to be considered when analysing soil herbivore–plant interactions.

Keywords: *Agriotes obscurus*, *Agriotes sputator*, root feeding, soil herbivory, trophic interaction

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Introduction

Insect herbivores feeding belowground can exert a profound effect not only on vegetation composition and community dynamics (Bever 1994; De Deyn *et al.* 2003; Johnson & Murray 2008) but on ecosystem processes in general (Johnson & Murray 2008). Among soil-living insects, external root-chewing generalist herbivores usually show the strongest impact on plants (Zvereva & Kozlov 2012). They primarily consume the fine roots

that are important in taking up water and nutrients, essential for plant growth. In mixed plant communities such as grasslands, the effect of root herbivory is often less obvious compared with species-poor sites (Zvereva & Kozlov 2012).

Generalist root herbivores are presumed to feed on a wide variety of plants, which can happen either unselectively or selectively: while unselective feeders choose their food upon availability, the food choice of selective feeders depends on plant-specific traits, leading to a preference for certain plants. These traits include nutrient composition (Behmer 2009) such as nitrogen content (Mattson 1980) as well as plant defence via mechanical

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and chemical deterrents (Kessler & Baldwin 2002). So far, the focus of studies examining the influence of plant-specific traits on the food choice of generalist herbivores has largely been on aboveground systems (e.g. Bernays & Minkenberg 1997; Behmer 2009; Franzke *et al.* 2010). For the generalist herbivore, dietary mixing is beneficial to dilute any of these plant secondary compounds and to increase the overall diet-quality by improving the nutrient balance. There is a paucity of studies on belowground herbivory compared with plant-feeding aboveground, mainly due to difficulties in examining what is eaten (Miles 1945; Brown & Gange 1990; Hunter 2001). This is especially true for soil-living arthropods, which consume their food in liquid state, and where identification of semi-digested plant tissue in the intestine is challenging. This lack of knowledge hampers a deeper mechanistic understanding of how the herbivores interact with plant communities (Hiltpold & Turlings 2012). So far, specific insect-plant model systems (e.g. *Diabrotica virgifera virgifera* and maize) have shown that this interaction is complex, but comparable studies for other belowground herbivores and in natural scenarios are currently missing. Fortunately, recent progress in molecular analysis of herbivory allows unravelling which roots insect herbivores eat and whether they consume specific plants in field situations (Soininen *et al.* 2009; Valentini *et al.* 2009; Pompanon *et al.* 2012). Among these molecular methods, diagnostic multiplex PCR provides a rapid means for identification of ingested plant taxa (Staudacher *et al.* 2011; Wallinger *et al.* 2012).

Grasslands occur naturally on almost all continents thus comprising some 26% of total land area and 80% of arable land (Boval & Dixon 2012). In vegetation, they are a mixture of different grass species, legumes and herbs that occupy land continuously for more than 1 year. Temperate grasslands harbour a large and diverse invertebrate fauna and wireworms, the larvae of click-beetle (Elateridae, Coleoptera) are among the dominant belowground macroinvertebrates (Jones & Jones 1984). Within the elaterid family, *Agriotes* larvae are the most abundant generalist root herbivores, externally chewing on subterranean plant parts. They exhibit a long-lasting development in the soil of up to 5 years (Parker & Howard 2001). Feeding on a wide range of plants including arable crops and vegetables (Hill 1987; Parker & Howard 2001; Traugott *et al.* 2008) they have significant agricultural importance (Benefer *et al.* 2012; Staudacher *et al.* 2013). While *Agriotes*-induced damage and wireworm-feeding behaviour are well studied in arable fields (e.g. Vernon 2005; Traugott *et al.* 2008; Schallhart *et al.* 2012; Staudacher *et al.* 2013), their feeding behaviour in temperate grasslands, their native habitat, remains fairly unknown. A long-distant detection

of potential attractants or deterrents in the rhizosphere is unlikely, so that *Agriotes* larvae are presumed to primarily feed on what they find on site (Chaton *et al.* 2003). Consequently, the larvae are probably to feed on the most abundant plant species in their habitat (Griffiths 1974; Sonnemann *et al.* 2012), summoning their generalist status. However, recent findings show that – in maize cropping systems – the food choice of *Agriotes* wireworms is not a simple matter of supply and demand but affected by both plant identity and diversity (Schallhart *et al.* 2012; Staudacher *et al.* 2013). Feeding experiments with selected plant species indicate that also among grassland plants, *Agriotes* wireworms do prefer some species over others (Hemerik *et al.* 2003; Johnson & Gregory 2006; Wallinger *et al.* 2013). But such laboratory-derived outcomes suffer compounding limitations when reconciling the observed behaviour under natural conditions.

In the present study, we remedy this situation by examining the feeding behaviour of *Agriotes* larvae under field conditions. We aimed to validate their putative generalist status in temperate grasslands and tested the following five hypotheses applying diagnostic multiplex PCR: (i) *Agriotes* wireworms have two feeding-active phases, one in spring and one in late summer, as suggested by earlier work of Parker & Howard (2001); (ii) Grasses are the most preferred food source because they are representing the dominant plant group in temperate grassland habitats and *Agriotes* larvae should have co-evolutionary adapted to this food source; (iii) Preference for specific plant species changes during the season; (iv) *Agriotes* species differ in their dietary preferences occupying separate trophic niches; and (v) early-instar larvae feed on different plant species than late-instar larvae due to different dietary requirements.

Materials and methods

Species and sampling design

For the assessment of the dietary choice during the season (full-season data set), two perennial grasslands were selected in the valley of the river Inn (Tyrol, Austria): grassland 1 (611 m a.s.l.; N 47.306305°; E 11.106283°; WGS 84) and grassland 2 (539 m a.s.l.; N 47.31245°; E 11.6450917°; WGS 84). In 2008, twenty parcels (10 × 10 m; 10 parcels in a row, two rows per grassland) were established per site. Within each parcel, six plots of 1 m² each were set up, leaving a buffer zone of 1 m around them. At the first sampling date, the plant cover and abundance of each plant species was estimated on the first of these six plots per parcel according to the method of (Braun-Blanquet 1964) using the 9-degree scale of scale of Wilmanns (1993).

Four soil cores (20 cm in diameter, ~15 cm in depth) centred within the 1-m² plot (Fig. 1), leaving a buffer zone of 20 cm around them were taken. *Agriotes* wireworms were hand-collected, individually placed in 1.5-ml reaction tubes and freeze-killed at -28 °C on the very same day. According to the catching results on this initial sampling, those ten parcels of the twenty harbouring the highest numbers of wireworms, were selected for further sampling in the following months. In doing so, we were able to maximize the numbers of larvae for the analysis because they are known to be patchily distributed in the field. At each of the monthly sampling dates, for each of the ten selected parcels, both, the estimation of plant cover and the wireworm-sampling took place on a consecutive plot of the remaining five plots. At the end of May (grassland 1) and beginning of June (grassland 2), data loggers Tinytag Ultra 2 (Gemini data loggers, West Sussex, UK) were installed to measure soil temperature and moisture during the study period in 2008. Time between sampling varied due to weather conditions and mowing events; sampling took place on 28 April–15 May, 26 May–10 June, 26 June–2 July, 30 July–1 August, 2–4 September, and 1–6 October 2008.

For the summer data set, four regions were selected throughout Tyrol, where vegetation was recorded and wireworms were sampled in June and July 2009 on 21 1-m²-plots as described previously.

Food supply was derived from aboveground plant cover, because it is difficult to obtain species-specific measurements of root biomass in the field. The lack of information on belowground plant distributions is due to major experimental difficulties. They originate from the fact that roots are so intermingled in the soil and cannot be easily traced back to an individual plant of known identity (Mommer *et al.* 2008). As a result, there is little comprehensive information on the distribution of root systems among coexisting species and grasslands possess large numbers of species with morphologically

indistinguishable roots (Frank *et al.* 2010). But the target plants within this study exhibit a medium degree of root penetration in the soil, and they are comparable in their root formation (Kutschera & Lichtenegger 1992), at least in the 0–15 cm soil layer. This is where 70% of the total root biomass and the feeding-active wireworms are situated.

DNA extraction

All reaction tubes containing frozen *Agriotes* wireworms were checked for regurgitates; if present, the wireworms were transferred to fresh tubes, so that regurgitates and wireworms could be DNA extracted and screened separately. As regurgitates are much smaller samples than whole wireworms and because they are probably to contain mainly food DNA and only few consumer DNA (Waldner & Traugott 2012), each regurgitate was immediately dissolved in extraction buffer and incubated for 2 h at 58 °C.

Larval stage of wireworms was assessed. To prevent the amplification of plant material potentially sticking on the wireworms' surface, each larva was washed in 1 mL of 1–1.5% sodium hypochlorite solution (Sigma-Aldrich, St. Louis, USA) for 30 s and then rinsed twice with molecular-grade water and then they were DNA extracted as a whole, including any plant DNA present within their guts, following a CTAB-based protocol (for details see: Wallinger *et al.* 2013).

All extractions were conducted in a pre-PCR laboratory using a UV-equipped laminar flow hood. Extraction negative controls were included in each batch of wireworms to check for cross-contamination of samples.

PCR and visualization of PCR products

The wireworm and regurgitate samples were screened for ingested plant DNA employing a two-step approach: first, they were tested with the PCR assay described

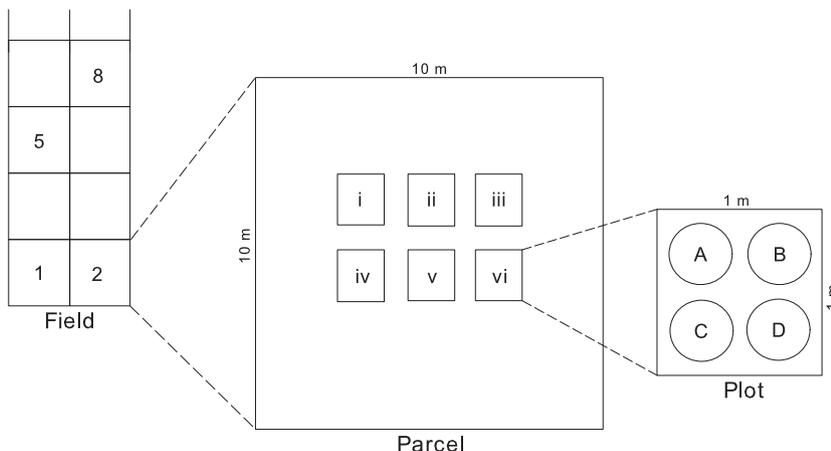


Fig. 1 Sampling design at the two grassland sites in 2008. Of twenty parcels, sampled on the first day, those 10 providing the highest numbers of *Agriotes* larvae were selected for further sampling. Within each parcel, six plots (à 1 m²: i–vi) were established and successively sampled from May–October 2013: After recording of the vegetation, four soil cores (A–D) were taken per plot to collect larvae.

in Wallinger *et al.* (2013) using general plant primers targeting the trnL region in cpDNA to check whether any plant DNA was detectable in the insects at all. This assay results in amplicons of similar length (120–123 bp) in all plants that equals their chance to be detected in the guts of the wireworms. Several positive (highly diluted target plant DNA) and negative controls (PCR-grade water instead of DNA) were run within each PCR to check for amplification success and DNA carry-over contamination, respectively. Samples which did not amplify in the first run were re-tested to account for the low numbers of target DNA. In a second step, in all wireworm/regurgitate samples that tested positive with the general plant primers, the ingested plant DNA was identified by applying specific PCR assays described by Wallinger *et al.* (2013): a multiplex PCR (multiplex 'TAT') targeting DNA of *Taraxacum officinale*, *Achillea millefolium* and two *Trifolium*-species (*T. pratense* and *T. repens*), as well as three different singleplex PCR assays targeting DNA of the Poaceae, Apiaceae and the genus *Plantago*, respectively. We stood aback from combining all seven plant targets within a single multiplex PCR in this step, to keep the difference in amplicon length as short as possible (100 bp as a maximum). Moreover, with a maximum length of 220 bp, the specific amplicons are well below the 300 bp recommended for molecular gut content analysis (King *et al.* 2008). The targeted plants represent the most abundant plants in the studied grasslands. Again, samples that did not amplify in a first run were re-tested in a second PCR. PCR products were visualized on a QIAxcel System (Qiagen) with the AL320 separation protocol and scored using BIOCALCULATOR FAST Analysis Software version 3.0 (Qiagen). All samples with signals exceeding 0.1 relative fluorescent units were deemed to be positive. For further analysis, the PCR results of wireworms and their corresponding regurgitates were combined, that is, individuals were counted positive if at least one of the samples (larva or regurgitate) was positive for a specific target plant.

Data analysis

Vegetation data were analysed by correspondence analyses (CA) with Canoco 4.5 (Ter Braak 1991), and plant associations were characterized if possible. The nomenclature of plant associations follows Mucina *et al.* (1993).

For the *Agriotes* wireworms, the mean number of different food types detected per individual analysed was calculated. The proportion of *Agriotes* larvae testing positive for a target plant was compared between plants on the basis of 95% tilting confidence intervals (TCI) derived from 9999 bootstrap resamples (Spotfire

S + 8.1 for Windows, TIBCO Spotfire, Somerville, USA). Following a conservative interpretation, nonoverlapping TCI were interpreted as significant difference.

To estimate selective feeding of the *Agriotes* wireworms the Manly's alpha preference index for a constant prey population was calculated for each target plant (Manly 1994). This index is linking the DNA detection rate (proportion of positive individuals) with plant food supply. An index value above or below the '1/m-threshold' (here 0.14; m, number of available plant species) indicates a preference or avoidance, respectively. If the value is above 1/m, it indicates that the plant species is consumed disproportionately high and thus is preferred; if it is below 1/m, then the plant species is avoided. Note, however, that the values for the preference indices are normalized, meaning that their sum is constant ($\Sigma = 1$). As a result, if one plant species is preferred, another one has to be avoided.

Results

Characterization of the grasslands

The two grasslands repetitively sampled for the full-season data set were classified as *Poa-Trisetum* Knapp ex Oberdorfer 1957 (grassland 1) and *Poa pratensis-Lolium perennis* Gams 1927 (grassland 2). These two plant communities are both part of the fertilized grasslands and pastures (Mucina *et al.* 1993). Apart from the eponymous indicator species *Poa pratensis*, *Lolium perenne*, and *Trisetum favesces* (all Poaceae), the grass *Dactylis glomerata* and the herbs *Taraxacum officinale*, *Achillea millefolium* (Asteraceae), *Trifolium repens*, *T. pratense* (Fabaceae) and *Plantago lanceolata* (Plantaginaceae) were highly abundant. The same held true for members of the plant family Apiaceae, that is, *Carum carvi*, *Pimpinella mayor*, *Heracleum sphondylium* and *Anthriscus sylvestris*. The species number per plot was between 13 and 17 and the mean plant cover 90% (± 13.2 SD). While the grasses exhibited a mean cover of 62% (± 18.7 SD), herbal plant species made up for 28% (± 15.1 SD) on average. The two plant communities outlined in the classification above are separating in a correspondence analysis (CA; Fig. S1, Supporting information).

The species composition in the grasslands of the summer data set was similar to those of the full-season data set, which was also reflected in the same plant species numbers. Mean plant cover was 93% (± 10.5 SD), with 51% (± 18.9 SD) for grasses and 41% (± 18.7 SD) for herbs. However, the different grasslands sampled could not be separated in a CA, where they were evenly distributed in the ordination space (data not shown),

indicating that the sites were similar in their plant species composition.

Feeding activity of wireworms during the season

Between one and all seven target plants were detected within a single wireworm and the feeding intensity was $2.2 (\pm 1.4 \text{ SE})$ target-plant detections per individual on average. For the assessment of the feeding activity, detection rates of plant DNA in wireworms collected in both grasslands were combined (Fig. 2). Over the season, mean DNA detection rates ranged between 40% and 80%. The *Agriotes* population was feeding-active in large parts throughout the entire vegetation period with a slight decrease in mid-summer. In that period of time, soil temperature increased significantly from 16 °C to above 20 °C, while it decreased towards temperatures around 12 °C at the end of the season in early October. Relative soil humidity remained almost constant across the vegetation period.

Feeding preferences of wireworms and seasonal changes

Regarding all wireworms collected, *Plantago lanceolata*, which was the only *Plantago* species present in the investigated fields, was the most frequently detected plant taxon of the seven specific plant targets (mean detection rate combining both data sets = 18.5%). The mean detection rates of *T. repens* (4.3%) and the Apiaceae (6.2%), on the contrary, were significantly lower than those of all other target plants, except for *T. pratense* (9.6%), which had an intermediate position.

In general, detection rates for specific plants were higher in the wireworms sampled for the full-season data set than in the one of the summer data set only (Fig. 3a). The detection rates for specific plants within the gut content of the *Agriotes* larvae were significantly different between some taxa in the two data sets: while *P. lanceolata* (24.6%) and *A. millefolium* (20.5%) were the most frequently detected target plants in wireworms

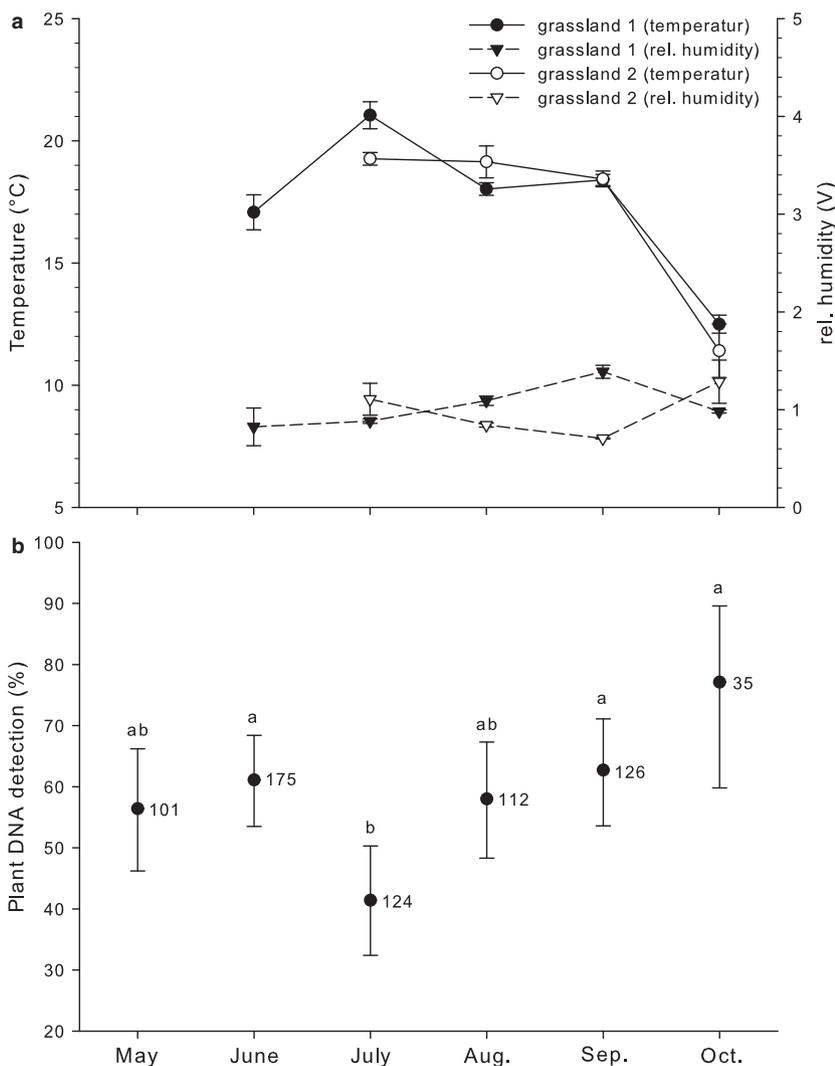


Fig. 2 (a) Soil temperature and relative humidity (mean \pm SD) recorded during the study period in 2008 in grassland 1 and 2. (b) Seasonal feeding activity of the *Agriotes* larvae in plots of grassland 1 and 2 represented by plant DNA detection rate ($\pm 95\%$ TCI). The different letters are standing for significant differences. Wireworm numbers per date are put beside the symbols.

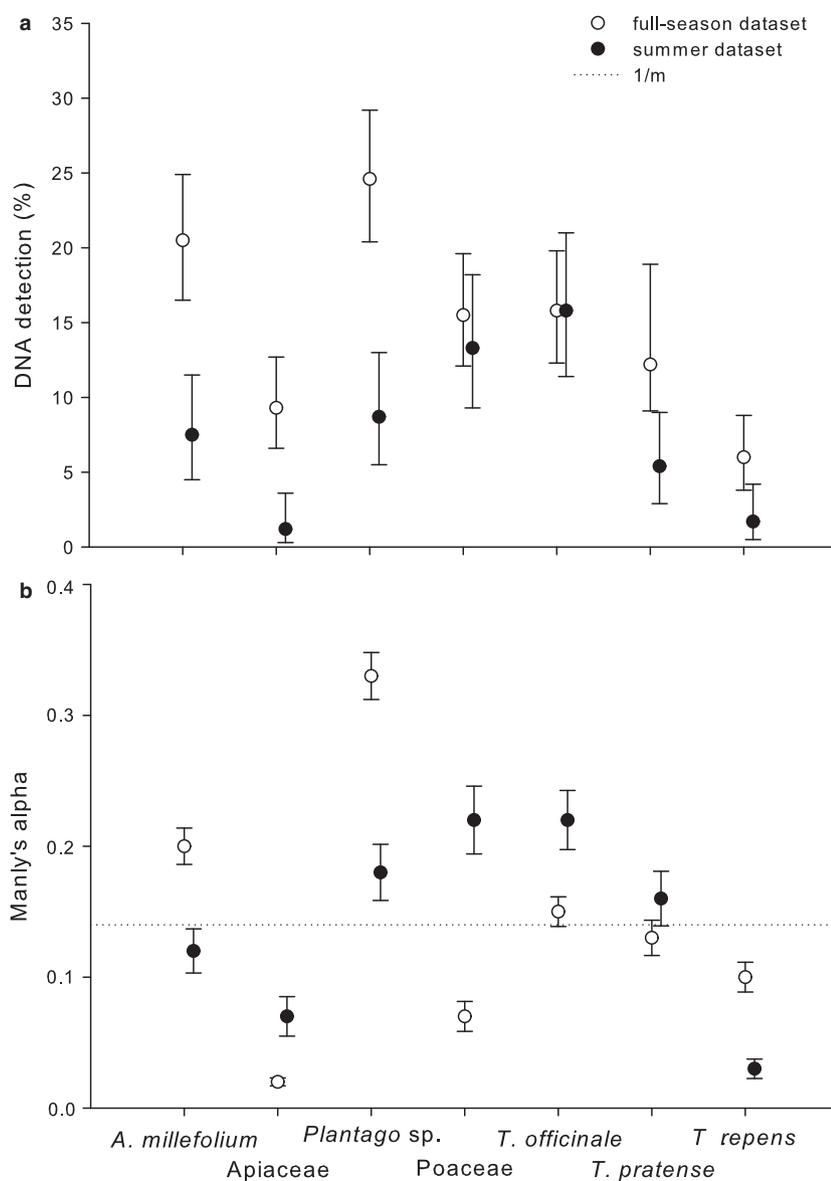


Fig. 3 (a) DNA Detection rates ($\pm 95\%$ TCI) of the seven target plants in the *Agriotes* wireworms separately for the two data sets (full-season, 2008 and summer, 2009). (b) Manly's alpha preference indices (mean \pm SE) for the seven targeted plant taxa consumed by *Agriotes* larvae in grassland plots in the two data sets. The dashed line displays the $1/m$ threshold, where m is the number of available plant species; values exceeding this threshold indicate preference, those below it avoidance for the respective plant species. Total number of individuals in full-season data set was $n = 386$, in the summer data set $n = 241$.

collected during the full-season, in the summer data set detection rates for *P. lanceolata* were significantly higher than all other targeted plant taxa. In the summer-caught wireworms, *T. officinale* (15.8%) and *Poa* sp. (13.3%) exhibited the highest detection rates, albeit being not significantly different from the rest of the plant targets. Generally, the Apiaceae and *T. repens* were only rarely detected; the detection rates of the Apiaceae were significantly lower in summer-caught than in wireworms collected over the full-season.

Taking the on-site food supply (i.e. plant abundance) into account, the most frequently detected plant taxa in general exhibited disproportional high preference indices (Fig. 3b). *P. lanceolata* and *A. millefolium* were the preferred plant species when the full-season was

considered, whereas the Poaceae, the Apiaceae and the two *Trifolium* species were avoided. In the summer-caught wireworms, *P. lanceolata*, *T. officinale*, Poaceae and *T. pratense* were preferred while again, the Apiaceae and both *Trifolium* species were avoided.

The preference of the *Agriotes* larvae for specific plant taxa changed over the season (full-season data set only; Fig. 4): *P. lanceolata* was preferred throughout the investigated period in both grassland sites, except for one sampling date in August in grassland 1. *Taraxacum officinale* and *A. millefolium* were preferred most of the time, whereas for *T. repens* and *T. pratense* the opposite was true. Poaceae, albeit highly abundant, were preferred at a few dates only, in grassland 1 (May–July and October) and Apiaceae were not liked at all. When

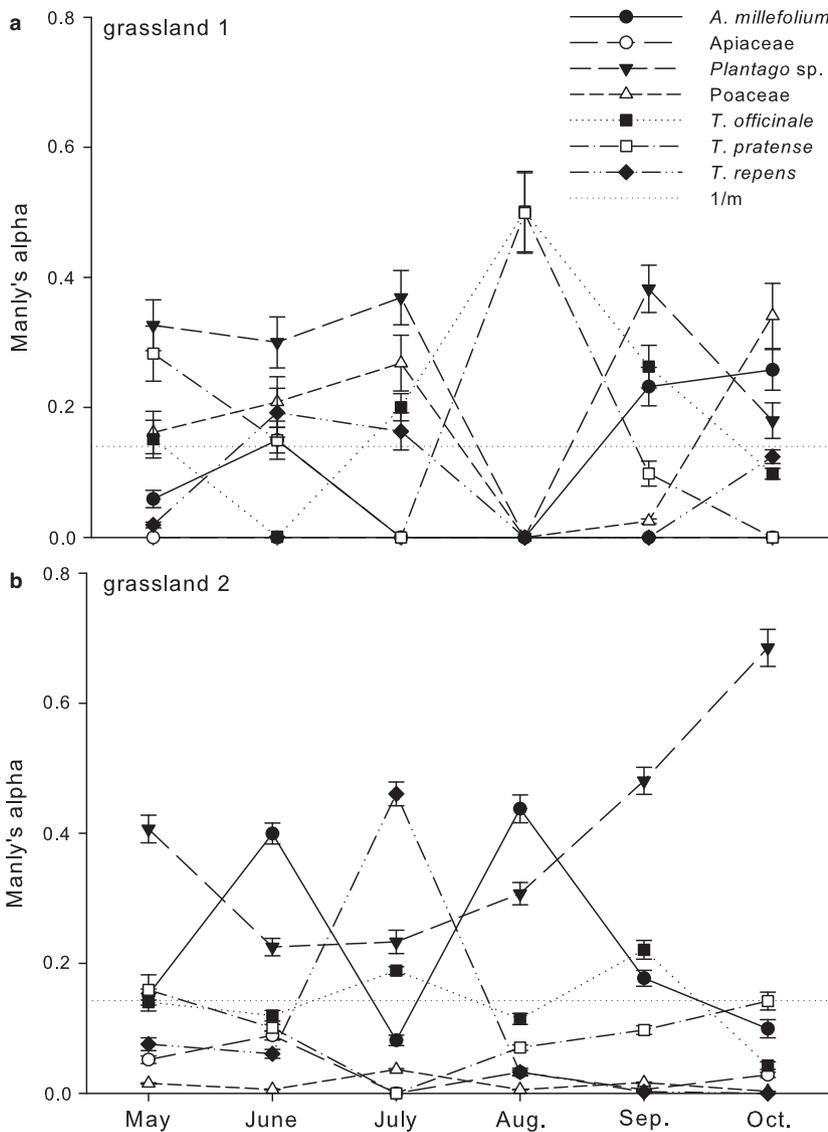


Fig. 4 Manly's alpha preference indices (mean \pm SE) for the seven target plants index (mean \pm SE) consumed by *Agriotes* larvae on the six different sampling dates over the season in grassland 1 (a) and 2 (b) in 2008. The dashed line displays the $1/m$ threshold, Where m is the number of available plant species; values exceeding this threshold indicate preference, those below it avoidance for the respective plant species.

comparing the general pattern of specific preferences on the targeted plant taxa between the two grasslands, there was more alternation in the dietary choice of wireworms in grassland 1 so that in the end all target plants but the Apiaceae were preferred at least at one date. In grassland 2, the preference pattern was more uniformly with a preference for *P. lanceolata* during the entire season and an avoidance of the Poaceae and Apiaceae.

Species- and instar-specific differences in plant food choices

The feeding intensity was similar in the two *Agriotes* species investigated here: on average, *Agriotes obscurus* and *Agriotes sputator* exhibited 2.2 (± 1.4 SE) and 2.1 (± 1.5 SE) target plant detections per individual,

respectively. Likewise, the preferences for the different plant targets were largely the same (Fig. 5a), with the exception of the Poaceae, which were preferred by *A. sputator* and avoided by *A. obscurus*.

The feeding intensity was 2.3 (± 1.4 SE) target plant detections in early-instar wireworms (<4th instar) and 2.1 (± 1.5 SE) detections in late-instar ones (4th–7th instar). Both groups of larvae significantly preferred *P. lanceolata* and *A. millefolium* over all other plants and avoided the Apiaceae and the two *Trifolium* species (Fig. 4b). Their feeding preference was controversial for *T. officinale*, which was avoided by the late-instar wireworms.

Discussion

Contrastingly to our first hypothesis, the *Agriotes* larvae did not exhibit two main feeding-active phases during

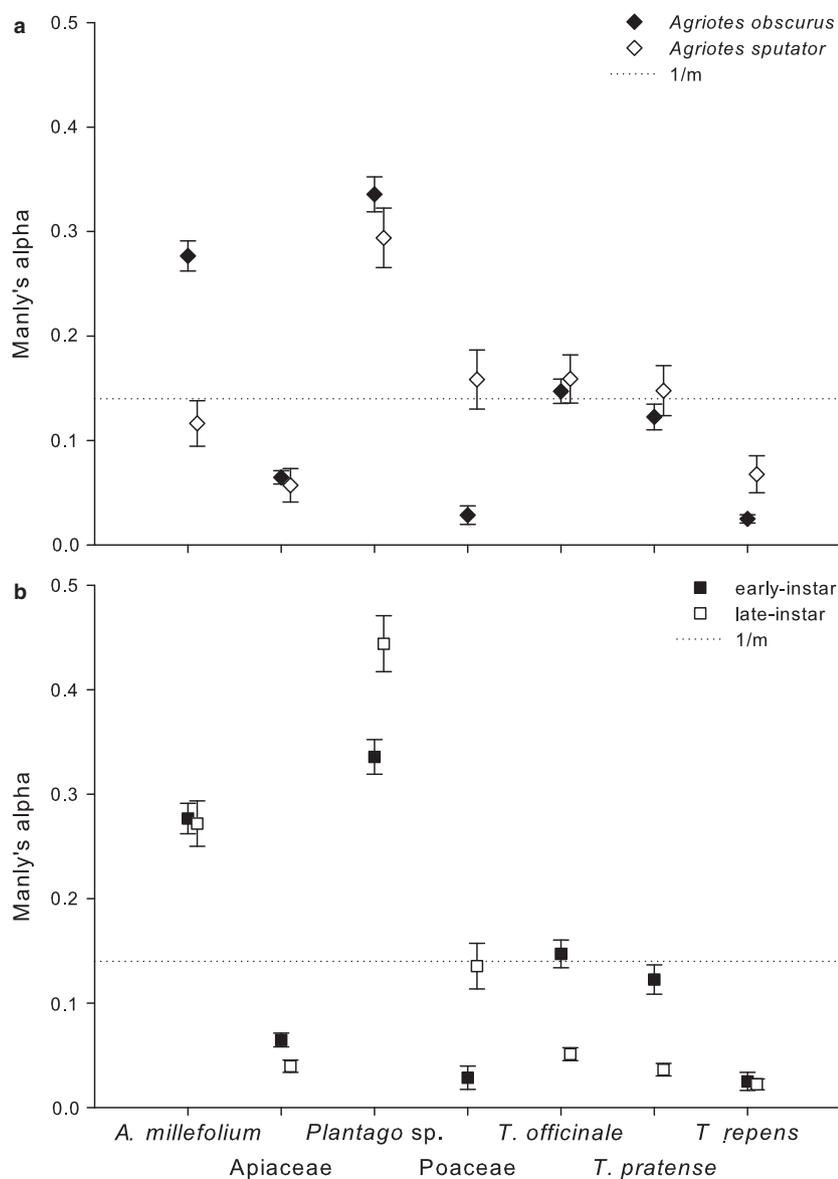


Fig. 5 (a) Overall Manly's alpha preference indices (mean \pm standard error) for the seven targeted plant taxa consumed by *Agriotes sputator* and *Agriotes obscurus* larvae in grassland plots. The dashed line displays the $1/m$ threshold above which preference is indicated. (b) Manly's alpha preference indices (mean \pm standard error) for the seven target plants separately for early- (<4th instar) and late-instar (4th–7th instar) *Agriotes* larvae.

the season as suggested earlier (Parker & Howard 2001), but they were feeding-active during the entire season. Still, when the soil temperatures were highest, the proportion of feeding-active larvae was lowest, indicating that *Agriotes* larvae decrease their feeding activity when temperatures increase above a certain level.

The current results indicate that *Agriotes* larvae are selective feeders and that they mix different plant species occurring in temperate grasslands in their diet. Due to their long-lasting larval development, *Agriotes* wireworms often have to cope with an altered spectrum of food plants, where a generalistic feeding behaviour is advantageous for survival. Our findings support this hypothesis, but they also emphasize that there is active food plant selection when there is a choice. While some

plants, such as the Apiaceae, were avoided by the *Agriotes* wireworms, others were preferred (i.e. *Plantago lanceolata*). Contrary to our expectations, the grasses (Poaceae) did not represent the preferred food source of the larvae despite their abundance. Preferences for particular food plants have been shown also in other generalist – mainly aboveground feeding – herbivorous insects, including lepidopterans, hemipterans and grasshoppers (e.g. Bernays & Minkenberg 1997; Singer *et al.* 2004; Franzke *et al.* 2010). In cafeteria experiments, *Agriotes* wireworms exhibited preferences towards some plants (e.g. wheat and onions), while others (mustard, cabbage, French marigold, clover and flax) were less frequently attacked (Griffiths 1974; Hemerik *et al.* 2003). Hemerik *et al.* (2003) indicate a preference of *A. obscurus* for plant species typical for nutrient-rich grasslands,

which may constitute a better food source for these insects than plants from nutrient-poor ones.

Active selection can counteract putative quality deficiencies of particular food plants (Franzke *et al.* 2010) and dilute potential phytochemical toxins. In *A. lineatus* larvae, for example, survival rates ranged between 3% and 50% when fed on different host plants (Kabanov 1975). Diet-mixing is especially important when relevant differences exist in nutrient composition or toxin content among plants. Wild plant populations are usually characterized by high intra-specific variation in most traits (Karban 1992), which in turn can be crucial for the development of herbivorous insects (Mody *et al.* 2007). Apart from enhancing their growth performance – the physiological efficiency hypothesis (Jeffries & Lawton 1984), insect herbivores may eat a mixture of plant species to enhance resistance against their consumers by incorporating plant toxins (enemy-free space hypothesis), which was observed in lepidopteran larvae (Singer *et al.* 2004). The latter may not apply for *Agriotes* wireworms, because the species composition of plants occurring in temperate grasslands exhibit rather low concentrations in deterrent or toxic compounds (Barbosa 1993).

The *Agriotes* larval feeding behaviour appears to be mediated by short distance mechanisms involving dietary preference. When wireworms reach the roots, contact chemosensory cues act as either 'phagostimulants' (most of the compounds being sugars) or feeding 'deterrents' (notably phenolic compounds) (Johnson & Gregory 2006). Carbon dioxide is one of the potential attractants towards they may orientate (Doane *et al.* 1975; Chaton *et al.* 2003), but its role in belowground systems is equivocal (Johnson & Gregory 2006). Apart from CO₂, roots are known to exude a range of other chemicals in the rhizosphere which attract or repel subterranean herbivores (Johnson & Nielsen 2012). Among these, sugars are reported to enable *Agriotes* wireworms to locate host-plants (Morgan & Crumb 1928; Thorpe *et al.* 1947; Olsson & Jonasson 1995; Bagheri & Nematollahi 2007). And finally, secondary-plant constituents are playing an indisputable role for selective root-feeders in mixed plant communities. They have even been regarded as one of the driving forces in macro-evolution (Gottlieb 1983) and may also represent the trigger for the larval food choice observed in the present study. The preference for *P. lanceolata* observed in this study is in accordance with results of earlier feeding experiments (Wallinger *et al.* 2013). It may be rooted in its high content of sugar alcohols sorbitol, which acts as its major osmotic regulator in contrast to, for example, grasses where the amino acid proline performs this role. Sorbitol has 60–70% the sweetness of sucrose and at the high levels present in *P. lanceolata* has the potential to

enhance palatability (Stewart 1996). Contrastingly, the low acceptability of *T. repens* may be ascribed to its cyanogenic glycoside contents, because the release of cyanide is known to be repellent for herbivores (Mølgaard 1986). The coumarins and essential oils present in the plant family of the Apiaceae seem to serve similarly deterrent. The inconsistency in the acceptance of the two Asteraceae *Taraxacum officinale* and *Achillea millefolium* between the two data sets and during the season may be explained by the fact that secondary-plant compounds (i.e. bitter principles and terpenes) show qualitative and quantitative variation among and within plant populations and even individuals. The latter two species were most frequently detected in feeding experiments, where only young plants had been used as food sources for wireworms (Wallinger *et al.* 2013).

The overall seasonal variation in the food preference of wireworms for specific plant taxa may be due to a lower concentration of herbivore-repellent secondary compounds in spring (Oates *et al.* 1977). A variation in secondary-plant compounds can affect the defence of plants to attacks by herbivores and conversely determine their selection for specific plants as food at a specific time point (Hiltpold & Turlings 2012). Moreover, plant-specific seasonal changes in root texture and size may have contributed to the observed feeding patterns and explain the discrepancy between the two data sets for specific plant taxa. To clarify this temporal relationships an examination of the variation in concentration of the specific secondary-plant constituents is a subject of further investigation.

Comparing the DNA detection rates with the Manly's alpha preference indices shows how important it is to include the food supply into the analysis for dietary preference of a given herbivore. Albeit exhibiting a comparable overall pattern, they differed greatly concerning some of the seven plant targets in detail. For instance, the DNA-detection rates of the Poaceae were rather high and almost similar in both data sets (full-season and summer data set). When looking at the Manly's alpha indices, however, there was a contrasting pattern, with some preference for the grasses in the full-season data set and avoidance in the summer data set. An interpretation of food preferences in *Agriotes* wireworms based on DNA detection rates alone would have led to a misinterpretation to some extent.

The sensitivity of the plant taxon-specific primers proved to be similar, which was also true when a high amount of *Agriotes* DNA was present (Wallinger *et al.* 2012), thus ensuring balanced amplification of plant DNA present in the guts. Still other factors could have affected postfeeding detection, such as that shorter amplicons may survive the digestive breakdown better than longer ones (Deagle *et al.* 2006). To minimize the

risk of this potential bias, we kept the difference in amplicon length as small as possible (max 100 bp). Moreover, we employed a two-step approach for the screening. In this standardized procedure, amplicon identity is probably to play a minor role because all samples had to pass the first filtering step with the same chances for every ingested plant to be detected. Apart from amplicon identity, the detectability between the targeted plant taxa may have differed due to plant-specific traits. For example, the density of chloroplasts and accordingly the cpDNA content in the root cells can vary among the different plant taxa, influencing the detection of DNA sequences located in the *trnF-trnL* region (the target region of the specific primers). However, similar to mitochondria, the number of plastids and cpDNA concentrations is known to be highly variable within and between tissues and can even change during cell growth and development (Cattolico 1978). This high intraspecific and development-specific variation may exceed potential plant-specific differences, a fact that is also indicated by the outcomes of earlier feeding experiments with *Agriotes* larvae (Wallinger *et al.* 2013), resulting in similar DNA detection rates for all but one plant species. Besides, digestibility of ingested tissues may also vary between the investigated plants. For example, grass grubs (Coleoptera: Scarabaeidae) more extensively digested *T. repens*-roots, with formation of partially digested xylem vessels, whereas those of ryegrass (*Lolium perenne*) appeared to undergo very little digestive breakdown (Bauchop & Clarke 1977). In the current study, a faster digestion of roots from *T. repens* could have contributed to the low overall detection rates of this plant. Digestion rates may be even different for the same plant species/group, for example, when comparing older roots to younger ones. Preceding feeding experiments, where four different *Agriotes* species were fed with the plants targeted in this study (Wallinger *et al.* 2013), indicate similar digestive breakdown of DNA in all but one species (*A. millefolium*). Moreover, plant DNA detection success was independent of digestion time for all plants for up to 72 h, except for wireworms which consumed *T. officinale* and the Apiaceae, where it significantly decreased with time postfeeding. Based on these findings, the overall low detection rates of Apiaceae observed in the field could also be accelerated by a faster digestive breakdown of the DNA of members of this plant family compared with others. Likewise, detection rates for *T. officinale* may have underestimated the actual consumption of dandelion in the field. The detection of *A. millefolium*, in contrast, may have been over-estimated compared with the Poaceae, Apiaceae and *T. pratense*, because detection rates of this species were significantly higher in the feeding experiments than in the other three plants

mentioned above. It is also important to consider that the current analysis does not allow for a quantification of the plant material consumed, that is, our interpretation is based on the assumption that a frequently detected plant taxon constitutes a major dietary component. Recent findings indicate that this assumption can hold true (Traugott M., Schallhart K., Staudacher K., Wallinger C., unpublished data), supporting the interpretation of the current findings.

We did not observe instar-specific differences in DNA detection rates for the plant targets and only small preferential differences concerning the seven targeted plant taxa. These findings reject the hypothesis that dietary needs change throughout larval development, which was suggested in earlier work (Langenbuch 1932). According to previous feeding experiments (Staudacher *et al.* 2011; Wallinger *et al.* 2013), plant DNA detection success is independent of the kind of *Agriotes* species, thus indicating similar digestion rates among them. Contrary to our hypothesis, *Agriotes obscurus* and *Agriotes sputator* showed similar dietary preferences in the grassland habitats. These findings suggest that larval instars and species exploit the similar trophic niche. In grassland systems, it is probably that the larvae are not limited by the availability of (specific) plant food, which might explain, at least partially, the lack in trophic niche separation. Still, it needs to be considered that the current study provides first insights and that further investigations, which include other *Agriotes* species and additional systems, are warranted before general conclusions can be derived.

This study provides conclusive evidence that *Agriotes* larvae are feeding-active throughout the vegetation period in their prime grassland habitat, resulting in continuous belowground feeding pressure in grassland habitats. The current findings also indicate that *Agriotes* wireworms do actively select among roots available, that grasses are not necessarily preferred and that the feeding preferences are changing during the season. Species- and instar-specific differences in dietary choice, however, were rather small. These results provide an important step towards a comprehensive understanding of the trophic ecology of generalist root herbivores, such as *Agriotes* larvae, in highly diverse plant systems under natural conditions. Knowing which plants are consumed and how feeding choice changes seasonally provides much needed information for future experimental work, elucidating the interplay between plants and their belowground herbivores.

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Data accessibility

Raw data of the field investigations: DRYAD doi:10.5061/dryad.f45q2.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Biplot of a canonical correspondence analysis of the plots of the full-field dataset according to their vegetational composition.