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Plant responses to variable timing of aboveground clipping and belowground herbivory depend on plant age

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Abstract

Aims
Plants use different types of responses such as tolerance and induced defense to mitigate the effects of herbivores. The direction and magnitude of both these plant responses can vary with plant age. However, most studies have focused on aboveground herbivory, whereas important feeding occurs belowground. Here, we tested the hypothesis that plant tolerance and defense following shoot damage or root herbivory depends on plant age.

Methods
In order to test our hypothesis, we exposed the perennial grass species *Holcus lanatus* to defoliation and root nematode inoculation at three growth stages (young, intermediate and old plants), and examined responses of plant traits related to tolerance (regrowth following defoliation) and defense (leaf and root nitrogen and phenolics).

Important Findings
Defoliation overall reduced plant shoot and root biomass as well as foliar concentrations of phenolics regardless of plant age at defoliation. In contrast, defoliation increased foliar N concentrations, but only when defoliation occurred at intermediate and old plant age. Inoculation with root-feeding nematodes reduced root N concentrations after a prolonged period of growth, but only when nematodes had been inoculated when plants were young. The relative shoot regrowth rate of plants increased immediately after defoliation but this was independent of the plant age at which defoliation occurred, i.e. was not stronger in plants that were defoliated at a more advanced age, as hypothesized. Similarly, relative root growth rates increased shortly after defoliation, but this was only observed for plants defoliated when they were young. We conclude that plant responses to aboveground and belowground herbivory in traits related to both defense and tolerance are affected by plant age, but do not generally change with plant age.

Keywords: plant age, above-belowground interaction, root-feeding nematodes, *Holcus lanatus*, defoliation

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INTRODUCTION

Plants can be exposed to various aboveground and belowground herbivores during their life. To reduce loss of fitness, plants have evolved strategies to reduce their damage or to mitigate the negative effects of their damage on plant fitness, for example by induced defense (Agrawal 2011; Chen 2008; Karban and Baldwin 1997) or tolerance (Fornoni 2011; Strauss and Agrawal 1999; Stowe *et al.* 2000). Induced defense enables plants to deter contemporary or future herbivores, e.g. by producing higher levels of secondary compounds, while plants can also reduce consumption by herbivores by lowering nutrient content of plant tissues (Agrawal 1998), although the latter may also result in compensatory feeding.

Other strategies of plants to deal with herbivores are tolerance of herbivore damage through mechanisms that reduce the negative fitness effects of damage incurred by the herbivores, e.g. compensatory regrowth of tissues consumed by herbivores (McNaughton 1983). Both defense and tolerance strategies are widely identified and incorporated in ecological and evolutionary theories on plant-herbivore interactions (Agrawal 2011; Leimu and Koricheva 2006; Strauss and Agrawal 1999; Tollrian and Harvell 1999).

The induction of plant defenses can vary with plant age as the priority of different plant organs to be defended changes during plant growth (Akiyama and Ågren 2012; Boege and Marquis 2005; Barton 2007; del-Val and Crawley 2005). Many studies have shown that the inducibility of metabolic compounds varies between ontogenetic stages of the plant (McArthur *et al.* 2010; Quintero and Bowers 2012), and that the ontogenetic trajectory can in fact be a more important determinant of observed variation in defense induction than the history of damage by herbivores (Quintero and Bowers 2011). For example, Quintero and Bowers (2011) investigated the chemical responses of *Plantago* spp. to a specialist foliar feeding insect herbivore and found that plant defense was only induced in juvenile and not in mature plants in response to herbivory. In contrast, other studies have shown that older plants are better defended against herbivores than young plants because they have accumulated higher amounts of plant defense compounds over a longer growth period (Boege 2005; Elger *et al.* 2009; Goodger *et al.* 2004).

Also, a plant's ability to tolerate herbivore damage can vary with plant age (Massad 2013), due to developmental changes in plant architecture, storage capacity and resource allocation (Boege 2005). In general, young plants possess fewer stored reserves and a lower capacity of resource acquisition, so that they are less capable of compensating herbivore damage than old plants (Bryant *et al.* 1992). Other studies have shown that plant growth can be more strongly suppressed by herbivory in older plants, because that coincides with the plant's shift in allocation of resources to reproduction (Nykänen and Koricheva 2004). Interestingly, intermediate-aged plants can be more vulnerable than both young and old plants due to the lack of seed-stored reserves in comparison to young plants and the lack of photosynthetic area in comparison to older plants (del-Val and Crawley 2005). Plants usually show ontogenetic changes in many intrinsic traits such as the ability to store and translocate resources (Trumble *et al.* 1993) or the ability to increase photosynthesis (Nykänen and Koricheva 2004) and growth rates (Tiffin and Inouye 2000). These traits can determine a plant's tolerance to herbivory and the ontogenetic pattern of these traits may reflect adaptive plasticity under variable herbivore pressure (Pankoke *et al.* 2013).

The majority of studies on plant responses to herbivory have focused on effects of aboveground herbivory, whereas studies on plant responses to belowground herbivory have emerged only more recently (e.g. Rasmann and Agrawal 2008; Robert *et al.* 2014; van Dam 2009; Watts *et al.* 2011). Root-feeding nematodes are major root herbivores of many plant species (Perry and Moens 2006), and their role in plant-herbivore interactions has frequently been studied (Mateille 1994; Soriano *et al.* 2004; van Dam *et al.* 2003; Zinov'eva *et al.* 2004). Plant parasitic nematodes often have devastating effects on crop yield, although pot studies commonly report modest and variable effects of root-feeding nematodes on plant growth (Seastedt *et al.* 1987; Verschoor *et al.* 2002). Exposure to root-feeding nematodes has been shown to reduce (Brinkman *et al.* 2008; Ingham and Detling 1990; Stanton *et al.* 1981) but also to enhance plant biomass (Stanton 1983) depending on environmental condition and the studied plant and nematode species (Brinkman *et al.* 2015; Verschoor *et al.* 2002).

The presence of root-feeding nematodes can also greatly alter the meleolite profile of host plants, in particular, the concentration and composition of plant defense compounds, both in roots and in shoots (Biere and Goverse 2016; de la Peña and Bonte 2014; Hofmann *et al.* 2010; Hol *et al.* 2010; Kammerhofer *et al.* 2015; Kaplan *et al.* 2008; Kyndt *et al.* 2012; van Dam *et al.* 2005; Wondafrash *et al.* 2013). In addition, nematodes can modulate how the concentrations of plant primary or secondary compounds change in response to the presence of other organisms (e.g. Lohmann *et al.* 2009). The response of plant primary and secondary plant compounds to nematode herbivory further varies with nematode species (Vaast *et al.* 1998), plant traits (Verschoor *et al.* 2002) and soil conditions (De Ruijter and Haverkort 1999), suggesting a specific interplay between plant- and root-feeding nematodes. However, in contrast to ontogenetic patterns in responses to defoliation (Akiyama and Ågren 2012; Boege 2005; Ilmarinen *et al.* 2005), ontogenetic patterns in plant responses to belowground herbivory are largely unknown.

In this study, we investigate how plant responses to nematode inoculation in the presence and absence of defoliation change with plant age in the perennial grass *Holcus lanatus*, a plant species that has evolved both tolerance and defense to cope with biotic stresses (Tiffin and Inouye 2000). We used defoliation (shoot clipping) as a surrogate for herbivory, acknowledging that clipping differs from herbivory as it is not selective and lacks effects of elicitors and effectors from herbivore oral secretions. As plant response traits, we focused on plant biomass and on plant traits that are putatively related to defense (shoot and root phenolics and N) and, for responses to defoliation, traits related to tolerance (the ability to regrow biomass). Responses to defoliation were studied in plants exposed to nematodes to create more challenging conditions for regrowth. Plants were inoculated by two species of root-feeding nematodes at young, intermediate and old age to assess ontogenetic responses to nematodes, and half of the nematode-exposed plants were defoliated to assess effects on tolerance to aboveground defoliation. We hypothesized that: (i) Clipping and nematode addition will induce a higher level of general plant defense (phenolics), and a lower nutritional value (tissue N concentration), but that the extent to which the levels of these metabolites respond to the treatments will depend on plant age; (ii) The ability of nematode-challenged

plants to regrow following defoliation will increase with plant age, as older plants have more stored resources available for biomass regrowth.

MATERIALS AND METHODS

Soil, plants and nematode inoculation

We collected soil from a restoration grassland on former arable land (De Mossel, Ede, The Netherlands, 52.04°N 5.44°E) at 5–20 cm below the soil surface. The soil was a sandy loam with 4.5% organic matter. In the laboratory, the soil was sieved through a 5 mm mesh, homogenized and sterilized by gamma irradiation (>25 KGray). Our focal grass species *H. lanatus* is a perennial grass species that commonly occurs in most European grasslands on various soil types (Beddows 1961). It is used as a host by a variety of plant-feeding nematode species (Verschoor *et al.* 2001). Seeds of *H. lanatus* were purchased from a commercial wild-seed supplier (Cruydthoeck, Nijeberkoop, the Netherlands). The seeds were surface sterilized with sodium hypochlorite (1%) for 1 min and rinsed 4 times with demineralized water, sown on moist glass beads and then placed in an incubator (16/8 h light/dark, 25/20°C day/night temperature) until germination. Nematodes were collected from a field that has been in agricultural cultivation since 1955 (Vredepeel, The Netherlands; Korthals 2014). *Holcus lanatus* naturally occurs in the field margins and vicinity of this field at high abundance, although it is rarely observed in the field due to weed control practices. The soil is composed of 1.1% clay, 3.7% silt and 94.9% fine sand. The nematode inoculum was dominated by root-feeding nematodes and was mainly comprised of root lesion nematodes, *Pratylenchus penetrans* and stunt nematodes, *Tylenchorhynchus dubius* (together 98.4% of the root-feeding nematodes). These species are commonly found in grasslands, have a broad host range, and are likely to frequently encounter *H. lanatus* as a potential host, although the status of their co-evolutionary history is unclear. As the study was designed to repeatedly inoculate nematodes (see below) these nematodes had been collected at different times from February to April in 2014. Each time, soil samples were collected from the same location within the agricultural field to maintain the similarity of these nematode collections. However, because the nematodes were collected at different times during the season, the composition of the nematode communities that we used as inoculum in the experiment varied slightly between inoculation dates. By adjusting the inoculation volume, densities of plant-feeding nematodes that we inoculated per plant were similar throughout the experiment.

Experimental setup

We set up the experiment with a total of 320 pots filled with 800 g fresh soil each (described above) and one seedling of *H. lanatus* was planted into each pot. All pots were randomly distributed within a greenhouse (16/8 h light/dark, 21/18 ± 2°C day/night). The pots were then assigned to one of the following treatments: (i) control plants, (ii) addition of nematodes, to study plant responses to belowground herbivory (BG) and (iii) addition of nematodes followed by defoliation 1 week later, to study plant responses to one aspect of aboveground herbivory (removal of leaf biomass) in the presence of nematodes (AG + BG). The plants were defoliated 1 week after the inoculation of nematodes in order to minimize the potential influence of defoliation on the establishment of nematodes. The treatments were applied at three different plant ages, starting 3, 6 and 9 weeks after transplantation, (hereafter young, intermediate and old, respectively). At each of these growth stages, plants were still in their vegetative stage, but had attained vastly different sizes (see online supplementary Fig. S1).

Young plants

Three weeks after transplanting (experimental week 0), each of 110 randomly chosen pots was inoculated with 5 ml nematode suspension that was composed of a mixture of extractions from 20 February and 13 March 2014 due to a shortage of nematode individuals from a single extraction. The suspension contained on average 100 (standard error [SE] = 6.8) individuals of *P. penetrans* and *T. dubius* and was inoculated into two 1-cm-deep small holes made in the soil. The holes were immediately covered using the surface layer of the soil in the pot. One week later (experimental week 1), 10 randomly selected plants were harvested and used to examine nematode recovery rates (see online supplementary Fig. S2). At the same time, 50 of the remaining 100 plants were defoliated by clipping the tillers at 4 cm above the soil surface (AG + BG-Y treatment). This way, most of the aboveground biomass was removed to maximize the defoliation impact, without running the risk of destroying the meristematic tissues for regrowth. The other 50 plants were only exposed to the nematode treatment (BG-Y treatment).

Intermediate-aged plants

Six weeks after transplanting (experimental week 3), each of 90 randomly chosen pots was inoculated with 5-ml nematode suspension extracted at 7 April 2014 that contained 100 (SE = 15.7) individuals of *P. penetrans* and *T. dubius* as described above. One week later (experimental week 4), 10 of the plants were harvested to check nematode survival (see online supplementary Fig. S2) and 40 plants were defoliated (AG + BG-I treatment) as previously described while the other 40 plants were only exposed to the nematode treatment (BG-I treatment).

Old plants

Nine weeks after transplanting (experimental week 6), each of 70 randomly chosen plants were inoculated with 5-ml nematode suspension extracted at 28 April 2014 containing 100 (SE = 5.7) individuals of *P. penetrans* and *T. dubius*. One week later (experimental week 7), 10 plants were harvested to check nematode survival (see online supplementary Fig. S2) and 30 plants were defoliated (AG + BG-O treatment) while the other 30 plants were not defoliated (BG-O treatment).

Control plants (Ctrl treatment) were each inoculated with 5-ml clean tap water at experimental weeks 0, 3 and 6, using the same method as described for nematode inoculation. For all treatment and plant age combinations, 10 plants were harvested every 3 weeks following defoliation and nematode inoculation until week 15 ('sequential harvests') (Fig. 1).

Plants were watered three times per week. Once a week, the soil moisture content was adjusted to 12.3% (w/w) with demineralized water and the pots were rotated weekly within the greenhouse. Nutrients were added once a week using Hoagland solution (Hewitt 1966). The nutrient strength and dosage were gradually increased over time to meet growth demands of *H. lanatus* (Van der Putten *et al.* 1988) according to ontogenetic measurements of nitrogen concentration of *H. lanatus* (Bezemer *et al.* Unpublished data). A quarter-strength Hoagland was added in week 1–4 (from 12.5 ml to 50 ml per week in steps of 12.5 ml), half-strength solution was added in week 5–9 (from 60 ml to 100 ml per week in steps of 10 ml) and full-strength solution was added in week 10–14 (from 60 to 100 ml per week in steps of 10 ml). The experiment was carried out in a greenhouse with natural daylight supplemented by 400-W metal halide lamps when light levels dropped below 225 µmol m^{-2} s^{-1} photosynthetically active radiation.

Plant harvest and chemical analysis

Biomass

At each harvest, the shoots were separated from the roots with scissors and the soil was carefully washed off the roots.

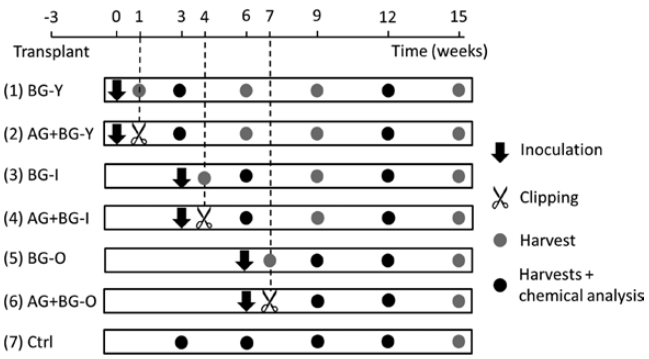

Figure 1: schematic overview of treatments and harvests of the experiment. Belowground (BG) treatments: plants were inoculated with the root-feeding nematode species *P. penetrans* and *T. dubius* at weeks 0 (young; capital used for abbreviating), 3 (intermediate) and 6 (old). Belowground and aboveground (BG + AG) treatments: plants were defoliated 1 week after inoculation with root-feeding nematodes. The control (Ctrl) treatment refers to plants that were neither inoculated with nematodes nor defoliated. Plants were harvested every 3 weeks after nematode inoculation until the last harvest at week 15. Arrows indicate the nematode inoculation events. Scissors indicate the defoliation events and grey circles indicate the harvests within treatments. The black circles indicate treatments selected for subsequent chemical analysis. One week after inoculation 10 plants were harvested to check nematode survival (dashed lines). There were 10 replicates for plant biomass measurements and 7 replicates for plant chemical measurements in each treatment.

Shoot and root biomass were oven-dried at 40°C for a minimum of 5 days before weighing (see online supplementary Fig. S1). Seven randomly selected plants from each of the treatments harvested at 3 weeks after each of the inoculations, and from all treatments harvested at week 12 ('fixed harvest') were used for further chemical analysis (see Fig. 1).

N concentration

The youngest tillers of dried shoots and a subset of upper, middle and lower part of roots of the selected plants were cut off and ground to a powder, and 1 mg of these fully mixed powdery materials was weighed into tin capsules. Nitrogen (N) concentration was measured using a CN analyzer (Flash EA 1112, Interscience, Breda, The Netherlands).

Total phenolics

Total phenolic content was determined using the Folin-Ciocalteu method (Medina-Remón *et al.* 2009). Twenty-five mg of powdery dry plant material was extracted with 5 ml 50% methanol for 2 h in a water bath at 90°C. The samples were centrifuged for 10 min at 5000 rpm and the supernatant was analyzed. Two-hundred microlitre supernatant was mixed with 200 µl Folin-Denis reagent and 1.0 ml Na_2CO_3 solution and centrifuged at 12 000 rpm for 5 min. The supernatant was measured photospectrometrically using Synergy HT Multi-detection reader (Vermont, USA) at a wavelength of 750 nm. Tannic acid was used as a standard. The bioactivity of phenolic compounds in terms of defensive properties can strongly differ among individual compound and will also depend on the identity of the herbivore that is encountered. Therefore, tissue concentrations of total phenolics content can only be used as a crude indicator of the defensive status of a plant; depending on the composition of compounds that make up the total phenolics content, the bioactivity may vary (e.g. Salminen and Karonen 2011).

Data analysis

To examine the effects of defoliation and nematode addition at different plant ages, we used data from the harvests in experimental weeks 3, 6 and 9 for plants inoculated at young, intermediate and old age, respectively, i.e. 3 weeks after nematode inoculation and 2 weeks after defoliation. We used a two-way analysis of variance (ANOVA) to analyze plant biomass as well as N and phenolic concentration in plant shoots and roots. Damage treatment ('Damage': Ctrl, BG and AG + BG) and plant age ('Age': young, intermediate and old) were used as fixed factors. Tukey HSD *post hoc* tests were used for multiple comparisons when treatment effects were significant. Further, we analyzed these biomass and chemistry data of plants of all treatments harvested at week 12 ('fixed harvest') using a one-way ANOVA. Subsequently, we used a Dunnett *post hoc* test to compare the control treatment with each of the BG or AG + BG treatments applied at each of the three plant ages. In addition, we used a two-way ANOVA to test whether defoliation ('Damage': BG vs.

AG + BG) and plant age ('Age': young, intermediate and old) affected plant growth and chemical responses by excluding the control treatment, because the control treatment had not been assigned to different plant ages and a full-factorial test including this treatment was not feasible.

We also calculated temporal changes in plant shoot and root biomass following the start of the different treatments. Shoot and root relative growth rates were calculated as RGR = $(B_x - B_{x-3})/B_{x-3}$, where B_x is the biomass (shoot or root) of an individual plant at weeks x = 6, 9, 12 and week 15. Note that these estimates are based on untransformed biomass data and not on log-transformed data as more commonly done in growth analyses. We used these relative root and shoot growth rates as proxies for the ability to regrow following defoliation as an aspect of tolerance. As we destructively harvested plants and thus could not match individual plants between two harvests, we used a random pairing approach. We randomly paired the 10 plants from the same treatment from two consecutive harvests to calculate RGR values and analyzed the data using a two-way ANOVA with damage treatment ('Damage', AG + BG, BG and Ctrl) and time ('Time': week 3–6, week 6–9, week 9–12 or week 12–15) as fixed factors. We repeated this procedure 1000 times, which yielded 1000 ANOVA results. The number of significant occurrences of each main factor and interaction (at $\alpha = 0.05$) out of 1000 replicates were summed and the proportion of non-significant occurrences was calculated (*Pr* = number of non-significant occurrences out of 1000). The factors (Damage and Time) with *Pr* <0.05 were considered significant. To determine whether two treatments differed, a 'contrast test' was used in which we compared the BG treatment with the Ctrl treatment, and the AG + BG treatment with both the BG treatment and the Ctrl. The 'contrast tests' were also replicated 1000 times and the *Pr* values were calculated as previously described for each contrast.

Shoot and root biomass from the sequential harvests was log-transformed to meet the assumptions of heterogeneity of variance in ANOVA tests. All analyses were performed using the R statistical package, version 3.1.3 (R Core Team 2014).

RESULTS

Plant biomass

Three weeks after inoculation (i.e. week 3, 6 and 9 for plants inoculated at early, intermediate and old age, respectively), plant shoot biomass was not significantly affected by nematode addition (comparison: BG vs. Ctrl) but roughly reduced by half following defoliation that took place 2 weeks earlier (comparison: AG + BG vs. BG), and this was independent of plant age at defoliation (Table 1; Fig. 2a). The reduction of shoot biomass by defoliation had not yet been compensated at week 12 ('fixed harvest'), 11, 8 and 5 weeks after defoliation of young, intermediate and old plants, respectively (Table 1; Fig. 2b). The extent of compensation was largest for plants that had been defoliated at young age, which had experienced the longest time period to compensate losses (Table 1, Damage × Age, $P = 0.008$). There were no significant nematode addition effects on shoot biomass at week 12 regardless of plant age at nematode addition (contrasts: Ctrl vs. BG-Y, Ctrl vs. BG-I, Ctrl vs. BG-O, Dunnett tests: all $P > 0.10$, Table 1; Fig 2b). By contrast, root biomass was reduced by nematode addition, but only when the nematodes were inoculated at intermediate plant age (contrast: BG-I vs. Ctrl, Dunnett test: $P = 0.045$, Fig. 2c). Root biomass was reduced by defoliation both when measured 2 weeks after defoliation and at week 12 (comparison: BG vs. AG + BG; Table 1; Fig. 2c and d).

Primary and secondary metabolites

Three weeks after inoculation, leaf N concentrations were unaffected by nematode inoculation (comparison: BG vs. Ctrl; Fig 3a), but increased following defoliation (comparison: AG + BG vs. BG; Fig. 3a). However, the latter increase was only observed for plants defoliated at intermediate and old age (Table 2; Fig. 3a). At week 12, neither nematode additions (contrasts: Ctrl vs. BG-Y, Ctrl vs. BG-I, Ctrl vs. BG-O; Dunnett tests: all $P > 0.10$, Fig 3b) nor defoliation (comparison: BG vs. AG + BG; Table 2; Fig. 3b) resulted in a significant change in leaf % *N*. Root % *N* was neither influenced by nematode

Table 1: ANOVA results for the effects of damage and plant age at the time of damage (age) on shoot and root biomass

	Sequential harvests					Fixed harvest				
		Shoot		Root			Shoot		Root	
Sources	*df*	*F*	*P*	*F*	*P*	*df*	*F*	*P*	*F*	*P*
Damage[b]	2	163.02	**<0.001**[a]	68.90	**<0.001**	1	82.30	**<0.001**	9.48	**0.003**
Age[c]	2	477.45	**<0.001**	289.93	**<0.001**	2	7.04	**0.002**	0.07	0.933
Interaction	4	0.30	0.878	4.95	**0.001**	2	5.28	**0.008**	0.41	0.669
Error	81					54				

Left columns are analyses of plant biomass 3 weeks after nematode inoculation ('sequential harvests') and right columns plants harvested at 12 weeks ('fixed harvest').

[a]Bold *P* values indicate significant effects at $P < 0.05$.

[b]Damage indicates treatments assigned to 'Ctrl' (no damage), 'BG' (only nematodes) and 'AG + BG' (both nematode and defoliation) in 'Sequential harvests' and to 'BG' and 'AG + BG' in 'Fixed harvest', respectively.

[c]Age indicates treatments with nematode inoculation at week 0, 3 and 6 over experiment.

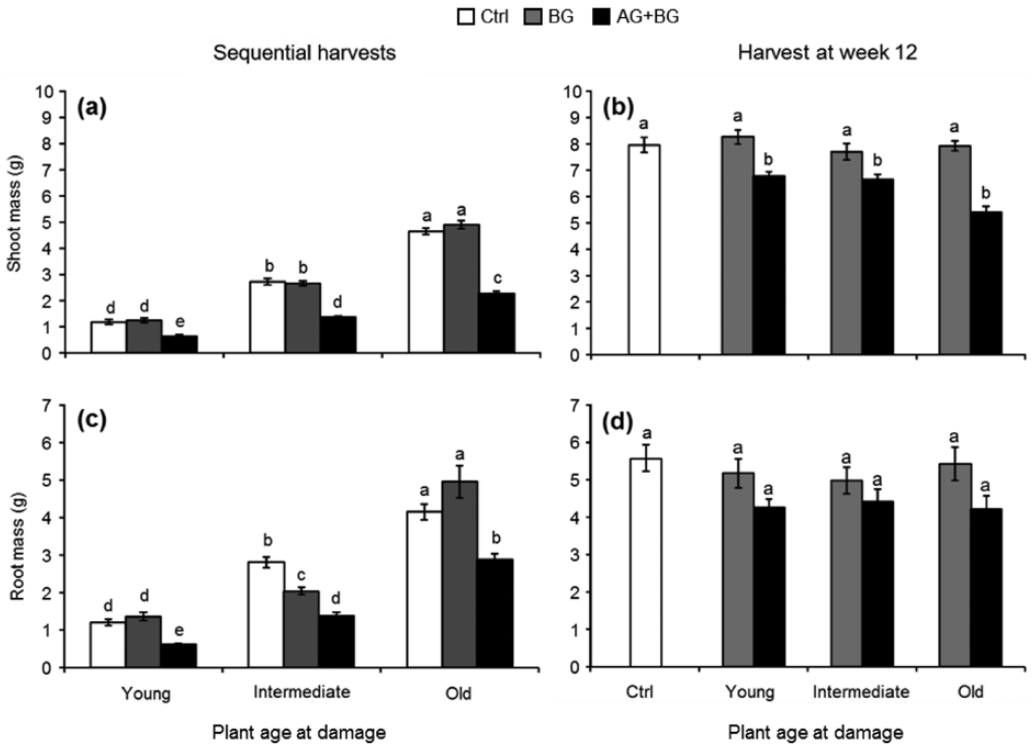

Figure 2: shoot (**a** and **b**) and root (**c** and **d**) biomass of plants exposed to nematodes (BG, grey bars) and both nematodes and defoliation (AG + BG, black bars) or plants neither exposed to nematodes nor defoliation (Ctrl) at different plant ages (young, intermediate and old). Biomass of plants were sequentially harvested 3 weeks after each inoculation (panels a and c) and at week 12 (panels b and d). Within panels, bars with identical letters are not significantly different ($P < 0.05$) based on a Tukey HSD test.

additions (comparison: BG vs. Ctrl) nor by additional defoliation (comparison: AG + BG vs. Ctrl), but it declined with plant age in both of these treatments, as measured 3 weeks after inoculation (Table 2; Fig. 3c). In contrast, Root % *N* was both reduced by nematode addition and by an additional defoliation in the longer run (at week 12) when plants had been inoculated at young age (contrast: Ctrl vs. BG-Y and Ctrl vs. AG + BG-Y, respectively; Dunnett tests: both $P = 0.004$, Fig. 3d).

Three weeks after inoculation, total phenolic concentrations in shoots were unaffected by nematode inoculation, but were significantly reduced by defoliation (comparison: AG + BG vs. BG) irrespective of plant age (Table 2; Fig. 4a). By contrast, at week 12 the concentrations of total phenolics in plant shoots were not significantly affected by the treatments (Table 2; Fig. 4b). On the other hand, the concentrations of total phenolics in roots were only enhanced by defoliation in old, nematode-exposed, plants when measured 2 weeks after defoliation (comparison: BG vs. AG + BG, Fig. 4c), and not affected by any treatment at week 12 (Table 2; Fig. 4d).

Relative shoot and root growth following defoliation

Young plants showed an enhanced shoot relative growth rate following defoliation (Fig. 5a), but this enhancement was not maintained, resulting in a significant Damage × Time interaction (contrast: AG + BG vs. BG, $Pr < 0.05$, Fig. 5a). A similar defoliation effect on relative shoot growth was observed in intermediate and old aged plants (contrast: AG + BG vs. BG, both $Pr < 0.05$, Fig. 5b and c). By contrast, nematode addition alone did not affect relative shoot growth rate (contrasts: BG vs. Ctrl, all $Pr > 0.10$, Fig. 5a–c).

Defoliation also caused a significant enhancement of the relative root growth rate when defoliation occurred at a young plant age, but, similar to what was observed for shoot regrowth, the effect disappeared over time (Damage × Time interaction: $Pr < 0.05$, contrast AG + BG vs. BG, Fig. 5d). Defoliation alone did not significantly affect the relative root growth rate in plants defoliated at the two later time points (AG + BG vs. BG: $Pr > 0.10$, Fig. 5e and f). Only the combination of nematode addition and defoliation enhanced the relative root growth rate in plants of intermediate age (AG + BG vs. Ctrl: $Pr < 0.05$, Fig. 5e), whereas it did not in plants treated at older age (AG + BG vs. Ctrl: $Pr > 0.10$, Fig. 5f). Nematode addition alone also did not affect relative root growth rate at any of the plant ages (BG vs. Ctrl: all $Pr > 0.10$, Fig. 5d–f).

DISCUSSION

We exposed the grass species *H. lanatus* to aboveground clipping and root-feeding nematodes at multiple plant ages to investigate plant defense and tolerance responses to

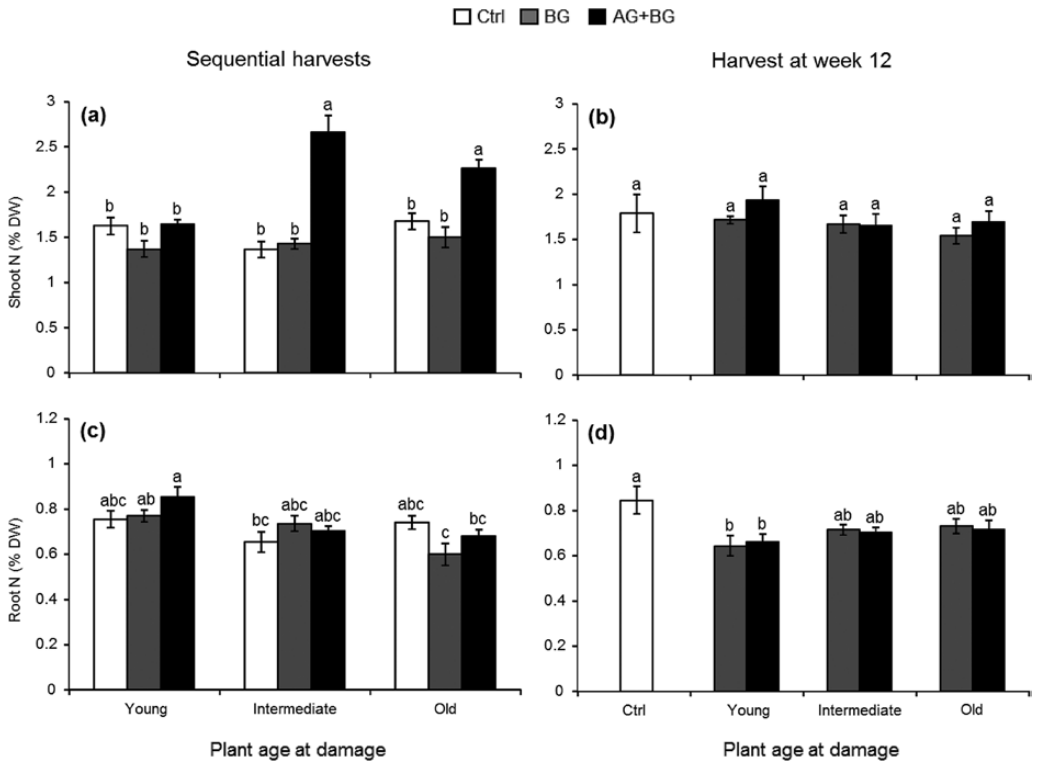

Figure 3: percentage N in shoots (**a** and **b**) and roots (**c** and **d**) of plants exposed to nematodes (BG, grey bars) and both nematodes and defoliation (AG + BG, black bars) or plants neither exposed to nematodes nor defoliation (Ctrl, open bars) at different plant ages (young, intermediate and old). Both % *N* in plants sequentially harvested 3 weeks after inoculation (panels a and c) and at week 12 (panels b and d) are shown. Within panels, bars with identical letters are not significant ($P = 0.05$) based on a Tukey HSD test.

Table 2: ANOVA results for the effects of damage and plant age at the time of damage (age) on plant chemistry (%*N*, and total phenolics)

	% *N* (DW)					Total phenolics (mg/g DW)			
		Shoot		Root		Shoot		Root	
Sources	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sequential harvests									
Damage[b]	2	43.21	**<0.001**[a]	1.10	0.341	7.93	**0.001**	1.64	0.203
Age[d]	2	5.27	**0.008**	8.96	**<0.001**	1.37	0.263	0.27	0.763
Interaction	4	8.38	**<0.001**	3.01	**0.026**	0.44	0.779	2.87	**0.032**
Error	54								
Fixed harvest									
Damage[c]	1	1.66	0.206	0.02	0.905	0.90	0.349	0.01	0.942
Age[d]	2	1.94	0.159	2.39	0.106	2.89	0.069	0.42	0.663
Interaction	2	0.56	0.577	0.14	0.866	0.69	0.510	4.14	**0.024**
Error	36								

Upper rows are analyses of plant chemistry 3 weeks after damage ('sequential harvest') and lower rows are analyses of plant chemistry at the same time (12 weeks) of growth ('fixed harvest').

[a]Bold *P* values indicate significant effects at $P < 0.05$.

[b]In 'Sequential harvests', damage indicates treatments assigned to 'Ctrl' (no damage), 'BG' (only nematodes) and 'AG + BG' (both nematodes and defoliation).

[c]In 'Fixed harvest' at week 12, damage indicates treatments assigned to 'BG' and 'AG + BG'.

[d]Age indicates treatments with nematode inoculation at week 0, 3 and 6 over experiment.

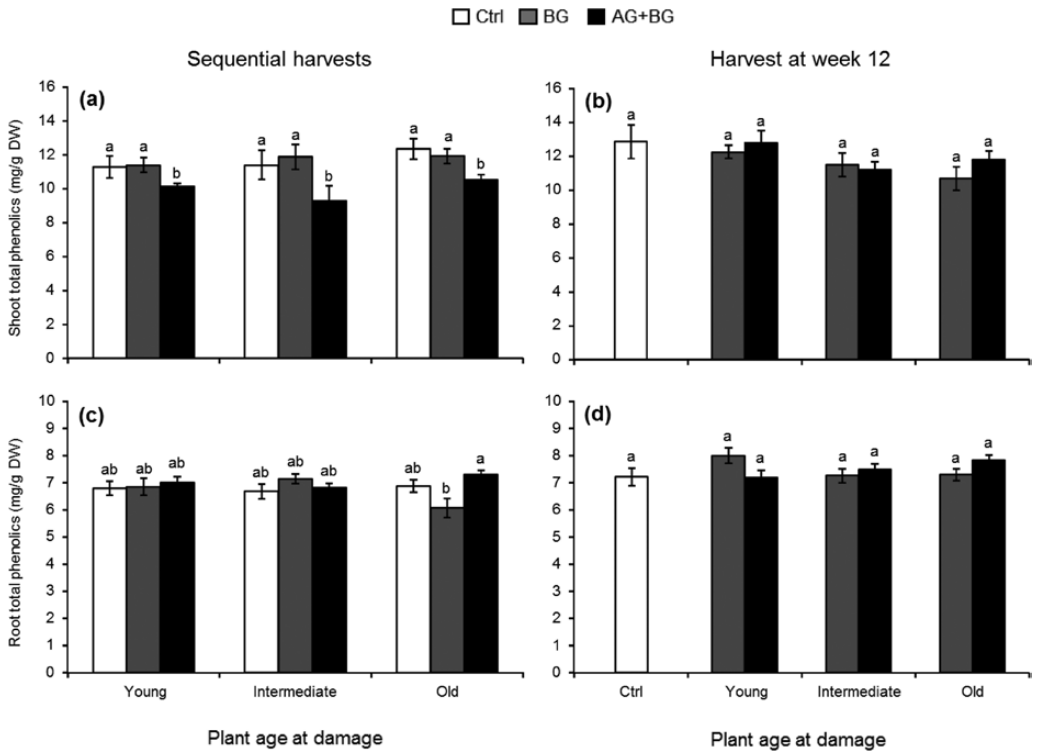

Figure 4: shoot (**a** and **b**) and root (**c** and **d**) concentration of total phenolics in plants exposed to nematodes (BG, grey bars) and both nematodes and defoliations (AG + BG, dark bars) or in plants neither exposed to nematodes nor to defoliation at different plant ages (young, intermediate and old). Both total phenolics concentration in plants sequentially harvested 3 weeks after inoculation (panels a and c) and at week 12 (panels b and d) are shown. Within panels, bars with identical letters are not significant ($P < 0.05$) based on a Tukey HSD test.

defoliation and belowground herbivory at various plant ages. Even though clipping does not really mimic herbivory, we did find that defoliation of nematode-challenged plants transiently increased leaf nitrogen concentrations of regrown foliage and reduced the concentration of total phenolics in these leaves, which might result in defoliation-induced plant susceptibility to generalist herbivores. Contrary to our first hypothesis, the defoliation-induced change in these plant chemicals was not affected by plant age at defoliation. Inoculation of root-feeding nematodes did not have a significant effect on plant chemistry except a decrease in the root nitrogen concentrations in the longer term, which was also independent of the timing of root inoculation. Defoliated plants showed enhanced relative shoot growth rates shortly (2–5 weeks) after defoliation, but these rates declined afterwards, and in contrast to our hypothesis they were independent of plant age at defoliation.

Plant age effects on induced defense following clipping and nematode exposure

Plants often respond to herbivory by inducing defense compounds (Karban and Baldwin 1997). By contrast, we observed that leaf concentrations of phenolics were transiently lower in defoliated plants. This can be due to the fact that plant responses to defoliation represent only one aspect of the spectrum of plant responses to actual herbivores, and will for instance not capture any response to salivary elicitor molecules. On the other hand, also studies using real herbivores have reported that foliar herbivory can result in a local temporary decrease in the concentration of secondary compounds (Barton 2008), particularly when available resources for metabolite synthesis are limited and simultaneously competed for by other functions such as growth and storage (Reudler *et al.* 2013; Thomson *et al.* 2003). Following defoliation plant compensatory growth and defense are expected to compete for plant stored resources (e.g. carbohydrates) when photosynthesis is limited (Boege 2005). Hence, it is not surprising that carbon limitations as a result of defoliation may restrict available resources for the synthesis of phenolics, directly leading to a decrease of this defense metabolite in plant tissues. We expected that foliar concentrations of plant defense metabolites after a defoliation event would be affected by plant age at defoliation (van Dam and Baldwin 2001). However, this effect was not observed for total phenolics in the present study, although we cannot exclude that the composition rather than concentration of phenolics could have been altered across plant ages at defoliation, which can also affect plant defenses against herbivores (Donaldson *et al.* 2006).

Root-feeding nematodes can also significantly change the concentration of secondary plant compounds in plants (e.g. Kammerhofer *et al.* 2015; Kyndt *et al.* 2012; van Dam *et al.* 2005). Moreover, nematodes can significantly reduce plant

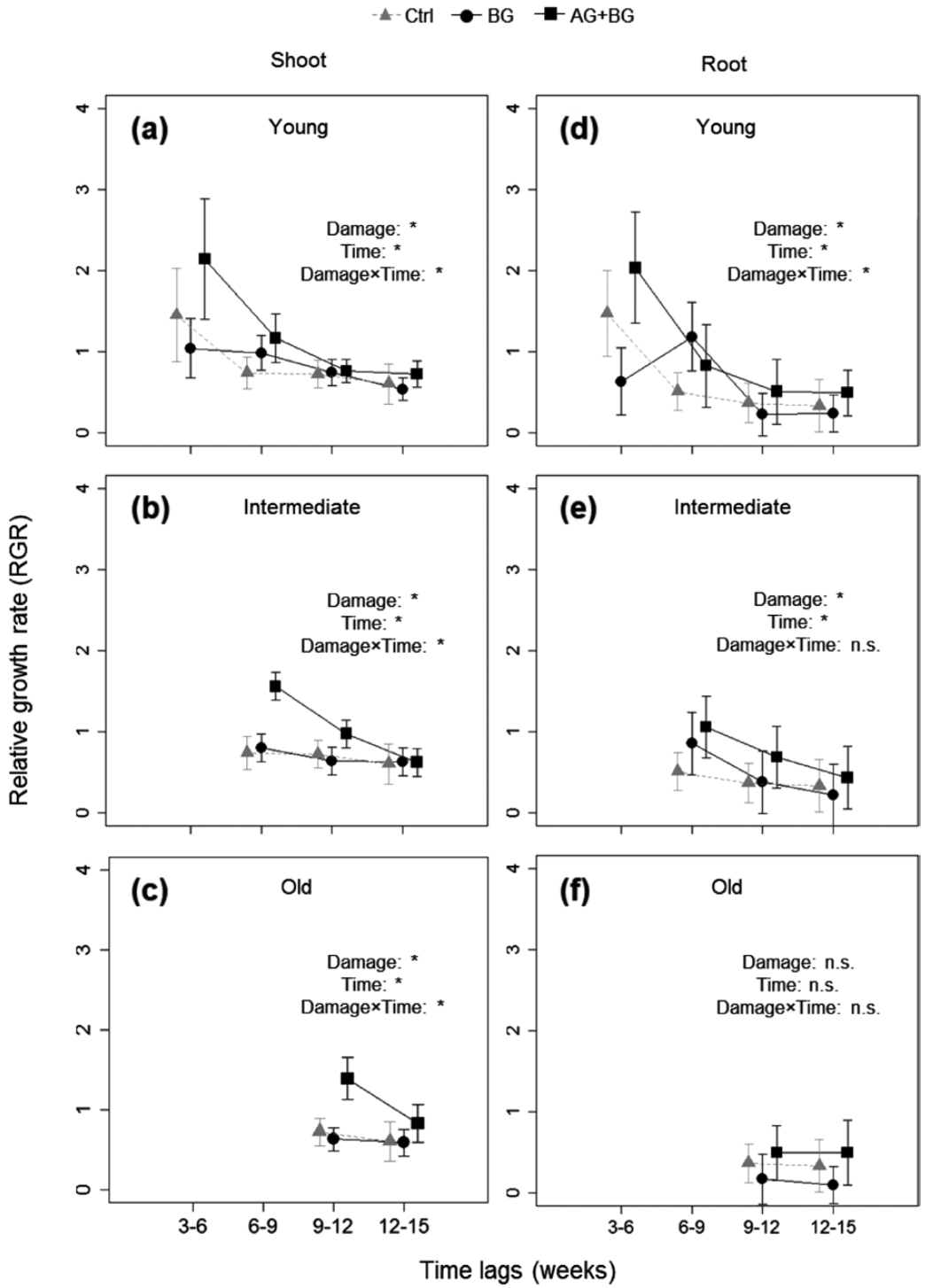

Figure 5: relative growth rate (RGR, RGR = $(B_x - B_{x-3})/B_{x-3}$, B_x indicates biomass at week x in shoots (panels **a**, **b**, **c**) and roots (panels **d**, **e**, **f**) over time ('Time', time lags after each defoliation) when plants were exposed to nematodes (BG) or both nematodes and defoliation (AG + BG) at young (a and d), intermediate (b and e) and old ages (c and f). The asterisks indicate statistical significances at $Pr < 0.05$ based on a bootstrap analysis and n.s. means non-significant. The graphs present the mean and 95% confidence intervals that were calculated from 1000 replicate analyses (see data analysis).

root biomass, resulting in a decrease of nutrient uptake. A decrease of nutrient availability to plants is usually accompanied by a relative excess of C that can be available for synthesis of C-based defense compounds (Cronin and Lodge 2003; Larson 1986), although this can be counteracted by an increased sink strength of local feeding structures of sedentary

endoparasitic nematodes (Cabello *et al.* 2014). In the current study, nematode addition only reduced the root biomass in *H. lanatus* when inoculated at intermediate plant age, and the concentration of total phenolics in roots and shoots was unaltered by nematode inoculation. One explanation for the weak response of plants to nematode inoculation may be the slow build-up of the nematode population during the experiment. Initial survival of nematodes was low (see online supplementary Fig. S2), resulting in low initial densities. Densities increased to around 50–100 nematode individuals per gram root (up to 1000 individuals per pot) towards the end of the experiment (reported in Wang *et al.* 2017, see Figs. 2a, d and 3a, d for nematode densities in the overlapping treatment between the experiments, *viz.* inoculated young plants). However, we basically lack information on minimum nematode densities required for evoking biochemical changes in the leaves of the host plant (Kammerhofer *et al.* 2015). So, alternatively the results could indicate that in our experiments *H. lanatus* was not strongly responsive to these nematodes or that the nematodes invoked other plant responses than the measured defense metabolites (Barton and Koricheva 2010).

Plant nitrogen responses to clipping and nematode addition during ontogeny

Plant regrowth after defoliation primarily relies on mobilization of available photosynthates. Photoassimilates are preferentially allocated to active shoot sinks (Briske and Richards 1995). The assimilate allocation to shoot sinks can occur at the expense of root growth (Ryle and Powell 1975). Indeed, in our experiment, 2 weeks after defoliation root biomass was reduced by half compared to non-defoliated plants. A decrease of resource allocation to roots following defoliation can lead to root mortality and a reduction in root growth and nitrogen uptake (Boege 2005; Deslauriers *et al.* 2015; Kosola *et al.* 2001). As a result of reduced N uptake the N concentration could be reduced in defoliated plants. In contrast, we found that the foliar N concentration in regrown plant foliage was enhanced by defoliation 2 weeks after defoliation but that the enhancement only occurred in plants at intermediate and old age. The higher N concentration in plant shoots can be remobilized from remaining shoot tissues or allocated from the root system (Ourry *et al.* 1990). Following defoliation intermediate and old aged plants possess higher absolute amounts of remaining shoot tissues so they are likely to have more previously absorbed N than young plants. On the other hand, the enhanced N concentration in plant foliage may also result from a redirection of N from roots to shoots after defoliation (Millard and Robinson 1990; Ourry *et al.* 1990). Young plants have smaller N pools in roots and lower ability in N uptake after defoliation, which constrains the amount of root N that can be mobilized to shoots. This may also partly explain the higher N concentration observed in intermediate-aged and old plants. Opposite to an increase of N concentration in plants following defoliation above ground, we found a decrease of root N concentration in plants subjected to treatments with nematodes in the longer term at 12 weeks after transplantation. However, the decrease only occurred in young plants probably because young plants had suffered the longest feeding period by nematodes, resulting in a more severe suppression of plant N absorption and assimilation.

Plant biomass in response to clipping and nematode addition during ontogeny

In accordance with many other studies (e.g. McNaughton 1983; Oesterheld 1992; Painter and Belsky 1993), final plant biomass was significantly reduced by defoliation in our study. Removed biomass can often be compensated by the plant over time after defoliation (McNaughton 1983) and the extent of compensation depends on many factors including timing of defoliation (Boege 2005) and the amount of time available for recovery (Oesterheld and McNaughton 1991). In our study, 2 weeks after defoliation, plants had compensated shoot tissue loss due to defoliation by about half, independent of plant age at defoliation (Fig. 2a). This suggests that in our study, younger plants did not more rapidly compensate for removed shoot biomass than old plants, as suggested by other studies (e.g. Boege 2005). However, plants that had been defoliated at different ages (young, intermediate and old) and hence differed in the time that had elapsed since defoliation (11, 8 and 5 weeks, respectively) still significantly differed in the extent to which they had compensated shoot biomass relative to their non-defoliated counterparts 12 weeks after the start of the experiment (Fig. 2b). This indicates that although plants defoliated at different ages may show similar rates of aboveground tissue loss compensation, old plants may not be able to mitigate size reduction to a similar extent as younger plants due to their shorter time for recovery. Similarly, defoliation reduced plant root biomass to a similar extent at different plant ages 2 weeks after defoliation. However, in the longer run, at 12 weeks after the start of the experiment, these defoliated plants had gained similar root biomass as non-defoliated plants, irrespective of plant age at defoliation (Fig. 2d). This suggests that plants more easily compensate for root than for shoot reduction following defoliation, regardless of the plant age when the defoliation occurs. Nematode addition significantly reduced root biomass but only of plants at intermediate age 3 weeks after inoculation. This may be because young plants can compensate the loss to herbivory to a larger extent while old plants can better tolerate herbivory because they had received a relatively lower density of nematodes per unit of root biomass due to their higher root biomass at the time of inoculation (Elger *et al.* 2009), both mitigating or even counteracting the negative effects of root-feeding nematodes on root biomass.

Growth of young and old plants differs after clipping and nematode addition

Relative changes in plant biomass were recorded following defoliation at variable plant ages because they can give

more detailed insight in the process of plant regrowth following herbivory. The relative shoot regrowth rate initially increased 2–5 weeks after defoliation compared to non-defoliated plants but the increase disappeared over time (Fig. 5a). This temporal increase in the relative shoot growth rate may be caused by a shoot sink created by defoliation. Following defoliation, plants usually prioritize current photosynthates to shoots for compensatory growth (Harber *et al.* 1989), which can be constrained over time as photosynthesis establishes and sink strength decays (Briske and Richards 1995). Patterns of relative shoot regrowth rates were independent of the age at which plants were defoliated, even though we expected that age at defoliation would strongly affect sink strength at the time of defoliation because the amount of biomass removed increased with age at defoliation. Plant root growth was also temporarily increased by defoliation in young plants but not in older plants. Relative root growth of old plants did not increase, indicating that only limited amounts of assimilates of these plants were diverted to roots. This is opposite to a recent study showing that young plants tend to invest new assimilates in shoots but old plants divert more to roots as a response to defoliation (Schmidt *et al.* 2015). This may be because old plants suffered the highest removal of biomass, creating an enormous sink in shoots that reduced the sink strength for root growth. It thus suggests that plant growth after defoliation may differ between shoots and roots, depending on when plants are defoliated during development.

CONCLUSION

We have shown that the grass *H. lanatus* exhibits different growth and nutrient responses when defoliated or exposed to nematodes at different ages, and that these responses have their own temporal dynamics. This nicely illustrates the dual temporal aspect of plant responses to belowground herbivory and defoliation: one is the effect of the timing of herbivory or defoliation on plant responses, the second is the temporal dynamics of the responses themselves. Plants enhanced N concentrations in the foliage following defoliation in the short term but reduced the N concentration of roots in response to nematode addition in the longer term. The changes in shoot N concentration only occurred at specific, older, plant ages. In contrast to our hypotheses, neither plant traits putatively related to defense (phenolic content), nor plant traits related to tolerance (ability to regrow biomass after defoliation) responded more strongly during later stages of vegetative growth. The plants tended to show an increase in shoot relative regrowth rate in response to defoliation that was strongest shortly after defoliation and became less pronounced over time, independent of the plant age at which the defoliation occurred. By contrast, the extent of the increase in relative root growth rate of defoliated plants depended on plant age at defoliation, and was strongest at young plant age.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

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Conflict of interest statement. The authors declare that they have no conflict of interests and the experiments comply with the current laws of the Netherlands where the experiments were performed.

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