Grazing intensity modulates carbohydrate storage pattern in five grass species from temperate grasslands

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Running head. Effect of grazing intensity on carbohydrate storage.

Declarations of interest: none.

Abstract

Regrowth after defoliation is an essential mechanism of plant tolerance to grazing. In grasses, non-structural carbohydrates (NSC) contained in tiller bases constitute a major substrate for regrowth after defoliation. Using a multi-specific approach, the present study aimed at testing the effect of grazing intensity on NSC concentration in tiller bases. We selected five grazing-tolerant grass species (*Agrostis stolonifera*, *Cynosurus cristatus*, *Hordeum secalinum*, *Lolium perenne* and *Poa trivialis*) and collected plants in a grassland subjected to two cattle grazing intensities (intensive *versus* moderate) for years. We measured NSC concentrations (starch, fructans, sucrose, glucose and fructose) in tiller bases. We found that fructan and sucrose concentrations before the grazing season (April) were higher under intensive than moderate grazing. By contrast, no significant effect of the grazing intensity on these NSC concentrations in tiller bases remained at the end of the grazing season (October). These results suggest that the level of reserves available before the onset of disturbance caused by grazing as well as the reserve replenishment capacity during the grazing season are modified by the intensity of grazing.

Key-words: Fructans, grassland, HPLC, non-structural carbohydrates, tolerance to grazing, tiller bases.

Abbreviations: DM: dry mass, HPLC: high performance liquid chromatography, NSC: non-structural carbohydrates, WSC: water-soluble carbohydrates

Introduction

Grazing acts as a complex factor on vegetation, inducing major changes in floristic and functional composition in grassland plant communities (Bullock *et al.*, 2001; Dìaz *et al.*, 2001, 2007; de Bello *et al.*, 2005; Marion *et al.*, 2010). It drives vegetation changes mainly through the consumption of above-ground biomass *i.e.*, defoliation (Kohler *et al.*, 2004). Defoliation affects individual plants directly, by removing part of photosynthetically active biomass (leaves and stems) or indirectly, through canopy opening and subsequent decrease in aboveground competition (Sala *et al.*, 1986; Richards, 1993; Bakker *et al.*, 2003; Bonis *et al.*, 2005; Veen *et al.*, 2008). Plant ability to survive and develop after defoliation depends on compensatory growth, mainly based on the plant ability to regrow after damage (McNaughton, 1983; Maschinski and Whitham, 1989), an essential mechanism of tolerance to grazing (Briske, 1996; Stowe *et al.*, 2000). Considering grazing as a filter over the local species pool, it generally favours plants capable of rapid regrowth after defoliation enabling the recovery of leaf area and the resumption of photosynthetic activity (Fulkerson and Donaghy, 2001).

Regarding the source of carbon, leaf regrowth after defoliation generally takes place into two phases. First, defoliation is followed by a transient time lag, during which stored carbohydrates are mobilized, a major mechanism that enables the plant to recover from losses of photosynthetic tissues (Richards, 1993; Morvan-Bertrand *et al.*, 1999*a*; Morvan-Bertrand *et al.*, 1999*b*; Schnyder and de Visser, 1999). Second, photosynthetic activity of newly produced tissues becomes the main source of assimilates, supporting growth and replenishment of reserve pools (Richards, 1993; de Visser *et al.*, 1997; Morvan-Bertrand *et al.*, 1999*b*). Consequently, tolerance to grazing, which depends on enhanced short-term ability of leaf area recovery after grazing, is likely to mainly rely on easily mobilized reserves (Richards, 1993).

Grazing occurs within a range of intensity: even in semi-natural grasslands the grazing pressure may vary from extensive to intensive, due to management choice but also to local heterogeneity in the animal choice (Marion *et al.*, 2010; Loucougaray *et al.*, 2004). Intensive grazing may corresponds to severe and/or frequent defoliation events, with respectively large amounts of biomass removed by each defoliation event and short time lags for reserve replenishment. Frequency between two consecutive defoliation events is a dimension of grazing intensity by which reserve pools may be substantially depleted (Beaulieu *et al.*, 1997; Kleijn *et al.*, 2005). Reserve making is considered to be costly, as it diverts resources from potential growth, decreasing growth rate and leading to smaller and less competitive plants (Kobe, 1997; van der Meijden *et al.*, 2000; de Jong and van der Meijden, 2000). Thus, reserve making and remobilization for compensatory growth are expected to be selected for in environments where their benefits outweigh these costs (Karban and Baldwin, 1997). In particular, the capacities to constitute large pools of reserves available before the beginning of a grazing season and to quickly remobilize and replenish these pools after defoliation (Lee *et al.*, 2010) are both expected to enable plants to cope with intensive grazing.

Storage of resources (carbon and other elements such as nitrogen) can take place in a diversity of plant organs such as roots, perennating organs and stems (van der Meijden *et al.*, 1988; Suzuki and Hutchings, 1997; Klimeš and Klimešová, 2002; Kavanová and Gloser, 2005), but considering a wide range of species, the highest levels are found in leaves (Martínez-Vilalta *et al.*, 2016). In particular, grasses store large amounts of carbohydrates in tiller bases, which is composed of elongating leaves enclosed in sheaths of mature leaves (Morvan-Bertrand *et al.*, 1999*a*; Morvan-Bertrand *et al.*, 1999*b*). Carbohydrates contained in tiller bases are mobilized rapidly after defoliation and, due to their close proximity to the leaf growth zone, they are likely to play a key role in compensatory growth (Morvan-Bertrand *et al.*, 2001). Carbon reserves, mainly stored as non-structural carbohydrates (NSCs), have indeed been shown to

be of great importance in regrowth after defoliation. Starch constitutes the main form of long-term storage NSCs in many plant species (Manner, 1985). In grasses, starch is the major carbohydrate stored in seeds. By contrast, fructans (polymers of fructose) are the predominant compounds of carbon storage in vegetative parts in most of C3 grasses from temperate areas (Pollock and Cairns, 1991). As the main extracellular form of NSCs, sucrose (non-reactive disaccharide) is involved in source-sink carbon transport (Salerno and Curatti, 2003) and might act as a signalling compound in response to environmental cues (Hawker, 1985; Rosa *et al.*, 2009). Sucrose represents the dominant form of carbohydrate storage (Hawker, 1985) in some species, such as sugar beet (*Beta vulgaris*, Chenopodiaceae) or sugar cane (*Saccharum sp.*, Poaceae), and can also constitute a major form of carbon reserves in vegetative tissues of temperate C3 grasses beside fructans (Chatterton *et al.*, 1989).

The content and composition of carbon reserves can vary at several temporal scales from the day to the year. In temperate climates with seasonal variations, numerous studies reported annual dynamics, with the highest pools of reserves recorded in late summer or fall (e.g., Pollock and Cairns, 1991; Beaulieu *et al.*, 1997; Kleijn *et al.*, 2005; Asaeda *et al.*, 2006; Janeček *et al.*, 2011; Baptist *et al.*, 2013; Benot *et al.*, 2013b; Janeček *et al.*, 2015), after a period of slow growth or leaf senescence and nutrient recycling (Chapin *et al.*, 1990). Then, reserve pools are generally reported to decrease during winter and during the early phases of the growing period in spring, certainly due to the combination of reduced photosynthesis and of reserve remobilisation to support the maintenance of metabolism during winter and the spring regrowth outbreak (Bloom *et al.*, 1985; Fulkerson and Donaghy, 2001). In most grasslands from temperate climate and, more particulary in commonly grazed grasslands, which are widespread along the Atlantic coast, the grazing season generally expands from spring to autumn i.e., during the period of resource storage in plants. The seasonal processes of resource storage are thus likely to be altered by grazing.

The present study aimed at studying how the pattern of formation and replenishment of carbohydrate reserves in tiller bases is modulated by the grazing intensity. More precisely, we formulated the two following hypotheses. H1 (hypothesis of efficient formation of reserves): plant species tolerance to grazing is expected to rely on large pools of reserves available before the beginning of a grazing season. Thus, for plant species tolerant to grazing, the concentrations of carbohydrate reserves before the beginning of a grazing season are expected to be higher under intensive than under moderate grazing. H2 (hypothesis of efficient replenishment of reserves): plant species tolerance to grazing is expected to rely on efficient reserve replenishment between two consecutive defoliation events. Thus, regardless of the grazing intensity, plant species tolerant to grazing are expected to be able to quickly restore reserve pools, and to show equivalent concentrations of carbohydrate reserves at the end of the grazing season whatever the grazing intensity.

We tested these hypotheses for five Poaceae species from a grassland traditionally grazed for 6 months a year from early spring (April) to early fall (October), where cattle grazing has generated mosaics of vegetation patches (Marion *et al.*, 2010). Plants were collected in vegetation patches submitted to two contrasting cattle grazing intensities (moderate and intensive) for about 15 years, at two sampling dates: just after a 6-month grazing season (October) and just before the beginning of the following grazing season (April). NSC concentrations (starch, fructans, sucrose, glucose and fructose) were measured in tiller bases of individual plants.

Material and methods

Study site

This study was carried out on the most abundant species in the vegetation in a commonly-own grassland of Magnils–Reigniers (250 ha-large), in Marais poitevin (French Atlantic coast, 46° 28'N; 1° 13'W). This grassland has been reclaimed from the sea from the X^{th} century onwards. A topographical gradient consisting of depressions, intermediate slopes and higher-level flats (with a maximum altitudinal range of 0.7 m) occurs repeatedly within the grassland (Amiaud *et al.*, 1998) and discriminates three plant communities, depending on flooding duration: the hygrophilous, meso-hygrophilous and mesophilous communities, respectively (Marion *et al.*, 2010). As fructans have been suggested to promote plant resistance to hypoxia (Albrecht *et al.*, 1997), this study was conducted on species collected within the never-flooded mesophilous community, to avoid confounding effects of the flooding regime on fructan concentrations. The soil on flats are sodisols characteristic of pasture grasslands in the Marais poitevin (Amiaud *et al.*, 1998).

This grassland has traditionally been grazed by cattle and horses from April to October, a grazing season occurring during spring and summer (Amiaud *et al.*, 1998). An experimental design consisting in several paddocks has been set up in this grassland and has enabled to control the grazing intensity (from no to intensive grazing) and herbivore type (cattle and/or horses) since 1995 (Loucougaray *et al.*, 2004; Rossignol *et al.*, 2006). The present study only focused on vegetation grazed by cattle. Since 1995, cattle grazing has generated mosaics of vegetation patches submitted to contrasting grazing intensities, with stabilised composition and structure (Marion *et al.*, 2010). The study species were collected in moderately (G+) and intensively (G++) grazed patches within 1ha-large paddocks grazed by 2 heifers.ha^{-1} (*i.e.* about 685 kg ha^{-1}) and 4 heifers.ha^{-1} (*i.e.* about 1370 kg ha^{-1}, Ménard *et al.*, 2002) respectively

(see also Benot *et al.*, 2013a). Moderately grazed vegetation (G+) is characterised by diverse grass species such as *Elytrigia repens* (L.) Nevski, *Cynosurus cristatus* L. and *Hordeum secalinum* Schreb, whereas intensively grazed vegetation (G++) is dominated by the grass *Lolium perenne* L. (Marion *et al.*, 2010).

Study species

We selected five common perennial Poaceae species of the mesophilous plant community that are present under both moderate and intensive grazing (Table 1). *Agrostis stolonifera* L., *Cynosurus cristatus* L., *Hordeum secalinum* Schreb., *Lolium perenne* L. and *Poa trivialis* L. are forage species, frequently found in meadows and pastures (Grime *et al.*, 2007), with a high level of grazing tolerance (see Table 1 for species level of tolerance to grazing retrieved from the TRY database, Kattge *et al.*, 2011; original source: BiolFlor database, Kühn *et al.*, 2004). They are all tussock-forming and do not present organs potentially specialized in storage (e.g. tubers, rhizomes). *A. stolonifera* generally produces long creeping stems (stolons) but displays an important morphological plasticity. This species was included in the study as plants growing in the mesophilous community remain mainly tussock forming with only a few or short stolons (personal observations). Thus tiller bases were considered as the main storage organ for the five study species.

Although present under both grazing intensities, study species vary in abundance in the vegetation. In June 2008 *i.e.*, during the biomass peak, we estimated these variations by recording the relative cover of the five species in ten 0.5 m × 0.5 m plots randomly located within the mesophilous community in G+ and G++ vegetation patches (Table 1). Given the co-occurrence of the study species at very small scales (i.e., even less than 0.25 m², personal observations), these variations in abundance are very little likely to be caused by selective cattle grazing. On the contrary, leaf damages caused by cattle defoliation were commonly

observed for all of these species (personal observations) and variations in species abundance likely reflect differences in tolerance to grazing.

Plant collection and material

Eight non-flowering individuals of each species, composed of several connected tillers, were randomly collected within the mesophilous community under both grazing intensities (G+ and G++ patches), just after a 6-month grazing season (October 2008) and two days before the following grazing season (April 2009). In October, plants were collected about ten days after the heifers had left the pastures and showed traces of leaf damage, indicating former grazing. This time lag from the end of the grazing season was chosen to ensure that the phase of reserve remobilization was completed and reserve replenishment could have been initiated (Richards, 1993; Morvan-Bertrand *et al.*, 1999*a*). In contrast, plants collected in April were intact. At both dates, only non-flowering tillers were selected. Tiller collection lasted for two consecutive days, from 10am to 4pm. We paid attention to pick up tillers of all species all day long, in order to capture the daily variation of carbohydrate content within a species (Chapin *et al.*, 1990; Shewmaker *et al.*, 2006; Smith and Stitt, 2007; Haydon *et al.*, 2011). Each sample consisting in a few connected tillers was picked up with a knife, carefully washed and immediately frozen in liquid nitrogen. From the field to the lab, samples were transported in a freezer and kept at -80°C until they were freeze-dried. For each sample, a tiller was randomly selected and freeze-dried plants were dissected in order to separate tiller basis, composed of mature leaf sheaths and enclosed elongating leaves, from the rest of the tiller (*i.e.*, leaf blades and roots). As fructans accumulate mainly in the first centimeters of the tiller bases (Pollock and Cairns, 1991; Morvan-Bertrand *et al.*, 2001) and as tillers might differ in size, we considered NSC concentrations in 3-cm tiller bases as a relevant indicator of the amount of carbon resources stored and potentially available for biomass compensation per biomass unit.

The tiller basis was thus cut either at 3 cm above the rooting point or under the ligule of the older leaf for tiller bases shorter than 3 cm. Tiller bases were then marble-powdered.

Extraction, purification and separation of water-soluble carbohydrates (WSC)

Twenty ± 1 mg dry mass (DM) of powder were weighted. Water soluble carbohydrates (WSC: fructans, sucrose, glucose and fructose) were extracted from this powder in 80% ethanol at 80°C for 15 min. After ethanol extraction, the sample was centrifuged at 10,000 *g* for 10 min. The supernatant was preserved on ice and 2 mL of water were added to the pellet. The tube contents were mixed and incubated 15 min at 60°C. After the first aqueous extraction, the sample was centrifuged at 10,000 *g* for 10 min. The supernatant was preserved on ice and the aqueous extraction was repeated once with the pellet. The three supernatants were pooled, evaporated to dryness under vacuum and the residue was dissolved into 450 µL of ultra-pure water. Aliquots of WSC extract (100 µL) were passed through minicolumns (Mobicols from MoBITec, Göttingen, Germany) containing 150 µL of anion exchange resin (Amberlite CG-400 II, formate form, Fluka, Buchs, Switzerland) and 250 µL of cation exchange resin (Dowex 50W X8-400, H+ form, Sigma, Saint-Louis, MO, USA) to remove charged compounds. Between these two resins, 80 µL of PVPP (polyvinylpolypyrrolidone) were added to eliminate lipids, pigments and phenolic compounds.

Glucose, fructose, sucrose, and fructans were separated and quantified by high-performance liquid chromatography (HPLC). The eventual remaining impurities were removed by a pre-column Guard-PAK (Millipore Waters, Milford, MA, USA) and the WSC were then separated on a cation exchange column (Sugar-PAK I, 300 × 6.5 mm, Millipore Waters Milford, MA, USA) eluted at 0.5 $mL.min^{-1}$ with 0.1 mM CaEDTA at 85°C, and detected using a refractometer as a sugar detector (see also Supplementary Material in Benot *et al.*, 2013b).

The concentration of each NSC was calculated as the carbohydrate mass divided by the dry mass of tissue powder.

Starch measurement

Starch insoluble pellet remaining after WSC extraction was dissolved under agitation into 200 µL of dimethylsulfoxide (DMSO) and 50 µL of HCl (8N) at 60°C for 30 min. After centrifugation (10,000 *g* for 15 min), 200 µL of extract were dissolved into 500 µL of ultra-pure water added with 40 µL of NaOH (5M) and pH was adjusted to 4.5. Ultra-pure water was then added up to 1mL. After decantation, starch content was measured using enzymatic kits (Enzyplus® kit EZ0 942+ Starch, Raisio Diagnostics SpA, Rome, Italy). In short, starch was degraded by amyloglucosidase into glucose, which was quantified through NADPH production by spectrophotometry at 340 nm (Sulmon *et al.*, 2011). Starch concentration was calculated as its mass divided by the dry mass of tissue powder.

Statistical analyses

Variation in the concentration of each NSC was analysed by linear model ANOVAs. First, in order to check for seasonal patterns of NSC concentrations in tiller bases, and to test whether grazing intensity modified these patterns, a model was carried out with the date, species and grazing intensity as main effects. Then, in order to test for the effect of grazing intensity on NSC concentrations just after and just before the beginning of a grazing season, models with species and grazing intensity as main effects were carried out separately for October and April. Tukey HSD tests were used for post-hoc comparisons. When necessary, data were either log-transformed or arcsine square root-transformed to improve homoscedasticity and normality of the residuals. In cases where extreme values in the dataset limited the effect of transformations, these values were temporally removed and the new models were compared

with complete ones to evaluate the robustness of the results. Only complete models based on all data were kept for the study. Because of errors in laboratory measurements, a few replicates were lost (see Table S1 for final number of replicates per date × species × grazing intensity interactions). Statistical analyses were carried out with the R software version 3.1.1 (R Development Core Team, 2014, http://www.R-project.org) and post-hoc HSD test with the agricolae package (de Mendiburu, 2014).

Results

A general trend for higher NSC concentrations in October than in April was observed. However, this trend depended of the species and was affected by the grazing intensity (Table 2, Figure 1). While the effect of the sampling date was significant for all of the five NSCs, significant date × species interaction was recorded for all NSC but fructose ($F_{1,133}$ = 2.33, P = 0.06), a date × grazing intensity interaction for all NSC but glucose ($F_{1,133}$ = 3.29, P = 0.07) and even a significant date × species × grazing intensity for fructose ($F_{4,133}$ = 2.69, P = 0.03, see Table 2 for complete results of the model). Starch was the least abundant carbohydrate with concentrations in tiller bases lower than 3 mg g DM^{-1} in October and even than 1 mg g DM^{-1} in April (Figure 1A, F). Fructan concentrations in tiller bases were largely higher than that of the other NSCs, comprised on average between 80 and 400 mg g DM^{-1} depending on the species and the date (Figure 1B, G).

At the end of the grazing season (October), species effect was significant for all NSC concentrations (Figure 1A-E). For fructans, the highest concentrations were found in *L. perenne* (Figure 1B), which is the only species being much more abundant under intensive than moderate grazing (Table 1). *C. cristatus* showed the lowest fructan concentrations (Figure 1B). At this date, and considering the five species altogether, grazing intensity only

affected fructose concentrations, with generally larger concentrations under intensive grazing (Figure 1E).

Just before the following grazing season (April), a significant species effect for all NSCs except glucose was detected (Figure 1F-J). Fructan and sucrose concentrations were significantly affected by grazing intensity with higher values for plants submitted to intensive grazing (Figure 1G, H). In contrast, starch concentration was globally negatively affected by grazing intensity, despite a species-dependant effect. Considering hexoses, glucose concentration was not affected by the grazing intensity (Figure 1I), whereas grazing effect on fructose concentration depended on the species (Figure 1J).

Discussion

The ranks of NCS concentrations remained similar among the species investigated: fructans were the most abundant NSCs, followed by sucrose, glucose and fructose, while starch was only found as traces. The highly different concentrations of fructans and starch found in the present study confirmed the marginal role of starch as a reserve NSC in vegetative organs for most C3 grasses from temperate climates (Brocklebank and Hendry, 1989; Pollock and Cairns, 1991; Cairns *et al.*, 2002). Given their largely higher abundance in the study species, fructans and even sucrose emerged as the most relevant NSC in carbon storage.

Seasonal variations in NCS concentrations

We found that concentrations of reserve carbohydrates were globally higher at the end of summer than in early spring, providing further support to a commonly observed seasonal pattern (e.g., Steen and Larsson, 1986; Pollock and Cairns, 1991; Beaulieu *et al.*, 1997; Klimešová and Klimeš, 2003; Janeček *et al.*, 2011; Baptist *et al.*, 2013). However, this date effect depended on the species as well as on the grazing intensity, indicating that grazing

intensity modified the seasonal patterns of reserve formation. This seasonal variation in NSC concentrations can be explained by a higher rate of mobilization than of photosynthetic assimilation during winter and early spring. During winter, carbohydrate storage is likely to decrease because of lower photosynthetic activity due to shorter day length and lower incoming radiation and temperature (Fulkerson and Donaghy, 2001; Höglind *et al.*, 2011). Carbohydrate reserves can also be depleted during winter because reserves may sustain energy demand during unfavourable conditions (Bloom *et al.*, 1985). In addition, reserves may be depleted by early-spring consumption before the grazing season in order to support fast growth and tiller emergence (Chapin *et al.*, 1990; Pollock and Cairns, 1991; Beaulieu *et al.*, 1997; Kleijn *et al.*, 2005; Asaeda *et al.*, 2006). Consequently, the decrease observed in the present study in NSC concentrations and more particularly, in starch, fructan and even sucrose concentrations, between October and April may be due both to reduced production and remobilization.

Just before the grazing season: a prevailing effect of grazing intensity

April sampling was conducted to reflect carry-over effects of former grazing seasons. We detected no significant effect of the grazing intensity (moderate *versus* intensive grazing) formerly applied on the vegetation on starch concentration for three species (*C. cristatus*, *L. perenne* and *P. trivialis*) and this concentration was lower under intensive than under moderate grazing for two species (*A. stolonifera* and *H. secalinum*). By contrat, regardless of the species, fructan and sucrose concentrations in tiller bases were significantly higher in intensively grazed vegetation, supporting the hypothesis of efficient formation of reserves for plants submitted to intensive grazing (H1). The similar patterns obtained for sucrose and fructans suggest that both NSCs are involved in the delayed response to grazing intensity. For the five study grass species, sucrose could thus represent an alternative form of carbohydrate

storage. Fulfilling transport function and being easily hydrolysable (Salerno and Curatti, 2003; Amiard et al., 2004), this disaccharide could represent a mobile and “ready to use” source of carbon, potentially advantageous in a context of intensive grazing. Alternatively, these higher sucrose concentrations for plants previously submitted to intensive grazing could reflect a higher rate of photosynthetic assimilation during early spring under intensive grazing.

Several studies have already reported higher NSC concentrations in roots or storage organs of grass species in response to mowing, either by the end of the growing season (Bartoš *et al.*, 2011) or, as in our study, at the beginning of the following growing season (Baptist *et al.*, 2013; Benot *et al.*, 2013b). However, while NSC concentrations provide a reliable insight in the investment to storage at the tiller level (i.e., the ratio of biomass allocated to storage), the effects of grazing (i) on either NSC concentrations or quantities and (ii) at either the tiller or the tussock level must be considered distinctly (Bartoš *et al.*, 2011; Janeček *et al.*, 2015) as they may reflect different effects of defoliation on NSC pools. Similar NSC concentrations in tillers may hide differences in NSC quantities either at the tiller level (linked to differences in tiller biomass) or at the tussock level (linked to differences in tiller number). Several studies indeed reported negative effects of experimental defoliation or mowing on tiller biomass or tiller number at the tussock level (e.g., Donaghy and Fulkerson, 1998; Cullen *et al.*, 2006 Bartoš *et al.*, 2011; Baptist *et al.*, 2013; Benot *et al.*, 2013b). Similarly, we cannot exclude that the observed inter-specific differences in NSC concentrations can be correlated to inter-specific differences in the number of tillers per tussock, as suggested by previous measurements in undisturbed experimental garden conditions for the five study species (see Fig. S2 in Benot *et al.*, 2013a) or even in tiller turnover rates (as observed in *A. stolonifera* and *L. perenne* by Bullock *et al.*, 1994). Although we did not record tiller dynamics in the field, the absence of correlation between the number of tillers per tussock and experimental

defoliation severity previously observed for the five study species (Benot *et al.*, 2013a) suggests that grazing intensity might not have any effect on tiller density for those species. Similarly, Bullock *et al.* (1994) showed no effect of spring and summer grazing (ie., same grazing period as in the present study) on tiller densities for *L. perenne* and *A. stolonifera*, but these results indeed masked summer grazing effects on tiller turnover rates, suggesting increased tissue renewing under intense grazing. As advocated by Bartoš *et al.* (2011), considering both concentrations and quantities at the tiller as well as the tussock levels could provide deeper understanding of grazing effects on carbon economy and plant response to aboveground biomass loss.

Just after a grazing season: a predominant species effect on NSC concentrations

October sampling occurred ten days after the end of the grazing season. At that date, the composition and concentrations of NSC pools in tiller bases were primarily constrained by the species rather than by grazing intensity. Such inter-specific differences in carbohydrate allocation to storage have already been demonstrated for sets of grasses as well as non-grass species in leaves (Chatterton *et al.*, 1989), roots and diverse stem or root-derived storage organs (e.g., Janeček and Klimešová 2014). They may be influenced, at least partly, by inter-specific differences in leaf turnover rates and energy requirements for tissue renewing (Cullen *et al.*, 2006, Gastal *et al.*, 2010). The absence of difference in NSC concentrations (except fructose) between moderate and intensive grazing suggested that plants were able to efficiently restore a functional pool of reserves, regardless of the grazing intensity. Yet, intensive grazing is likely to generate more severe and/or frequent defoliation than moderate grazing, which could deplete more completely fructan pools (Beaulieu *et al.*, 1997; Fulkerson and Donaghy, 2001; Kleijn *et al.*, 2005; Lasseur *et al.*, 2007). In a previous study on two *L. perenne* varieties, Lasseur *et al.* (2007) observed that frequently defoliated plants showed a

373 higher increase of fructan synthesizing enzyme activities during the period of reserve
374 replenishment. In line with these findings, our results suggest that plants growing under
375 intensive grazing may efficiently synthesize NSC reserves between two consecutive
376 defoliation events (Lee *et al.*, 2010). The higher concentrations of fructose, which is the
377 product of fructan breakdown by fructan exohydrolase (FEH), under intensive than moderate
378 grazing ten days after the last defoliation suggested that fructan mobilization efficiency was
379 enhanced by grazing intensity. Indeed, it is well known that FEH activity is up-regulated after
380 defoliation (Morvan-Bertrand *et al.*, 1999a, 2001) and may remain at a high level during even
381 more than ten days following defoliation (Lasseur *et al.*, 2007). Altogether, these results
382 support the hypothesis of both efficient reserve remobilization and, thereafter, efficient
383 synthesis resumption for plants submitted to intensive grazing (H2).
384
385 **Potential complex grazing effects on NSC concentrations**
386 Grazing is a complex factor not only directly affecting plants through defoliation, but also
387 through other direct or indirect (e.g., modification of plant environment) effects on plants.
388 Thus, the NSC patterns recorded in the present study could reflect other grazing effects on
389 plant physiology or competitive environment. Firstly, grazing intensity might reduce energy
390 investment in seed production, which is expected to divert resources from vegetative storage
391 (Chapin *et al.*, 1990; Crone *et al.*, 2009). Although we paid attention to collect exclusively
392 vegetative tillers from non-flowering clonal fragments, such indirect effects of grazing on
393 NSC reserve concentrations cannot fully be excluded. Secondly, Liu *et al.* (2012) showed on
394 the grass *Lymus chinensis*, that sheep saliva addition to clipped plants decreased fructan
395 concentrations while favouring glucose and fructose accumulation in plant organs during ten
396 days following treatments. This suggests possible effects of grazing intensity on fructose
397 concentration through animal saliva. Finally, grazing might also modify the available

resources, both in terms of soil nutrients (Rossignol *et al.*, 2011) and light. For instance, by opening canopy, grazing could limit shading by taller plants and indirectly enhance photosynthesis and carbohydrate storage in smaller ones. Inter-specific differences in NSC concentrations in the present study could thus reflect differences in access to light. All of these potential indirect effects of grazing on reserve making were beyond the scope of the present study but should deserve more attention in the future.

Conclusion and perspectives

The mechanisms of reserve storage regulation and remobilization in response to defoliation have seemed to be clearly understood for long (see Introduction). Yet, two recent studies highlighted that carbon storage may in fact be more complex than we do believe (Martínez-Vilalta *et al.*, 2016; Klimešová *et al.*, 2017). Pathways and organs (e.g., source – sinks relationships) involved in carbon storage and remobilization in response to disturbance, as well as their timing and the effect of environmental conditions still remain to be clearly identified. As expected, our study showed that the concentrations of fructans and sucrose were significantly higher under intensive than moderate grazing in April (H1), whereas grazing intensity did not affect NSC concentrations, except for fructose, in October (H2). These results thus suggest that the composition and the concentrations of NSCs in tiller bases should be considered to better understand plant responses to grazing. Although field experiments may suffer from some limitations, for instance due to little spatial or temporal repetitions, we are convinced that they will help building a stronger understanding of plant carbon economy.

Authors' contribution

MLB, CM and AB concieved the idea. MLB, CM and JH collected plants in the field. MLB, AMB, JH, CS, MLD and MPP concieved and performed laboratory analyses. MLB and CM carried out statistical analyses. All authors discussed the results. MLB, AMB, CM, CS, MPP and AB wrote the manuscript.

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Table 1 Mean (± SE) species relative cover (%) under moderate (G+) and intensive (G++) grazing (n=10 sampling plots per grazing intensity) and grazing tolerance retrieved TRY database (Kattge *et al.* 2011; original source: BiolFlor database, Kühn *et al.* 2004).

	A. stolonifera	*P. trivialis*	*C. cristatus*	*H. secalinum*	*L. perenne*
Cover in G+ (%)	8.7 ± 3.5	10.5 ± 2.2	16.2 ± 2.2	7.49 ± 2.2	0.4 ± 0.3
Cover in G++ (%)	5.0 ± 2.7	9.4 ± 2.6	17.0 ± 3.51	12.3 ± 2.8	17.7 ± 1.6
Grazing tolerance*	9	6	7	7	8

* ranging from 1 (intolerant) to 9 (very tolerant to grazing).

Table 2 Result of the ANOVA testing for the impact of sampling date (*Date*), species (*Sp.*) and grazing intensity (*Graz.*) and their interactions on non-structural carbohydrate (NSC) concentrations in tiller bases. Significant P-values (<0.05) are in bold. Data transformations for the models are indicated (log: log-transformed data, asin-sqrt: arcsine square root-transformed data).

		Starch		Fructans		Sucrose		Glucose		Fructose	
	df	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
		n = 158		*n = 153*		*n = 153*		*n = 153*		*n = 153*	
Date	1	53.81	**<0.001**	14.72	**<0.001**	71.25	**<0.001**	31.21	**<0.001**	19.99	**<0.001**
Sp.	4	4.85	**0.001**	19.59	**<0.001**	48.16	**<0.001**	8.49	**<0.001**	12.66	**<0.001**
Graz.	1	1.98	0.16	0.91	0.34	43.42	**<0.001**	0.10	0.76	3.58	0.061
Date × Sp.	4	7.98	**<0.001**	2.95	**0.023**	3.07	**0.019**	3.28	**0.013**	2.33	0.059
Date × Graz.	1	9.74	**0.002**	5.21	**0.024**	11.37	**<0.001**	3.29	0.072	9.49	**0.002**
Sp. × Graz.	4	3.76	**0.006**	1.53	0.20	1.88	0.12	1.51	0.20	0.40	0.81
Date × Sp. × Graz.	4	0.75	0.56	1.48	0.21	0.76	0.55	1.19	0.32	2.69	**0.034**
Transformation		asin sqrt		log		log		asin sqrt		asin sqrt	

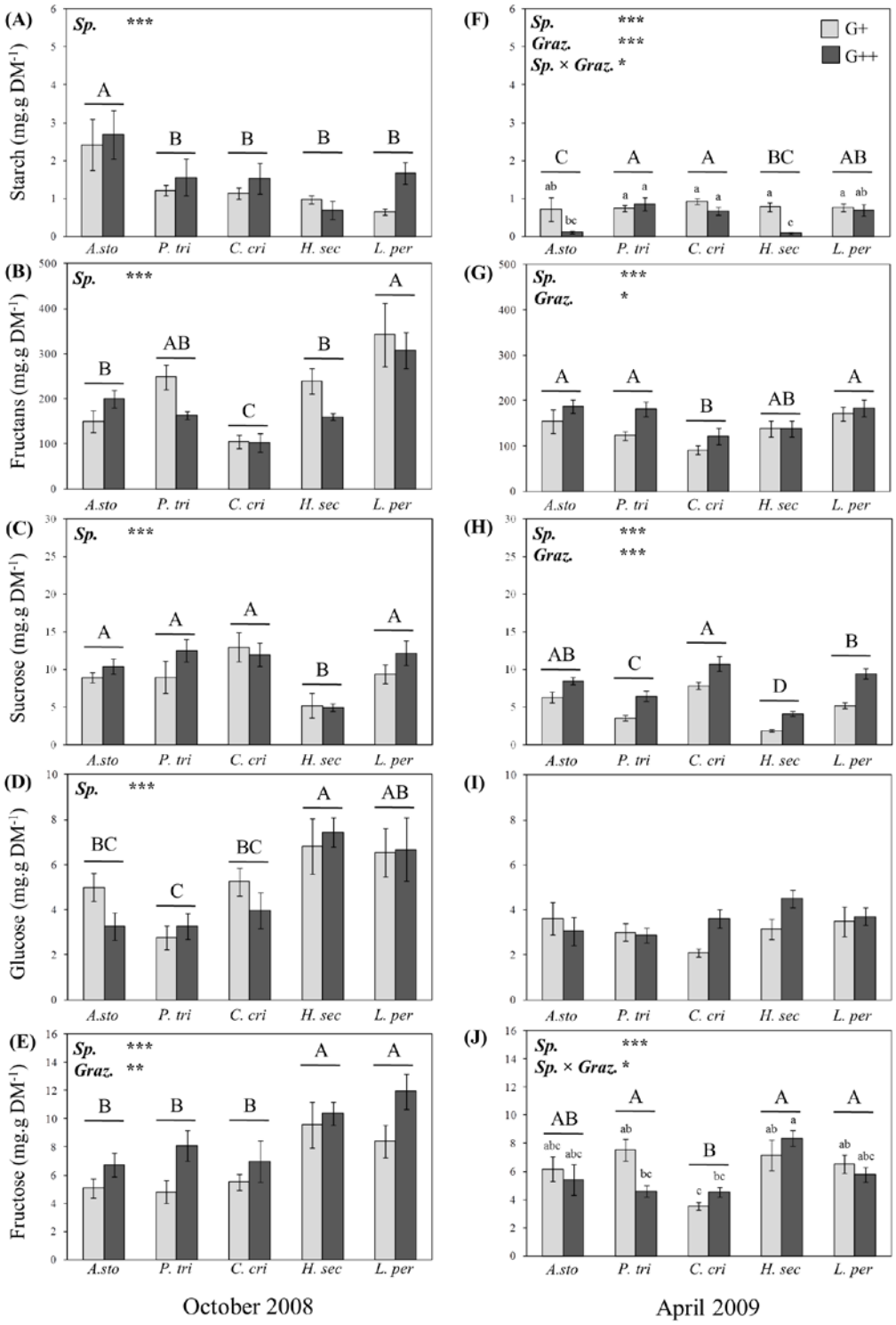

Figure 1 Mean (± SE) non-structural carbohydrate (NSC) concentrations (mg g DM^{-1}) in tiller bases in October 2008 (A-E) and April 2009 (F-J). Species are ranged according to increasing difference in relative cover between G+ and G++ (see Table 1). Only significant effects of species (*Sp.*), grazing

intensity (*Graz.*) and their interaction are indicated: * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Different letters indicate significant differences. G+ moderate grazing, G++ intensive grazing.