Genetic variation in non-structural carbohydrates in *Plantago lanceolata* is related to mowing intensity but not to regrowth ability

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Short title: Genetic variation in NSCs in ribwort plantain

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Abstract

- Non-structural carbohydrates (NSCs) are important storage reserves of plants, and they may play a key role in the plants' ability to recover from disturbance events such as drought, fire, or biomass removal. In managed grasslands, plants regularly experience aboveground biomass removal by grazing or mowing. If NSCs influence plant tolerances to these damages, then land-use intensification could lead to adaptive changes in NSC storage allocation.
- In a common garden experiment, we quantified NSC storage in the taproots of *Plantago lanceolata* plants from 63 grassland populations that covered a broad range of land-use intensities, and we tested if pre-clipping levels of NSCs correlated positively to land-use intensity and the regrowth ability of plants after aboveground biomass removal.
- We found significant genetic variation in NSC storage among populations, but in contrast to our expectation NSC storage correlated negatively with mowing intensity and its temporal variation. This suggests that plants from frequently or irregularly mown plots have been selected against investing assimilates into their taproot but rather into aboveground biomass. That only relationships of NSC with mowing, and not with grazing, were detected, could be due to more predictable and homogeneous effects of mowing in contrast to grazing.
- As NSC variation was unrelated to the regrowth ability of *P. lanceolata* after aboveground biomass removal, this suggests that this common grassland plant is not C-limited. We conclude that NSCs possibly have only short-term effects on regrowth ability which we did not measure.

Introduction

In order to grow and maintain metabolic functions plants assimilate carbon through photosynthesis. If photosynthesis surpasses the actual carbon demand of a plant, assimilated carbon can be stored in the form of non-structural carbohydrates (NSCs) in stems, leaves, and roots as well as in specialized organs such as storage roots (Janeček and Klimešová 2014; Martínez-Vilalta et al. 2016) for later use. Depending on the plant species, NSCs include low-molecular weight sugars (fructose, sucrose, glucose) and starch (in some herbs and grasses also fructans) (Chapin et al. 1990; Martínez-Vilalta et al. 2016; Landhäusser et al. 2018). Starch that is stored during the day is used to maintain growth and metabolism during the night (Smith and Stitt 2007). However, plants can also mobilize stored carbon during episodes of low carbon availability that would otherwise result in net carbon depletion and decreased photosynthetic activity, e.g. during spring regrowth (Heilmeier et al. 1986), flowering and fruit production (Horibata et al. 2007), periods of abiotic stress such as drought (Hartmann et al. 2013), or during regrowth after biomass removal (Greub and Wedin 1971; Richards and Caldwell 1985a; Li et al. 2002; Carpenter et al. 2008).

In temperate European grasslands, plants are usually subjected to regular aboveground biomass removal through grazing or mowing. These management practices remove much of the photosynthetically active tissue, and plants need to regrow. The ability of plants to compensate for a loss of biomass, which is related to the concept of grazing tolerance (McNaughton 1983), is not only based on the production of new leaves (Richards and Caldwell 1985a; Visser et al. 1997; Morvan-Bertrand et al. 1999), but often also on the mobilization of stored carbon reserves (NSC) that enhance the recovery of photosynthetically active tissue (Morvan-Bertrand et al. 1999; Schnyder and de Visser 1999). Several previous studies showed that stored carbohydrates are indeed mobilized from storage tissue after defoliation and translocated to newly produced shoots (Danckwerts and Gordon 1987;

Morvan-Bertrand et al. 1999; Schnyder and de Visser 1999). The abilities of building up carbon reserves and quickly mobilizing them after biomass loss therefore seem crucial for plant fitness and should thus be selected for.

In managed grasslands, the frequency of grazing or mowing, as well as the intensity of grazing in terms of the duration and type of animals, can vary considerably among different grasslands. If there is genetic differentiation in NSC storage among grasslands that results from natural selection, then we would expect adaptation to management intensity in three main ways: (1) in the amount of carbon stored before the start of management in spring, (2) in the degree of carbon mobilization after disturbance, and (3) in the replenishment of carbon reserves after disturbance. A few previous studies showed that NSC storage of plants can indeed adapt to the severity of biomass loss (Palacio et al. 2012; Benot et al. 2019). For instance, after natural defoliation of *Pinus nigra* by the pine processionary moth, NSC content in needles and stems decreased but replenished proportional to defoliation intensity in just one growing season, with more heavily defoliated trees re-accumulating more NSC (Palacio et al. 2012). Moreover, Benot et al. (2019) showed that cattle grazing intensity influenced early-season NSC content in five grass species, with intensely grazed plants showing higher NSC concentrations than moderately grazed ones. In addition, all plants replenished NSC storage at the end of the grazing period to pre-grazing levels irrespective of the grazing intensity (Benot et al. 2019). All of these studies indicate that biomass loss, through natural defoliation or land use, may exert selection on NSC storage patterns.

In contrast to the influence of biomass removal on NSC storage, the influence of stored NSC on grazing tolerance in terms of regrowth has gained much less attention. So far, no consensus exists about whether higher NSC concentrations before biomass removal increase regrowth after disturbance (Davies 1965; Richards and Caldwell 1985a; Hogg and Lieffers 1991). For example, a study on NSCs and regrowth of *Lolium perenne* found that only fructans influenced early regrowth, whereas at later stages this relationship between pre-defoliation levels of NSC and regrowth vanished (Morvan-Bertrand et al. 1999). In contrast, *Populus* saplings compensated better when cut in late fall, when root starch content is high, compared to saplings cut in spring, when root starch content is lower (Landhäusser and Lieffers 2002). Also, the saplings cut in late fall replenished their root starch to pre-disturbance levels, while spring-cut saplings achieved only 20% recovery (Landhäusser and Lieffers 2002).

While the research described above has demonstrated links between biomass removal and NSC storage, so far no study has, to our knowledge, tested for population differentiation in NSC storage, i.e. demonstrated that NSC storage differences between populations are heritable, and related to the intensity and/or timing of grassland management. We also know that grassland plants show phenotypic adaptations towards variation in grassland management, for instance through prostrate growth forms (Warwick and Briggs 1979; Díaz et al. 2007), phenological escape strategies (Völler et al. 2013, 2017) or increased grazing tolerance (Rosenthal and Kotanen 1994; Louault et al. 2005; Díaz et al. 2007). Hence, we hypothesize that variation in biomass removal through grazing and/or mowing should also select for different levels of NSC storage.

Here, we tested for population differentiation in NSC storage along a gradient of land-use intensity, specifically grazing and mowing intensities, in *Plantago lanceolata*. We expected that higher land-use intensity correlates with higher NSC storage as this would allow plants to regrow better after more frequent biomass removal. Additionally, as farmers might change land-use practices among years, we also tested for the relationship between NSC storage and the degree of inter-annual temporal variation in land-use intensity. The short-lived perennial *P. lanceolata* is widespread in the northern hemisphere and stores NSC mainly in its taproot. The most important carbohydrates for this species are raffinose-family oligosaccharides, sorbitol and glucose (Janeček et al. 2011). NSC content in *P. lanceolata* decreases after defoliation (Lee et al. 2015) and re-accumulation of total NSCs appear to be higher in damaged than undamaged plants under nutrient-rich conditions (Latzel et al. 2014). We also tested if pre-clipping levels of NSC positively affected the regrowth ability of *P. lanceolata*. For this, we used data from another common garden experiment, conducted with the same populations of *P. lanceolata*, in which we tested the effects of land-use intensity on the regrowth ability of *P. lanceolata* after a clipping treatment (Kirschbaum et al. 2021).

Specifically, we were interested in the following questions: 1) Is there genetic variation in the concentration of non-structural carbohydrates (NSCs) in *P. lanceolata* taproots along a land-use gradient? 2) Does NSC storage in *P. lanceolata* correlate positively with among-population variation in land-use intensity, in particular with grazing and mowing? 3) Does NSC storage in *P. lanceolata* correlate with inter-annual temporal variation in land-use intensity, in particular with grazing and mowing? 4) Do pre-defoliation levels of NSC explain variation in regrowth ability in *P. lanceolata*?

Methods

Study area

Our study was conducted within the framework of the Biodiversity Exploratories, a large-scale and long-term project investigating relationships between land use, biodiversity and ecosystem processes. The project consists of a network of study sites in three regions of Germany – the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas, and the UNESCO Biosphere Area Schwäbische Alb – with 50 grassland plots (50 × 50 m) in each region. Together, the three regions span a north-south transect of about 800 km in Germany, and the distances between plots within each region range from less than a km to about 30 km, with a mean distance of 13.4 km. All grasslands are continuously managed; they cover broad land-use gradients from unfertilized and lightly grazed grasslands to strongly fertilized meadows and pastures that are heavily mown or grazed several times per year. In each plot, the type and intensity of management are monitored annually (Vogt et al. 2019), and intensity of management is calculated using the LUI calculation tool (Ostrowski et al. 2020) implemented in BExIS (http://doi.org/10.17616/R32P9Q), as follows: (1) fertilization intensity as the amount of nitrogen applied per hectare (kg N ha^{-1}), (2) grazing intensity as the units of livestock per hectare, multiplied with grazing period and weighted by type of livestock (horse, cattle, sheep, and goat), and (3) mowing intensity as the number of cuts per year (Blüthgen et al. 2012). In our study, we used 11 years of monitoring data (2006 – 2016) to calculate average land-use intensities for all plots.

Seed material

Between May and September 2017, we collected seeds of *Plantago lanceolata* from all plots in the Biodiversity Exploratories where the species occurred. Depending on seed maturity (influenced by land-use management), we visited all plots up to three times and collected ripe seeds of 12 individuals per plot. Below, we refer to these as seed families (= all seeds from one individual) in contrast to populations (= all individuals from one plot). The 12 individuals were selected randomly but with at least 1 m distance between each. We were able to collect seed material from 63 plots. We dried all seeds at room temperature in paper bags and subsequently stored them in the dark at 4°C until further use.

Common garden experiment

To investigate heritable variation in NSC content in the taproots of *P. lanceolata* we used the same seedlings as in an outdoor common garden experiment conducted from April-October

2018 in Tübingen (Kirschbaum et al. 2021). Depending on germination success, we used one seedling from five to seven seed families per population, altogether 370 plants. The procedures for sowing and germination (cold stratification), pots (1 L), soil type (sand-soil mixture), fertilization (equivalent of 60 kg N ha^{-1}), watering (*ad libitum* but at least twice per week) and random placement on the field site were identical to the aforementioned common garden experiment (for details see Kirschbaum et al. 2021). Six weeks after transplanting the seedlings to pots and placing them outside in our experimental garden, we recorded if plants flowered, measured rosette diameter as a proxy for plant size, and harvested belowground biomass for NSC analysis.

Non-structural carbohydrate analysis

As we were only interested in the taproot, we removed all secondary roots with scissors and scalpels. The morphology of the taproot – with the largest diameter at the top and narrowing downwards – allowed us to define the total length of the taproot as the point where the diameter became less than 1 mm. The processed and cleaned taproots were kept on ice until later, on the same day, when we heated the taproots in a microwave oven at 900 W for two times 30 s with cooling of 5 s in between the heating repetitions, to halt activity of NSC-modifying enzymes. After that, we dried the roots for three days at 60°C and subsequently weighed them. We ground the dried root material in metal grinding jars (10 mL, stainless steel, Retsch GmbH, Haan) with two grinding balls (Ø 7 mm, stainless steel, Retsch GmbH, Haan) in a mixer mill (MM 400, Retsch GmbH, Haan) at 20 Hz until all material was pulverized. We then transferred the ground root material into 2 mL Eppendorf tubes (Eppendorf AG, Hamburg) and stored them over silica gel until further use.

To estimate non-structural carbohydrates (NSCs), defined as free, low molecular weight sugars (glucose, fructose, sucrose) plus starch, we employed a slightly modified protocol by Landhäusser et al. (2018). After heating approximately 15 mg of grinded root sample with distilled water, the soluble fraction was treated with invertase (from baker's yeast; Grade VII, ≥300 U/mg, I4504-1G, Sigma-Aldrich Corp, Saint Louis, Missouri) and isomerase (from baker´s yeast; Type III, ammonium sulfate suspension, ≥400 U/mg, P5381-5KU, Sigma-Aldrich Corp., Saint Louis, Missouri) to digest fructose and sucrose to glucose (see protocol S1 in Landhäusser *et al.* 2018). The glucose was then quantified by spectrophotometry after enzymatic conversion to gluconate-6-phosphate (see protocol S4 in Landhäusser et al. 2018). The insoluble starch fraction was treated with α-amylase (from *Bacillus licheniformis*; lyophilized powder, 500 – 1500 U/mg, A4551-100mg, Sigma-Aldrich Corp., Saint Louis, Missouri) and amyloglucosidase (from *Aspergillus niger*; ROAMYGLL – 3500 U, 6 U/mg, Roche Diagnostics GmbH, Mannheim) to convert the starch into glucose (see protocol S2 in Landhäusser et al. 2018), which was then quantified as above.

Statistical analysis

To obtain a measure of absolute NSC content per plant, we calculated NSC content as the sum of sugar and starch content multiplied by the dried root weight. We then divided this measure of absolute NSC content by the rosette diameter of that plant to obtain a measure of NSC content relative to plant size, henceforth termed relative NSC content. We calculated relative sugar and relative starch contents in the same manner. To test for population differentiation in relative NSC content, we fitted a linear model with relative NSC content as response variable and region of origin and population nested within regions as fixed effects. Since some plants had started flowering before our harvest, we included flowering as a binary covariate (yes – 1 / no – 0) in our model.

To investigate the effects of land-use intensity on relative NSC contents we fitted linear mixed-effects models with flowering (binary), region of origin and one of three land-use factors – grazing, mowing or a combination of both variables, further referred to as total damage, calculated as the square-root of their summed standardized values – as fixed effects and population as random effect. To test for the effects of inter-annual temporal variation in land-use intensity we further calculated the standard deviations of mowing and grazing intensity over eleven years (2006-2016), and for total damage the square root of the sum of the two standard deviations. We applied linear mixed-effects models with relative NSC content as response variable, flowering (binary), region of origin and one of the measures of temporal land-use variation as fixed effects, and population as random effect. We repeated the same analyses separately for relative sugar and relative starch content. The statistical assumptions of normality and homoscedasticity of residuals were achieved through a log-transformation of the response variable in all linear mixed effects models. Since our analyses of the different NSC measures and land-use factors constituted multiple testing, we used the Benjamini-Hochberg correction for false discovery rates (Benjamini and Hochberg 1995).

Finally, we tested if relative NSC content was related to the regrowth ability of *P. lanceolata* after a clipping treatment. For this we used data from a common garden experiment with the same plant material where we had previously tested for plasticity of regrowth ability after a clipping treatment, and had calculated plasticity as the log response ratio of clipped versus unclipped plants in terms of their total and reproductive biomass at the end of the growing season (for details see Kirschbaum et al. 2021). We fitted linear mixed-effects models with plasticity of total biomass of *P. lanceolata* as response variable, region of origin and flowering-corrected residuals of relative NSC content as fixed effects, and population as random effect. Additionally, we fitted a linear model with the plasticity of reproductive biomass of *P. lanceolata* as a response variable, and region of origin and flowering-corrected residuals of relative NSC content as explanatory variables. We used population-level plasticity values of reproductive biomass, as plasticity calculations at the seed family level yielded infinite values because most plants did not reproduce, and hence there was no need for including population as a random factor. We repeated the same analyses for relative sugar and relative starch content. In all models, the assumptions of normality and homoscedasticity of residuals were met without transformation of the response variable.

The statistical analyses described above were done with R version 3.6.1 (R Core Team 2019), the *lme4* package (Bates et al. 2015) and the *lmerTest* package (Kuznetsova et al. 2017).

Results

The relative NSC content of *P. lanceolata* differed significantly among regions of origin and populations (Figure 1, Table 1), and the patterns were similar for relative sugar and relative starch content (Table 1). We also found that all three carbohydrate variables were strongly influenced by whether a plant had been flowering during the experiment or not.

We found a significant negative relationship between the mowing intensity of the grasslands of origin and relative NSC content as well as relative sugar content of the *P. lanceolata* plants (Table 2, Figure 2), while there were no relationships with relative starch content, or with the other two measures of mean management intensity (Table 2). The pattern was similar for temporal variation in land-use: the temporal variation in mowing intensity was negatively related to relative NSC as well as relative sugar content (Table 2, Figure 3), but we found neither a relationship with relative starch content nor with the other two measures of temporal land-use variation.

The plasticity in regrowth ability of both total biomass and reproductive biomass of *P. lanceolata* biomass was unrelated to relative NSC, sugar or starch content (Table 3).

Discussion

The ability of plants to store non-structural carbohydrates (NSC) – low-molecular weight sugars and starch – can buffer the effects of environmental variability and disturbances such as drought, fire or herbivory, because it allows plants to mobilize stored NSCs during periods of carbon shortage. Here, we show that NSC storage of *Plantago lanceolata* exhibits genetic differentiation across 63 grassland populations with different land-use intensities, with the amount of NSC storage negatively related to mowing but not grazing intensity of the populations of origin. The observed negative relationship was surprising since we expected plants to invest more into NSC storage with increasing land-use intensity. As NSC levels were also uncorrelated to the regrowth ability of *P. lanceolata* after damage tested in another experiment, our study suggests that NSC storage does not play a large role for the recovery of *Plantago lanceolata* after disturbance.

Genetic variation in NSC storage

Generally, after accounting for the variance explained by flowering and region of origin, relative NSC, sugar and starch contents still exhibited significant genetically based variation among populations. This observed population differentiation is particularly remarkable since *P. lanceolata* is a wind-pollinated obligate outcrosser, which should lead to high rates of gene flow and thus strongly reduce the potential for population differentiation. If in such a situation, phenotypes are still significantly differentiated, this usually indicates strong selective forces that are able to counterbalance strong gene flow. Our findings corroborate previous studies that also found population differentiation in *P. lanceolata*: For instance, Gáspár et al. (2019) found genetic and epigenetic variation among an overlapping set of *P. lanceolata* populations. In another study Janeček and colleagues (2014) showed that levels of carbohydrate storage of *P. lanceolata* plants were higher in mown plots than in abandoned plots, which already indicated a potential for population differentiation of NSC in *P.*

lanceolata. Clearly, there is potential for population differentiation and adaptation of *P. lanceolata* in various phenotypic traits, and in NSC storage.

Relationships with land use

When relating our measures of NSC storage to land-use intensity, however, we found that only mowing intensity and temporal variation in mowing intensity explained variation in NSC and sugar concentrations, but that there were no such relationships with grazing intensity. In contrast to our expectations, plants originating from more intensively mown plots as well as from more variable mowing regimes had *decreased* NSC and sugar contents. The observed negative relationship with mowing intensity was largely driven by populations from the Schwäbische Alb that also generally encompassed a broader range of mowing intensities than the populations from the other two regions.

Prior to our study, we had predicted that plants from more intensively managed populations would store more NSC because we expected this to be beneficial for the recovery from recurrent damage. Our expectation was supported by a study of Benot et al. (2019) that found that pre-grazing-season fructan and sucrose concentrations of five grass species (*Agrostis stolonifera, Cynosurus cristatus, Hordeum secalinum, Lolium perenne* and *Poa trivialis*) were higher in intensively grazed plots compared to moderately grazed plots, suggesting an adaptation of NSC to grazing intensity. A similar pattern was found in a study on *Pinus nigra* trees where trees that experienced 11 years of repeated defoliations through insect herbivory accumulated more NSCs in sapwood compared to a non-defoliated control group (Palacio et al. 2012). In our study, we found the opposite: NSC and sugar content decreased at higher mowing intensity. One explanation for this could be that *P. lanceolata* plants from frequently mown plots do not invest into reserve storage but rather into aboveground biomass, possibly because reserve storage makes sense only when this energy can later be re-invested into aboveground biomass, e.g. directly after a mowing event. With too frequent mowing, however, the time between mowing events may become too short for such an investment to pay off. In other words, there could be selection for increased storage at intermediate damage frequencies but selection against it at high damage frequencies.

Interestingly, we found that NSC storage was correlated with mowing intensity but not with grazing intensity. This could be because from a plant's perspective mowing is more predictable than grazing. While mowing generally homogenizes grasslands, grazing creates heterogeneity through livestock trampling, dung deposition and selective grazing. Although this is believed to have many positive effects on biodiversity, it may also create less predictable conditions where NSC storage may be less advantageous than in regularly mown meadows. The importance of the predictability of biomass removal for the adaptation of carbon storage to grassland management is also indicated by the negative relationship between NSC and sugar concentrations and the temporal variation in mowing intensity. We found that *P. lanceolata* plants originating from more constant mowing regimes, with little or no management changes during the 11 years preceding our sampling, stored more NSC compared to those from more variable mowing regimes. Similar to grazing, plants under a less predictable mowing regime may not be able to anticipate when the next mowing event will take place, and therefore NSC storage may have been selected against, in favor of investment into aboveground biomass.

In our study, we analyzed variation in NSC storage before damage. However, it could be that, instead of pre-defoliation NSC levels, the replenishment after disturbance (not studied here) may be adapted to land-use intensity – analogous to constitutive versus induced herbivore defenses (Karban 2011). Several previous studies found damage intensity to be related to the levels or speed of NSC replenishment. For example, *Lolium perenne* plants depleted of their storage carbon through repeated defoliations showed increased synthesis of water soluble sugars one day after the last defoliation, suggesting a dynamic adjustment of NSC storage to repetitive carbon depletion (Lee et al. 2010). Also, carbon replenishment in *Pinus nigra* needles was proportional to insect herbivory intensity, with higher levels of end-of-season NSC in more strongly defoliated trees (Palacio et al. 2012). In a previous study with *Plantago lanceolata*, Lee et al. (2015) found pre-defoliation levels of NSC after five weeks of regrowth. Together, these studies highlight the importance of NSC replenishment and its possible adaptive significance at high damage intensities (Turner et al. 2006; Lee et al. 2010, 2015). It is conceivable, that for starch contents, and with regard to grazing intensity, where we did not find significant results for (constitutive) NSC levels, adaptation is through variation in post-damage replenishment – a hypothesis that remains to be tested in the future.

NSC storage and regrowth ability

NSC storage is expected to be beneficial because it facilitates recovery after damage. However, when we related our NSC storage data with regrowth data from a sister study (Kirschbaum et al. 2021) we found no relationship between the two. The role of NSCs for regrowth is still subject of debate. Some studies found a link between pre-defoliation levels of carbon reserves and regrowth ability (Hume 1991; Danckwerts 1993; Donaghy and Fulkerson 1998; Turner et al. 2006; Palacio et al. 2012), whereas others found none (e.g. (Ryle and Powell 1975; Richards and Caldwell 1985b). For example, *Lolium perenne* plants with higher levels of water soluble carbohydrates (WSC) showed higher grazing tolerance but also faster WSC replenishment (because of the earlier production of photosynthetic active tissue) (Donaghy and Fulkerson 1998). This, however, also indicates that stored carbohydrates may only be important for a short period after defoliation but assimilation through photosynthesis soon becomes the main factor of carbon supply (Richards and Caldwell 1985a; Hoogesteger and Karlsson 1992; Donaghy and Fulkerson 1997; Morvan-Bertrand et al. 1999; Lee et al.

2015). Indeed, previous studies showed that the mobilization of carbon reserves is highest in the first week post-defoliation and decreases shortly thereafter (Danckwerts and Gordon 1987; Visser et al. 1997; Morvan-Bertrand et al. 1999; Schnyder and de Visser 1999). For example, in perennial ryegrass, NSC significantly influenced early regrowth but after four weeks the NSC-biomass relationship had disappeared (Morvan-Bertrand et al. 1999). As the regrowth ability in the current common garden study (Kirschbaum et al. 2021) was evaluated 16 weeks after clipping, it is thus possible that we were unable to detect potential early correlations between NSC levels and regrowth ability. Moreover, NSCs may not be the only stored carbon compounds used for regrowth. Neutral lipids, which are also synthesized specifically for storage, and probably also hemicellulose contribute to the carbon storage pool and possibly to regrowth ability (Hoch et al. 2003; Schädel et al. 2010; Hoch 2015). Moreover, some NSC fractions such as soluble sugars may serve different functions in the plant metabolism besides regrowth. They are osmotically active and contribute to turgor maintenance and phloem transport, and they are also involved in signaling and in cold tolerance (Morgan 1984; Gibson 2005; Krasensky and Jonak 2012). This could make it more difficult for these types of NSCs to disentangle their effects on regrowth from other functions.

In this study, we investigated genetic variation in the taproot storage of non-structural carbohydrates (NSC) among 63 populations of *Plantago lanceolata*, and its relationship with land-use intensity and regrowth ability. We found genetic variation in NSC storage among the studied populations, with decreasing NSC storage at higher mowing intensities and at greater temporal variation in mowing intensity. There was no relationship between NSC storage and grazing intensity, however, probably because of the greater heterogeneity of grazing disturbance. We also found no evidence for a link between NSC storage and regrowth ability after damage, because NSCs possibly only have short-term effects there and we measured regrowth too late to capture this. Altogether our results indicate that there is little carbon limitation after biomass removal in the studied *P. lanceolata* populations.

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Authors' contributions: Declaration of authorship: JFS and OB conceived the experiment. AK, JFS and OB designed the experiment. AK conducted fieldwork, performed the experiment and collected data. AK conducted laboratory work under the supervision of GH. AK wrote the manuscript with input from JFS, GH and OB.

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Table 1. Results of linear models testing the effects of flowering state (y/n), region of origin and population on relative content of sugar, starch and total non-structural carbohydrates (NSC) as the sum of both, in *Plantago lanceolata*. Bold *P*-values are significant (< 0.05) after FDR correction. df = degrees of freedom.

	Sugar content			Starch content			Total NSC content		
	df	*F*-value	*P*-value	df	*F*-value	*P*-value	df	*F*-value	*P*-value
Flowering	1	5.70	**0.018**	1	16.70	**<0.001**	1	1.85	**0.004**
Region	2	7.46	**<0.001**	2	5.33	**0.005**	2	1.50	**0.002**
Population	59	1.50	**0.016**	59	1.39	**0.042**	59	0.34	**0.018**
Residuals	290			281			281		

Table 2. Results of linear mixed-effects models that test for relationships between land-use intensity (mowing intensity, grazing intensity, or total damage as the sum of the both), or its temporal variation, and stored non-structural carbohydrates (relative content (= absolute content divided by rosette diameter) of sugar, starch and total non-structural carbohydrates, the sum of both sugar and starch) in *Plantago lanceolata*. Each model includes flowering (y/n) as a binomial covariate, region as a fixed effect, and population of origin as a random effect. Bold *P*-values are significant (< 0.05) after FDR correction. df = degrees of freedom.

		Sugar content		Starch content		Total NSC content	
	df	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Flowering	1	1.33	0.250	6.96	**0.009**	2.66	0.104
Region	2	2.79	0.069	2.13	0.127	2.51	0.088
Mowing intensity	1	6.48	**0.014**	3.95	0.052	6.86	**0.011**
Flowering	2	1.25	0.265	11.40	**<0.001**	2.54	0.112
Region	1	4.70	**0.012**	1.09	0.343	4.36	**0.017**
Grazing intensity	1	0.50	0.498	0.96	0.332	0.62	0.434
Flowering	1	1.14	0.286	11.17	**<0.001**	2.39	0.123
Region	1	4.93	**0.010**	1.91	0.155	4.58	**0.014**
Total damage	2	2.22	0.142	0.24	0.626	1.93	0.170
Flowering	1	1.10	0.294	6.64	**0.010**	2.35	0.127
Region	2	7.14	**0.002**	4.46	**0.015**	6.33	**0.003**
Temporal variation in mowing intensity	1	8.13	**0.006**	1.27	0.265	6.41	**0.014**
Flowering	2	1.19	0.276	6.64	**0.010**	2.44	0.120
Region	1	4.92	**0.010**	3.53	0.035	4.5	**0.015**
Temporal variation in grazing intensity	1	0.04	0.850	1.11	0.298	0.2	0.658
Flowering	1	1.22	0.271	6.74	**0.010**	2.48	0.117
Region	2	5.34	**0.007**	3.28	0.044	4.71	**0.012**
Temporal variation in total damage	1	0.46	0.503	0.33	0.569	0.12	0.728

Table 3. Results of linear mixed-effects models testing for relationships between contents of relative (= absolute content divided by rosette diameter) sugar, starch and total non-structural carbohydrates (NSC), the sum of both sugar and starch, of 63 grassland populations of *Plantago lanceolata* and their phenotypic plasticity of total or reproductive biomass in response to experimental clipping. Each model includes region of origin as a fixed effect and population of origin as a random effect. The plasticity data is from Kirschbaum et al. (2021). None of the *P*-values is significant after FDR correction.

		Plasticity of total biomass		Plasticity of reproductive biomass	
	df	*F*-value	*P*-value	*F*-value	*P*-value
Region	2	0.94	0.391	3.38	0.041
Sugar content	1	0.15	0.701	0.00	0.998
Region	2	0.90	0.408	3.44	0.039
Starch content	1	1.03	0.310	0.95	0.334
Region	2	1.11	0.331	3.39	0.041
Total NSC content	1	0.00	0.966	0.13	0.724

Fig. 1 Variation in the tap-root contents of non-structural carbohydrates (NSCs) among 63 grassland populations of *Plantago lanceolata*. The values are flowering-corrected residuals of relative NSC content (= absolute NSC content divided by rosette diameter). The boxplots are ordered by their medians and colored by region of origin (Schwäbische Alb in red, Hainich in yellow, Schorfheide-Chorin in blue).

Fig. 2 Relationship between mowing intensity (average # cuts per year during 2006-2016), and the non-structural carbohydrates (NSC) content of *Plantago lanceolata* in grasslands of different land use intensity. Points represent individual values that are the residuals of relative NSC content (absolute NSCs divided by plant size) that have been corrected for the influence of plant flowering (Schwäbische Alb in red, Hainich in yellow, Schorfheide-Chorin in blue).

Fig. 3 Relationship between the temporal variation in mowing intensity (standard deviation of the number of cuts during 2006-2016) and the non-structural carbohydrates (NSC) content of *Plantago lanceolata*. Points represent individual values that are the residuals of relative NSC content (absolute NSCs divided by plant size) that have been corrected for the influence of plant flowering (Schwäbische Alb in red, Hainich in yellow, Schorfheide-Chorin in blue).

Figure 1

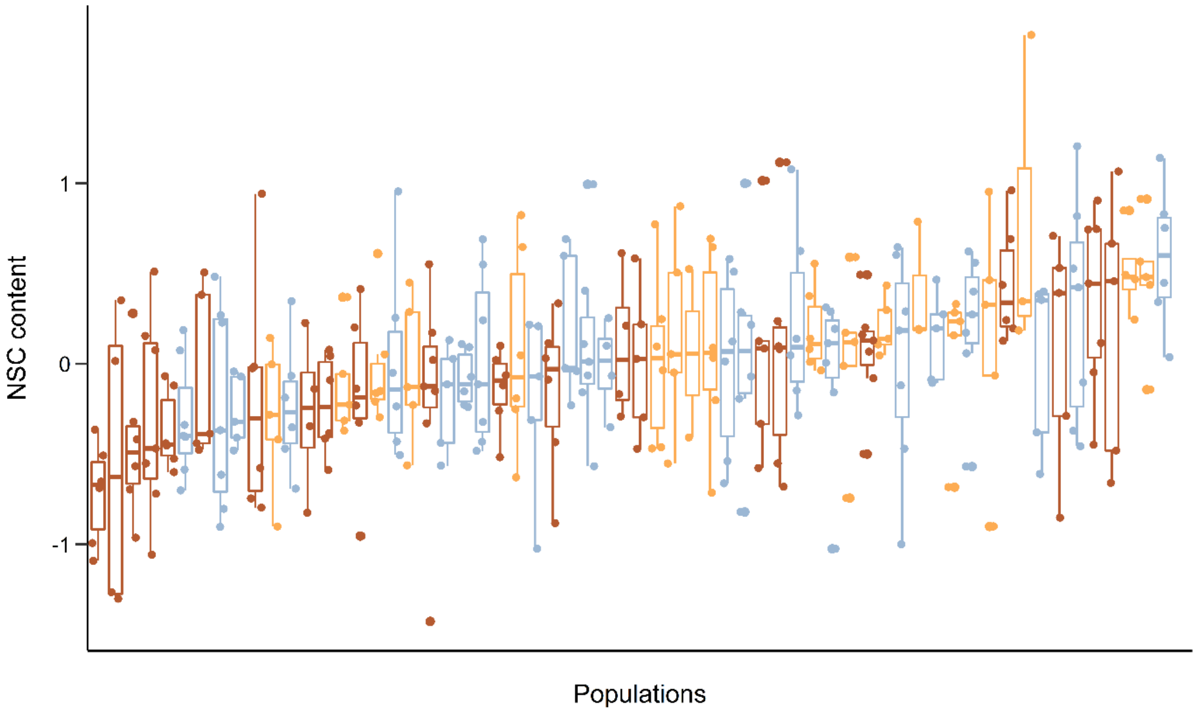


Figure 2

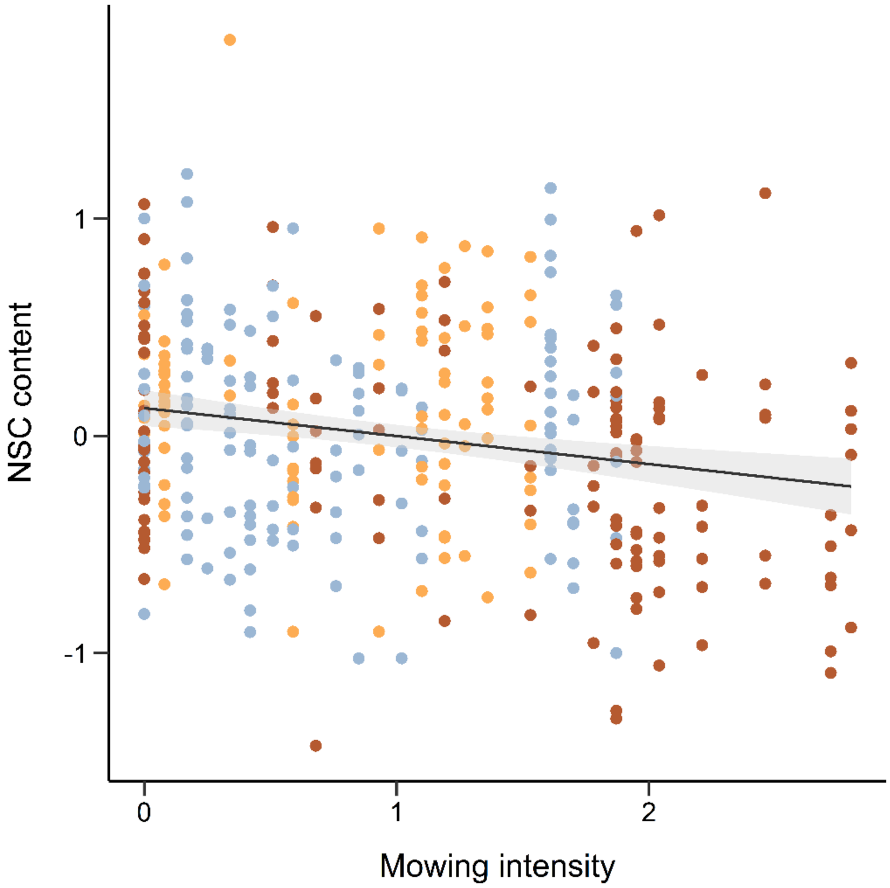
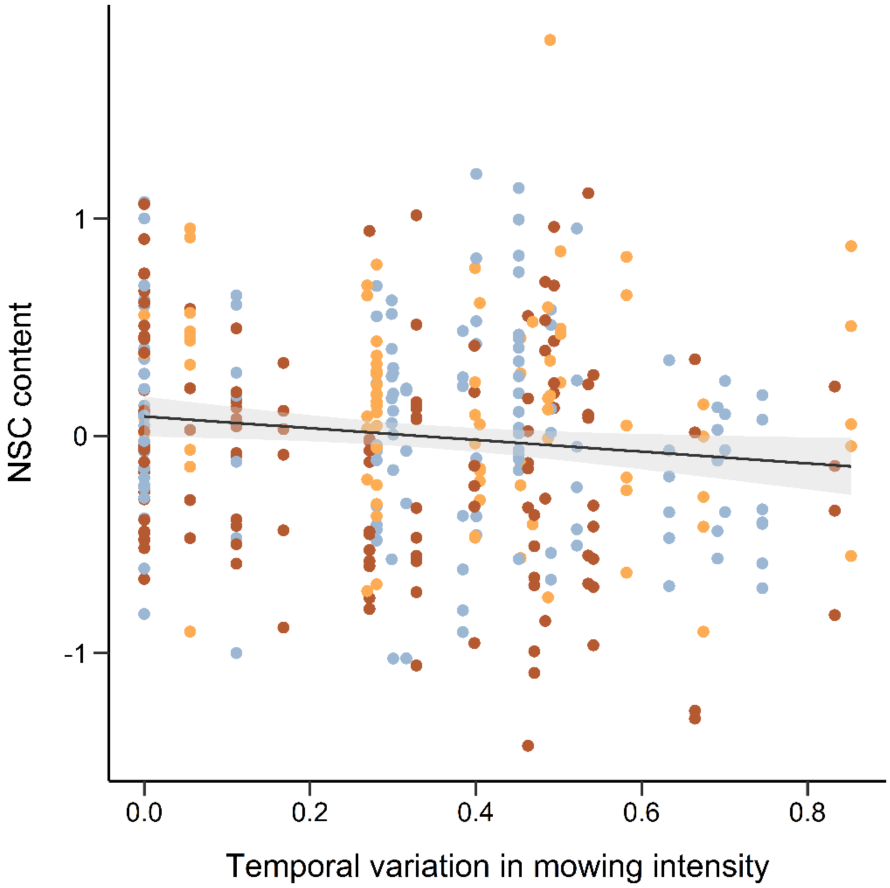


Figure 3



NSC content
1
0
-1
0.0
0.2
0.4
0.6
0.8
Temporal variation in mowing intensity