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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.

1 Abstract

2 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 3 4 regrowth after defoliation. Using a multi-specific approach, the present study aimed at testing 5 the effect of grazing intensity on NSC concentration in tiller bases. We selected five grazingtolerant grass species (Agrostis stolonifera, Cynosurus cristatus, Hordeum secalinum, Lolium 6 perenne and Poa trivialis) and collected plants in a grassland subjected to two cattle grazing 7 8 intensities (intensive versus moderate) for years. We measured NSC concentrations (starch, 9 fructans, sucrose, glucose and fructose) in tiller bases. We found that fructan and sucrose 10 concentrations before the grazing season (April) were higher under intensive than moderate 11 grazing. By contrast, no significant effect of the grazing intensity on these NSC 12 concentrations in tiller bases remained at the end of the grazing season (October). These 13 results suggest that the level of reserves available before the onset of disturbance caused by 14 grazing as well as the reserve replenishment capacity during the grazing season are modified 15 by the intensity of grazing.

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

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

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26 Introduction

27 Grazing acts as a complex factor on vegetation, inducing major changes in floristic and 28 functional composition in grassland plant communities (Bullock et al., 2001; Diaz et al., 29 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). 30 31 Defoliation affects individual plants directly, by removing part of photosynthetically active 32 biomass (leaves and stems) or indirectly, through canopy opening and subsequent decrease in 33 aboveground competition (Sala et al., 1986; Richards, 1993; Bakker et al., 2003; Bonis et al., 34 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 35 (McNaughton, 1983; Maschinski and Whitham, 1989), an essential mechanism of tolerance to 36 37 grazing (Briske, 1996; Stowe et al., 2000). Considering grazing as a filter over the local 38 species pool, it generally favours plants capable of rapid regrowth after defoliation enabling 39 the recovery of leaf area and the resumption of photosynthetic activity (Fulkerson and 40 Donaghy, 2001).

41 Regarding the source of carbon, leaf regrowth after defoliation generally takes place into two 42 phases. First, defoliation is followed by a transient time lag, during which stored 43 carbohydrates are mobilized, a major mechanism that enables the plant to recover from losses 44 of photosynthetic tissues (Richards, 1993; Morvan-Bertrand et al., 1999a; Morvan-Bertrand et 45 al., 1999b; Schnyder and de Visser, 1999). Second, photosynthetic activity of newly produced 46 tissues becomes the main source of assimilates, supporting growth and replenishment of 47 reserve pools (Richards, 1993; de Visser et al., 1997; Morvan-Bertrand et al., 1999b). 48 Consequently, tolerance to grazing, which depends on enhanced short-term ability of leaf area 49 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 50 51 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 52 53 grazing may corresponds to severe and/or frequent defoliation events, with respectively large 54 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 55 grazing intensity by which reserve pools may be substantially depleted (Beaulieu et al., 1997; 56 57 Kleijn et al., 2005). Reserve making is considered to be costly, as it diverts resources from 58 potential growth, decreasing growth rate and leading to smaller and less competitive plants 59 (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 60 61 environments where their benefits outweigh these costs (Karban and Baldwin, 1997). In 62 particular, the capacities to constitute large pools of reserves available before the beginning of 63 a grazing season and to quickly remobilize and replenish these pools after defoliation (Lee et 64 al., 2010) are both expected to enable plants to cope with intensive grazing.

65 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; 66 Suzuki and Hutchings, 1997; Klimeš and Klimešová, 2002; Kavanová and Gloser, 2005), but 67 68 considering a wide range of species, the highest levels are found in leaves (Martínez-Vilalta et 69 al., 2016). In particular, grasses store large amounts of carbohydrates in tiller bases, which is 70 composed of elongating leaves enclosed in sheaths of mature leaves (Morvan-Bertrand et al., 71 1999a; Morvan-Bertrand et al., 1999b). Carbohydrates contained in tiller bases are mobilized rapidly after defoliation and, due to their close proximity to the leaf growth zone, they are 72 73 likely to play a key role in compensatory growth (Morvan-Bertrand et al., 2001). Carbon 74 reserves, mainly stored as non-structural carbohydrates (NSCs), have indeed been shown to 75 be of great importance in regrowth after defoliation. Starch constitutes the main form of long-76 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 77 78 compounds of carbon storage in vegetative parts in most of C3 grasses from temperate areas 79 (Pollock and Cairns, 1991). As the main extracellular form of NSCs, sucrose (non-reactive 80 disaccharide) is involved in source-sink carbon transport (Salerno and Curatti, 2003) and 81 might act as a signalling compound in response to environmental cues (Hawker, 1985; Rosa et 82 al., 2009). Sucrose represents the dominant form of carbohydrate storage (Hawker, 1985) in 83 some species, such as sugar beet (Beta vulgaris, Chenopodiaceae) or sugar cane (Saccharum 84 sp., Poaceae), and can also constitute a major form of carbon reserves in vegetative tissues of 85 temperate C3 grasses beside fructans (Chatterton et al., 1989).

86 The content and composition of carbon reserves can vary at several temporal scales from the 87 day to the year. In temperate climates with seasonal variations, numerous studies reported 88 annual dynamics, with the highest pools of reserves recorded in late summer or fall (e.g., 89 Pollock and Cairns, 1991; Beaulieu et al., 1997; Kleijn et al., 2005; Asaeda et al., 2006; 90 Janeček et al., 2011; Baptist et al., 2013; Benot et al., 2013b; Janeček et al., 2015), after a 91 period of slow growth or leaf senescence and nutrient recycling (Chapin et al., 1990). Then, 92 reserve pools are generally reported to decrease during winter and during the early phases of 93 the growing period in spring, certainly due to the combination of reduced photosynthesis and 94 of reserve remobilisation to support the maintenance of metabolism during winter and the 95 spring regrowth outbreak (Bloom et al., 1985; Fulkerson and Donaghy, 2001). In most 96 grasslands from temperate climate and, more particulary in commonly grazed grasslands, 97 which are widespread along the Atlantic coast, the grazing season generally expands from 98 spring to autumn i.e., during the period of resource storage in plants. The seasonal processes 99 of resource storage are thus likely to be altered by grazing.

100 The present study aimed at studying how the pattern of formation and replenishment of 101 carbohydrate reserves in tiller bases is modulated by the grazing intensity. More precisely, we 102 formulated the two following hypotheses. H1 (hypothesis of efficient formation of reserves): 103 plant species tolerance to grazing is expected to rely on large pools of reserves available 104 before the beginning of a grazing season. Thus, for plant species tolerant to grazing, the 105 concentrations of carbohydrate reserves before the beginning of a grazing season are expected 106 to be higher under intensive than under moderate grazing. H2 (hypothesis of efficient 107 replenishment of reserves): plant species tolerance to grazing is expected to rely on efficient 108 reserve replenishment between two consecutive defoliation events. Thus, regardless of the 109 grazing intensity, plant species tolerant to grazing are expected to be able to quickly restore 110 reserve pools, and to show equivalent concentrations of carbohydrate reserves at the end of 111 the grazing season whatever the grazing intensity.

112 We tested these hypotheses for five Poaceae species from a grassland traditionally grazed for 113 6 months a year from early spring (April) to early fall (October), where cattle grazing has 114 generated mosaics of vegetation patches (Marion et al., 2010). Plants were collected in 115 vegetation patches submitted to two contrasting cattle grazing intensities (moderate and 116 intensive) for about 15 years, at two sampling dates: just after a 6-month grazing season 117 (October) and just before the beginning of the following grazing season (April). NSC 118 concentrations (starch, fructans, sucrose, glucose and fructose) were measured in tiller bases 119 of individual plants.

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125 Material and methods

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127 Study site

128 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, 129 46° 28'N; 1° 13'W). This grassland has been reclaimed from the sea from the Xth century 130 onwards. A topographical gradient consisting of depressions, intermediate slopes and higher-131 132 level flats (with a maximum altitudinal range of 0.7 m) occurs repeatedly within the grassland 133 (Amiaud et al., 1998) and discriminates three plant communities, depending on flooding 134 duration: the hygrophilous, meso-hygrophilous and mesophilous communities, respectively 135 (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-136 137 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 138 139 Marais poitevin (Amiaud et al., 1998).

140 This grassland has traditionally been grazed by cattle and horses from April to October, a 141 grazing season occurring during spring and summer (Amiaud et al., 1998). An experimental 142 design consisting in several paddocks has been set up in this grassland and has enabled to 143 control the grazing intensity (from no to intensive grazing) and herbivore type (cattle and/or 144 horses) since 1995 (Loucougaray et al., 2004; Rossignol et al., 2006). The present study only 145 focused on vegetation grazed by cattle. Since 1995, cattle grazing has generated mosaics of 146 vegetation patches submitted to contrasting grazing intensities, with stabilised composition 147 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. 148 about 685 kg ha⁻¹) and 4 heifers.ha⁻¹ (*i.e.* about 1370 kg ha⁻¹, Ménard *et al.*, 2002) respectively 149

(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).

154

155 Study species

We selected five common perennial Poaceae species of the mesophilous plant community that 156 157 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. 158 159 are forage species, frequently found in meadows and pastures (Grime et al., 2007), with a 160 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., 161 162 2004). They are all tussock-forming and do not present organs potentially specialized in 163 storage (e.g. tubers, rhizomes). A. stolonifera generally produces long creeping stems 164 (stolons) but displays an important morphological plasticity. This species was included in the 165 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 166 167 main storage organ for the five study species.

168 Although present under both grazing intensities, study species vary in abundance in the 169 vegetation. In June 2008 *i.e.*, during the biomass peak, we estimated these variations by 170 recording the relative cover of the five species in ten $0.5 \text{ m} \times 0.5 \text{ m}$ plots randomly located 171 within the mesophilous community in G+ and G++ vegetation patches (Table 1). Given the 172 co-occurrence of the study species at very small scales (i.e., even less than 0.25 m², personal 173 observations), these variations in abundance are very little likely to be caused by selective 174 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 abundancelikely reflect differences in tolerance to grazing.

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178 Plant collection and material

179 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 180 181 G++ patches), just after a 6-month grazing season (October 2008) and two days before the 182 following grazing season (April 2009). In October, plants were collected about ten days after 183 the heifers had left the pastures and showed traces of leaf damage, indicating former grazing. 184 This time lag from the end of the grazing season was chosen to ensure that the phase of 185 reserve remobilization was completed and reserve replenishment could have been initiated (Richards, 1993; Morvan-Bertrand et al., 1999a). In contrast, plants collected in April were 186 187 intact. At both dates, only non-flowering tillers were selected. Tiller collection lasted for two 188 consecutive days, from 10am to 4pm. We paid attention to pick up tillers of all species all day 189 long, in order to capture the daily variation of carbohydrate content within a species (Chapin 190 et al., 1990; Shewmaker et al., 2006; Smith and Stitt, 2007; Haydon et al., 2011). Each 191 sample consisting in a few connected tillers was picked up with a knife, carefully washed and 192 immediately frozen in liquid nitrogen. From the field to the lab, samples were transported in a 193 freezer and kept at -80°C until they were freeze-dried. For each sample, a tiller was randomly 194 selected and freeze-dried plants were dissected in order to separate tiller basis, composed of 195 mature leaf sheaths and enclosed elongating leaves, from the rest of the tiller (*i.e.*, leaf blades 196 and roots). As fructans accumulate mainly in the first centimeters of the tiller bases (Pollock 197 and Cairns, 1991; Morvan-Bertrand et al., 2001) and as tillers might differ in size, we 198 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. 199

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.

202

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

204 Twenty $\pm 1 \text{ mg}$ dry mass (DM) of powder were weighted. Water soluble carbohydrates (WSC: 205 fructans, sucrose, glucose and fructose) were extracted from this powder in 80% ethanol at 206 80°C for 15 min. After ethanol extraction, the sample was centrifuged at 10,000 g for 10 min. 207 The supernatant was preserved on ice and 2 mL of water were added to the pellet. The tube 208 contents were mixed and incubated 15 min at 60°C. After the first aqueous extraction, the 209 sample was centrifuged at 10,000 g for 10 min. The supernatant was preserved on ice and the 210 aqueous extraction was repeated once with the pellet. The three supernatants were pooled, 211 evaporated to dryness under vacuum and the residue was dissolved into 450 μ L of ultra-pure 212 water. Aliquots of WSC extract (100 µL) were passed through minicolumns (Mobicols from 213 MoBITec, Göttingen, Germany) containing 150 µL of anion exchange resin (Amberlite CG-214 400 II, formate form, Fluka, Buchs, Switzerland) and 250 µL of cation exchange resin 215 (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 216 217 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 precolumn 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 drymass of tissue powder.

226

227 Starch measurement

228 Starch insoluble pellet remaining after WSC extraction was dissolved under agitation into 229 200 µL of dimethylsulfoxide (DMSO) and 50 µL of HCl (8N) at 60°C for 30 min. After 230 centrifugation (10,000 g for 15 min), 200 µL of extract were dissolved into 500 µL of ultra-231 pure water added with 40 µL of NaOH (5M) and pH was adjusted to 4.5. Ultra-pure water 232 was then added up to 1mL. After decantation, starch content was measured using enzymatic 233 kits (Enzyplus® kit EZ0 942+ Starch, Raisio Diagnostics SpA, Rome, Italy). In short, starch 234 was degraded by amyloglucosidase into glucose, which was quantified through NADPH production by spectrophotometry at 340 nm (Sulmon et al., 2011). Starch concentration was 235 236 calculated as its mass divided by the dry mass of tissue powder.

237

238 Statistical analyses

239 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 240 241 grazing intensity modified these patterns, a model was carried out with the date, species and 242 grazing intensity as main effects. Then, in order to test for the effect of grazing intensity on 243 NSC concentrations just after and just before the beginning of a grazing season, models with 244 species and grazing intensity as main effects were carried out separately for October and 245 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 246 247 normality of the residuals. In cases where extreme values in the dataset limited the effect of 248 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, <u>http://www.R-project.org</u>) and post-hoc HSD test with the agricolae package (de Mendiburu, 2014).

255

256 **Results**

257 A general trend for higher NSC concentrations in October than in April was observed. 258 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, 259 significant date \times species interaction was recorded for all NSC but fructose ($F_{1,133} = 2.33$, P =260 0.06), a date \times grazing intensity interaction for all NSC but glucose ($F_{1,133} = 3.29, P = 0.07$) 261 and even a significant date \times species \times grazing intensity for fructose ($F_{4,133} = 2.69, P = 0.03$, 262 see Table 2 for complete results of the model). Starch was the least abundant carbohydrate 263 with concentrations in tiller bases lower than 3 mg g DM⁻¹ in October and even than 1 mg 264 g DM⁻¹ in April (Figure 1A, F). Fructan concentrations in tiller bases were largely higher than 265 that of the other NSCs, comprised on average between 80 and 400 mg g DM⁻¹ depending on 266 267 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).

282

283 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.

291

292 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 298 intensity modified the seasonal patterns of reserve formation. This seasonal variation in NSC 299 concentrations can be explained by a higher rate of mobilization than of photosynthetic 300 assimilation during winter and early spring. During winter, carbohydrate storage is likely to 301 decrease because of lower photosynthetic activity due to shorter day length and lower 302 incoming radiation and temperature (Fulkerson and Donaghy, 2001; Höglind et al., 2011). 303 Carbohydrate reserves can also be depleted during winter because reserves may sustain 304 energy demand during unfavourable conditions (Bloom et al., 1985). In addition, reserves 305 may be depleted by early-spring consumption before the grazing season in order to support 306 fast growth and tiller emergence (Chapin et al., 1990; Pollock and Cairns, 1991; Beaulieu et 307 al., 1997; Kleijn et al., 2005; Asaeda et al., 2006). Consequently, the decrease observed in the 308 present study in NSC concentrations and more particularly, in starch, fructan and even sucrose 309 concentrations, between October and April may be due both to reduced production and 310 remobilization.

311

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

313 April sampling was conducted to reflect carry-over effects of former grazing seasons. We 314 detected no significant effect of the grazing intensity (moderate versus intensive grazing) 315 formerly applied on the vegetation on starch concentration for three species (C. cristatus, 316 L. perenne and P. trivialis) and this concentration was lower under intensive than under 317 moderate grazing for two species (A. stolonifera and H. secalinum). By contrat, regardless of 318 the species, fructan and sucrose concentrations in tiller bases were significantly higher in 319 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 320 321 fructans suggest that both NSCs are involved in the delayed response to grazing intensity. For 322 the five study grass species, sucrose could thus represent an alternative form of carbohydrate 323 storage. Fulfilling transport function and being easily hydrolysable (Salerno and Curatti, 324 2003; Amiard et al., 2004), this disaccharide could represent a mobile and "ready to use" 325 source of carbon, potentially advantageous in a context of intensive grazing. Alternatively, 326 these higher sucrose concentrations for plants previously submitted to intensive grazing could 327 reflect a higher rate of photosynthetic assimilation during early spring under intensive 328 grazing.

Several studies have already reported higher NSC concentrations in roots or storage organs of 329 330 grass species in response to mowing, either by the end of the growing season (Bartoš et al., 331 2011) or, as in our study, at the beginning of the following growing season (Baptist et al., 332 2013; Benot et al., 2013b). However, while NSC concentrations provide a reliable insight in 333 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 334 335 the tussock level must be considered distinctly (Bartoš et al., 2011; Janeček et al., 2015) as 336 they may reflect different effects of defoliation on NSC pools. Similar NSC concentrations in 337 tillers may hide differences in NSC quantities either at the tiller level (linked to differences in 338 tiller biomass) or at the tussock level (linked to differences in tiller number). Several studies 339 indeed reported negative effects of experimental defoliation or mowing on tiller biomass or 340 tiller number at the tussock level (e.g., Donaghy and Fulkerson, 1998; Cullen et al., 2006 341 Bartoš et al., 2011; Baptist et al., 2013; Benot et al., 2013b). Similarly, we cannot exclude that 342 the observed inter-specific differences in NSC concentrations can be correlated to inter-343 specific differences in the number of tillers per tussock, as suggested by previous 344 measurements in undisturbed experimental garden conditions for the five study species (see 345 Fig. S2 in Benot et al., 2013a) or even in tiller turnover rates (as observed in A. stolonifera 346 and L. perenne by Bullock et al., 1994). Although we did not record tiller dynamics in the 347 field, the absence of correlation between the number of tillers per tussock and experimental 348 defoliation severity previously observed for the five study species (Benot et al., 2013a) 349 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 350 351 grazing period as in the present study) on tiller densities for L. perenne and A. stolonifera, but 352 these results indeed masked summer grazing effects on tiller turnover rates, suggesting 353 increased tissue renewing under intense grazing. As advocated by Bartoš et al. (2011), 354 considering both concentrations and quantities at the tiller as well as the tussock levels could 355 provide deeper understanding of grazing effects on carbon economy and plant response to aboveground biomass loss. 356

357

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

359 October sampling occurred ten days after the end of the grazing season. At that date, the 360 composition and concentrations of NSC pools in tiller bases were primarily constrained by the 361 species rather than by grazing intensity. Such inter-specific differences in carbohydrate 362 allocation to storage have already been demonstrated for sets of grasses as well as non-grass 363 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-364 specific differences in leaf turnover rates and energy requirements for tissue renewing (Cullen 365 366 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 367 efficiently restore a functional pool of reserves, regardless of the grazing intensity. Yet, 368 369 intensive grazing is likely to generate more severe and/or frequent defoliation than moderate 370 grazing, which could deplete more completely fructan pools (Beaulieu et al., 1997; Fulkerson 371 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 372

higher increase of fructan synthesizing enzyme activities during the period of reserve 373 replenishment. In line with these findings, our results suggest that plants growing under 374 intensive grazing may efficiently synthesize NSC reserves between two consecutive 375 376 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 support the hypothesis of both efficient reserve remobilization and, thereafter, efficient 382 383 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 concentration through animal saliva. Finally, grazing might also modify the available 397

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.

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405 **Conclusion and perspectives**

406 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 407 408 highlighted that carbon storage may in fact be more complex than we do believe (Martínez-409 Vilalta et al., 2016; Klimešová et al., 2017). Pathways and organs (e.g., source - sinks 410 relationships) involved in carbon storage and remobilization in response to disturbance, as 411 well as their timing and the effect of environmental conditions still remain to be clearly 412 identified. As expected, our study showed that the concentrations of fructans and sucrose were 413 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 414 415 results thus suggest that the composition and the concentrations of NSCs in tiller bases should 416 be considered to better understand plant responses to grazing. Although field experiments may 417 suffer from some limitations, for instance due to little spatial or temporal repetitions, we are 418 convinced that they will help building a stronger understanding of plant carbon economy.

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423 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.

428

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Table 1 Mean (\pm 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. stolonife	era P. trivi	P. trivialis		cristatus	H. secalinum		L. perenne	
Cover in G+ (%)	8.7 ± 3.5	5 10.5 ±	10.5 ± 2.2		5.2 ± 2.2	7.49 ± 2.2		0.4 ± 0.3	
Cover in G++ (%)	5.0 ± 2.7	9.4 ± 2	9.4 ± 2.6		.0 ± 3.51	12.3 ± 2.8		17.7 ± 1.6	
Grazing tolerance*	9	6	6		7	7		8	
* ranging fr	rom 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 nonstructural 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	Р	F	Р	F	Р	F	Р	F	Р
		<i>n</i> = <i>158</i>		<i>n</i> = 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 \times Sp.	4	7.98	<0.001	2.95	0.023	3.07	0.019	3.28	0.013	2.33	0.059
Date \times Graz.	1	9.74	0.002	5.21	0.024	11.37	<0.001	3.29	0.072	9.49	0.002
Sp. \times Graz.	4	3.76	0.006	1.53	0.20	1.88	0.12	1.51	0.20	0.40	0.81
Date \times Sp. \times 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 (\pm 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.