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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,
3 non-structural carbohydrates (NSC) contained in tiller bases constitute a major substrate for
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 grazing-
6 tolerant grass species (*Agrostis stolonifera*, *Cynosurus cristatus*, *Hordeum secalinum*, *Lolium*
7 *perenne* and *Poa trivialis*) and collected plants in a grassland subjected to two cattle grazing
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.

16

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

19

20 **Abbreviations:** DM: dry mass, HPLC: high performance liquid chromatography, NSC: non-
21 structural 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
30 through the consumption of above-ground biomass *i.e.*, defoliation (Kohler *et al.*, 2004).
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
35 compensatory growth, mainly based on the plant ability to regrow after damage
36 (McNaughton, 1983; Maschinski and Whitham, 1989), an essential mechanism of tolerance to
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).

50 Grazing occurs within a range of intensity: even in semi-natural grasslands the grazing
51 pressure may vary from extensive to intensive, due to management choice but also to local
52 heterogeneity in the animal choice (Marion *et al.*, 2010; Loucougaray *et al.*, 2004). Intensive
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
55 replenishment. Frequency between two consecutive defoliation events is a dimension of
56 grazing intensity by which reserve pools may be substantially depleted (Beaulieu *et al.*, 1997;
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
60 making and remobilization for compensatory growth are expected to be selected for in
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
66 of plant organs such as roots, perennating organs and stems (van der Meijden *et al.*, 1988;
67 Suzuki and Hutchings, 1997; Klimeš and Klimešová, 2002; Kavanová and Gloser, 2005), but
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
72 rapidly after defoliation and, due to their close proximity to the leaf growth zone, they are
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
77 carbohydrate stored in seeds. By contrast, fructans (polymers of fructose) are the predominant
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 particularly 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.

120

121

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123

124

125 **Material and methods**

126

127 **Study site**

128 This study was carried out on the most abundant species in the vegetation in a commonly-own
129 grassland of Magnils–Reigniers (250 ha-large), in Marais poitevin (French Atlantic coast,
130 46° 28'N; 1° 13'W). This grassland has been reclaimed from the sea from the Xth century
131 onwards. A topographical gradient consisting of depressions, intermediate slopes and higher-
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
136 (Albrecht *et al.*, 1997), this study was conducted on species collected within the never-
137 flooded mesophilous community, to avoid confounding effects of the flooding regime on
138 fructan concentrations. The soil on flats are sodisols characteristic of pasture grasslands in the
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
148 intensively (G++) grazed patches within 1ha-large paddocks grazed by 2 heifers.ha⁻¹ (*i.e.*
149 about 685 kg ha⁻¹) and 4 heifers.ha⁻¹ (*i.e.* about 1370 kg ha⁻¹, Ménard *et al.*, 2002) respectively

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

154

155 **Study species**

156 We selected five common perennial Poaceae species of the mesophilous plant community that
157 are present under both moderate and intensive grazing (Table 1). *Agrostis stolonifera* L.,
158 *Cynosurus cristatus* L., *Hordeum secalinum* Schreb., *Lolium perenne* L. and *Poa trivialis* L.
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
161 from the TRY database, Kattge *et al.*, 2011; original source: BiolFlor database, Kühn *et al.*,
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
166 only a few or short stolons (personal observations). Thus tiller bases were considered as the
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 m × 0.5 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

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

177

178 **Plant collection and material**

179 Eight non-flowering individuals of each species, composed of several connected tillers, were
180 randomly collected within the mesophilous community under both grazing intensities (G+ and
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
186 (Richards, 1993; Morvan-Bertrand *et al.*, 1999a). In contrast, plants collected in April were
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
199 carbon resources stored and potentially available for biomass compensation per biomass unit.

200 The tiller basis was thus cut either at 3 cm above the rooting point or under the ligule of the
201 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 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 μ L) were passed through minicolumns (Mobicols from
213 MoBITec, Göttingen, Germany) containing 150 μ L of anion exchange resin (Amberlite CG-
214 400 II, formate form, Fluka, Buchs, Switzerland) and 250 μ L of cation exchange resin
215 (Dowex 50W X8-400, H⁺ form, Sigma, Saint-Louis, MO, USA) to remove charged
216 compounds. Between these two resins, 80 μ L of PVPP (polyvinylpolypyrrolidone) were
217 added to eliminate lipids, pigments and phenolic compounds.

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

224 The concentration of each NSC was calculated as the carbohydrate mass divided by the dry
225 mass 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
235 production by spectrophotometry at 340 nm (Sulmon *et al.*, 2011). Starch concentration was
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
240 order to check for seasonal patterns of NSC concentrations in tiller bases, and to test whether
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
246 either log-transformed or arcsine square root-transformed to improve homoscedasticity and
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

249 with complete ones to evaluate the robustness of the results. Only complete models based on
250 all data were kept for the study. Because of errors in laboratory measurements, a few
251 replicates were lost (see Table S1 for final number of replicates per date \times species \times grazing
252 intensity interactions). Statistical analyses were carried out with the R software version 3.1.1
253 (R Development Core Team, 2014, <http://www.R-project.org>) and post-hoc HSD test with the
254 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
259 2, Figure 1). While the effect of the sampling date was significant for all of the five NSCs,
260 significant date \times species interaction was recorded for all NSC but fructose ($F_{1,133} = 2.33$, $P =$
261 0.06), a date \times grazing intensity interaction for all NSC but glucose ($F_{1,133} = 3.29$, $P = 0.07$)
262 and even a significant date \times species \times grazing intensity for fructose ($F_{4,133} = 2.69$, $P = 0.03$,
263 see Table 2 for complete results of the model). Starch was the least abundant carbohydrate
264 with concentrations in tiller bases lower than 3 mg g DM^{-1} in October and even than 1 mg
265 g DM^{-1} in April (Figure 1A, F). Fructan concentrations in tiller bases were largely higher than
266 that of the other NSCs, comprised on average between 80 and 400 mg g DM^{-1} depending on
267 the species and the date (Figure 1B, G).

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

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

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

282

283 **Discussion**

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

291

292 **Seasonal variations in NCS concentrations**

293 We found that concentrations of reserve carbohydrates were globally higher at the end of
294 summer than in early spring, providing further support to a commonly observed seasonal
295 pattern (e.g., Steen and Larsson, 1986; Pollock and Cairns, 1991; Beaulieu *et al.*, 1997;
296 Klimešová and Klimeš, 2003; Janeček *et al.*, 2011; Baptist *et al.*, 2013). However, this date
297 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 contrast, 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
320 plants submitted to intensive grazing (H1). The similar patterns obtained for sucrose and
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.

329 Several studies have already reported higher NSC concentrations in roots or storage organs of
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
334 effects of grazing (i) on either NSC concentrations or quantities and (ii) at either the tiller or
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.
350 Similarly, Bullock *et al.* (1994) showed no effect of spring and summer grazing (ie., same
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
356 aboveground biomass loss.

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
364 organs (e.g., Janeček and Klimešová 2014). They may be influenced, at least partly, by inter-
365 specific differences in leaf turnover rates and energy requirements for tissue renewing (Cullen
366 *et al.*, 2006, Gastal *et al.*, 2010). The absence of difference in NSC concentrations (except
367 fructose) between moderate and intensive grazing suggested that plants were able to
368 efficiently restore a functional pool of reserves, regardless of the grazing intensity. Yet,
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
372 *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

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

404

405 **Conclusion and perspectives**

406 The mechanisms of reserve storage regulation and remobilization in response to defoliation
407 have seemed to be clearly understood for long (see Introduction). Yet, two recent studies
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
414 intensity did not affect NSC concentrations, except for fructose, in October (H2). These
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.

419

420

421

422

423 **Authors' contribution**

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

428

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433

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439

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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).

	<i>A. stolonifera</i>	<i>P. trivialis</i>	<i>C. cristatus</i>	<i>H. secalinum</i>	<i>L. perenne</i>
Cover in G+ (%)	8.7 \pm 3.5	10.5 \pm 2.2	16.2 \pm 2.2	7.49 \pm 2.2	0.4 \pm 0.3
Cover in G++ (%)	5.0 \pm 2.7	9.4 \pm 2.6	17.0 \pm 3.51	12.3 \pm 2.8	17.7 \pm 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
	<i>n</i> = 158		<i>n</i> = 153		<i>n</i> = 153		<i>n</i> = 153		<i>n</i> = 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		

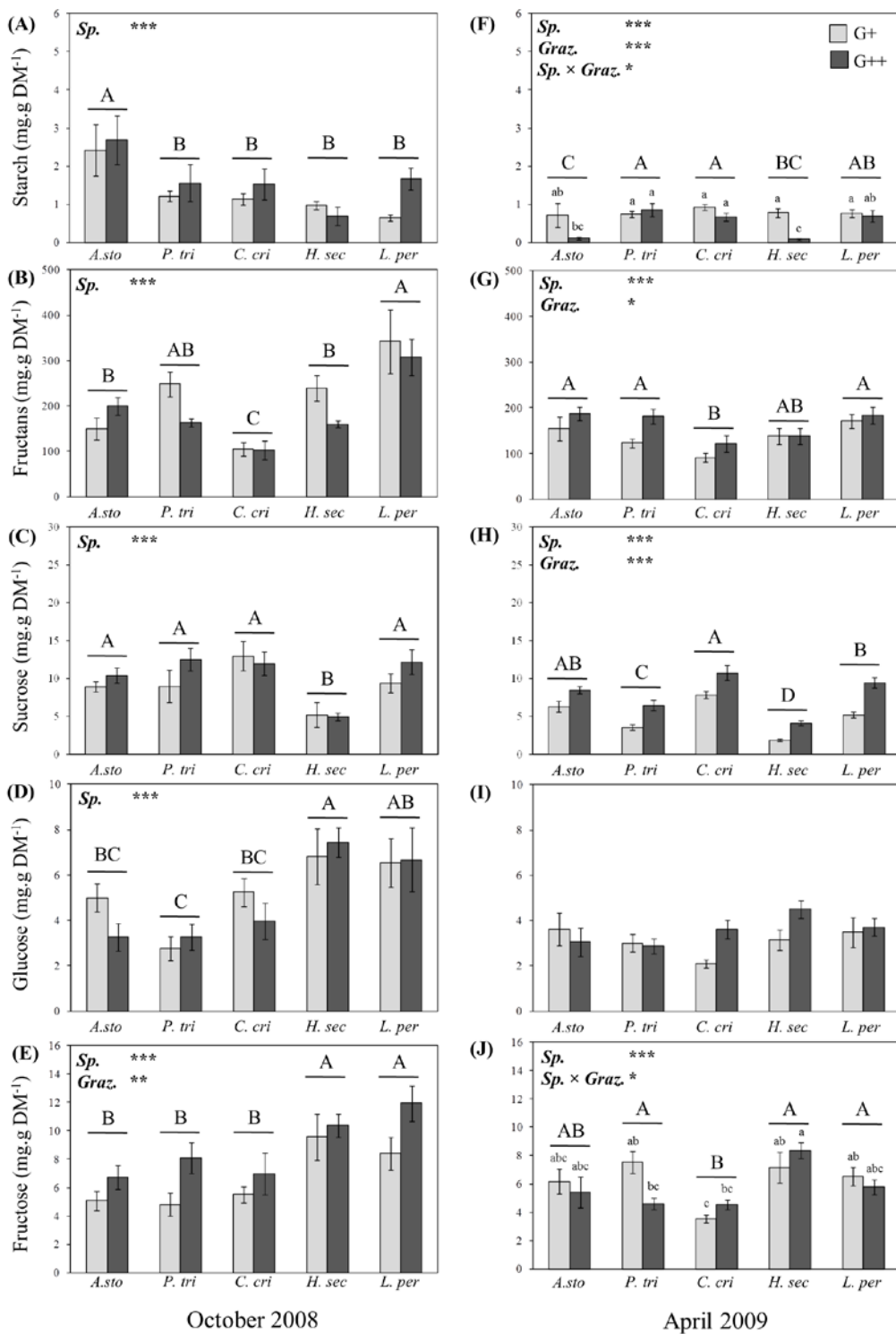


Figure 1 Mean (\pm SE) non-structural carbohydrate (NSC) concentrations (mg g DM⁻¹) in tiller bases in October 2008 (A-E) and April 2009 (F-J). Species are ranged according to increasing difference in relative cover between G+ and G++ (see Table 1). Only significant effects of species (*Sp.*), grazing

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.