High sugar grasses – harnessing the benefits of new cultivars through growth management

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Abstract

Perennial ryegrass cultivars with high levels of water-soluble carbohydrates (WSCs) have been proposed as a means to increase animal performance and nitrogen use efficiency in pasture-based animal production systems, with consequent environmental benefits. But this depends on a sufficient elevation of WSC in leaves. A gene x environment interaction (G x E) in the expression of the high sugar (HS) trait has been shown previously, with WSCs measured at a single stage in regrowth. Here we report a controlled environment study of how WSCs change over the duration of regrowth (cf defoliation management, M) in 5 ryegrass cultivars, under two temperature regimes. Overall, the UK cultivar 'AberDart', and a breeding line 'PG1113', maintained significantly higher levels of WSCs in blades, than UK and New Zealand control cultivars. This was true both in a 20°C/10°C (day/ night) temperature growth regime, where WSCs decreased substantially following defoliation before recovering to pre-defoliation levels, and in a 'colder' (10°C/10°C) regime, where the decrease in WSCs was less, notably in 'PG1113'. Any complexity in the change in WSCs during regrowth, and any gene x management (G x M) or G x E interaction, introduces uncertainty in assessing new plant traits under uncontrolled field conditions. This may go some way to explain some inconsistency in expression seen in field trials. Our results show simple guidelines for defoliation management are sufficient to ensure WSCs are high at the time of harvest. We also propose a method for presenting data on plant chemical composition that reveals reductions in fibre (and less so in crude protein) seen in HSGs may not be a fundamental change in plant structural composition, but largely a simple effect of 'dilution'.

Keywords: regrowth interval, defoliation, *Lolium perenne*, water soluble carbohydrates, plant composition

Introduction

In pasture fed ruminants, a large proportion (typically 50-70%) of the nitrogen (N) ingested in the diet is degraded by rumen microbes and excreted in urine as urea (Beever *et al*. 1986). This can reduce animal productivity during growth stages requiring high protein supply to the ruminant. It also has environmental implications, as urine N deposited on soil is a source of greenhouse gas emissions (as nitrous oxide), and increased nitrate leaching. One strategy proposed by forage breeders to recapture proteins degraded in the rumen into microbial protein, available in the small intestine, is to increase the energy supply to the rumen by elevating water soluble carbohydrates (WSCs) in grazed leaf blades (Turner *et al*. 2006).

There have been conflicting conclusions as to the efficacy of high sugar grasses (HSGs) to boost animal productivity and/or improve ruminal nitrogen use efficiency (NUE), but proof of concept was demonstrated in two recent reviews by combining data from multiple sources (Edwards *et al*. 2007a, b). The reviews suggested that to harness the benefits of HSGs, in improving NUE, depended on a sufficient elevation of WSC: crude protein (CP) ratio, from 0.6 to c. 1.2-1.5. A far greater elevation of sugars is therefore necessary in productive forage based systems (with high N content of herbage, >3% of DM) to achieve the same NUE (low urinary N loss) seen on low herbage N diets. In United Kingdom (UK) and European (EU) field trials, the HS trait was seen to be consistently expressed, with the concentrations of WSC in the cultivar 'AberDart' being c. 10-14% higher than in 'Fennema' (the EU control) (Halling *et al*. 2004). However, the amount of sugar harvested per ha (dry matter yield x concentration) was not greater. Initial field trials in New Zealand (NZ), using EU cultivars, revealed a gene x temperature (G x E) interaction that explained some concerns over a (then) less consistent expression of the trait in NZ compared to EU controls (Parsons *et al*. 2004). But sugar levels are also affected by defoliation management (M). Storage sugars, such as the fructose polymers (fructans) found mainly in leaf sheaths, are not only valuable as a long-term seasonal energy store in cool-temperate grasses, but can also be critical for regrowth following defoliation (Pollock & Cairns 1991; Pavis *et al*. 2001). Fructans may be consumed at early stages of regrowth after severe defoliation, when plant photosynthetic carbon/energy supply has been substantially reduced (Morvan-Bertrand *et al*. 2001). They are remobilised in the stubble, and transported

Figure 1 Biomass and amounts of WSCs per plant removed as leaf blades at the outset of regrowth (t = 0). Graphs show effects of cultivar: see (a) and (d); temperature: see (b) and (e), and the G x E interaction of cultivar with temperature: see (c) and (f), on biomass: see (a), (b), and (c) and amounts of total WSCs: see (d), (e), and (f) in plants grown at 10°C/10°C for 31 days or at 20°C/10°C for 21 days, and so at the outset of the subsequent regrowth. Grey bar areas indicate HMW, white bar areas LMW WSCs. Different letters indicate significantly different means according to THD test; stars indicate P-values (*** P<0.001, ** P<0.01, * P<0.05). Shown are untransformed means and standard errors (bars) of untransformed means.

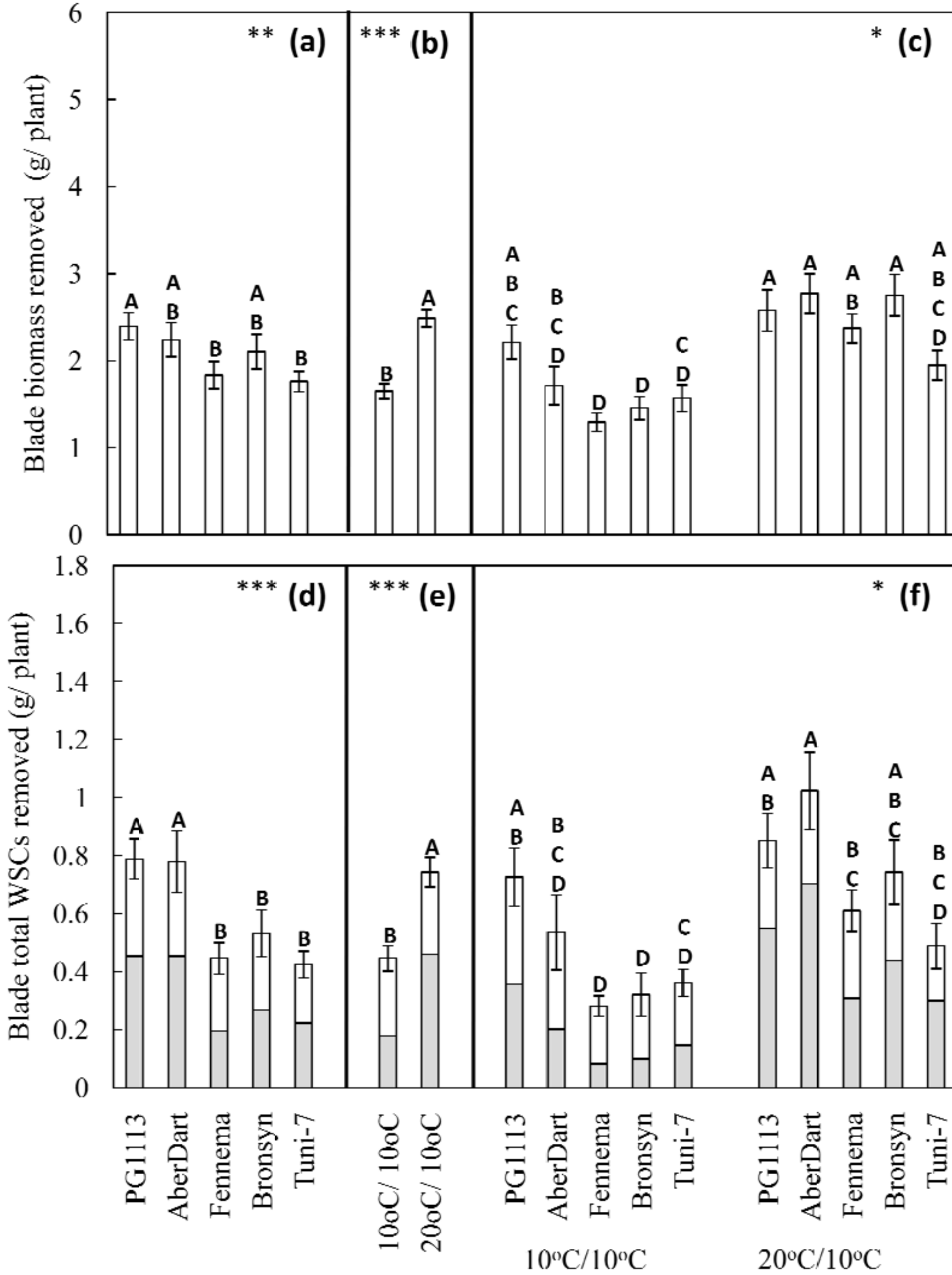


Figure 2 Biomass and amounts of WSCs per plant remaining in stubble at the outset of regrowth (t = 0). Graphs show effects of cultivar: see (a) and (d); temperature: see(b) and (e), and the G x E interaction of cultivar with temperature: see (c) and (f) on biomass: see (a), (b) and (c) and amounts of total WSCs: see (d), (e) and (f) in plants grown at 10°C/10°C for 31 days or at 20°C/10°C for 21 days at the outset of the subsequent regrowth. Grey bar areas indicate HMW, white bar areas LMW WSCs. Different letters indicate significantly different means according to THD test; *** = P<0.001. Shown are untransformed means and standard errors (bars) of untransformed means.

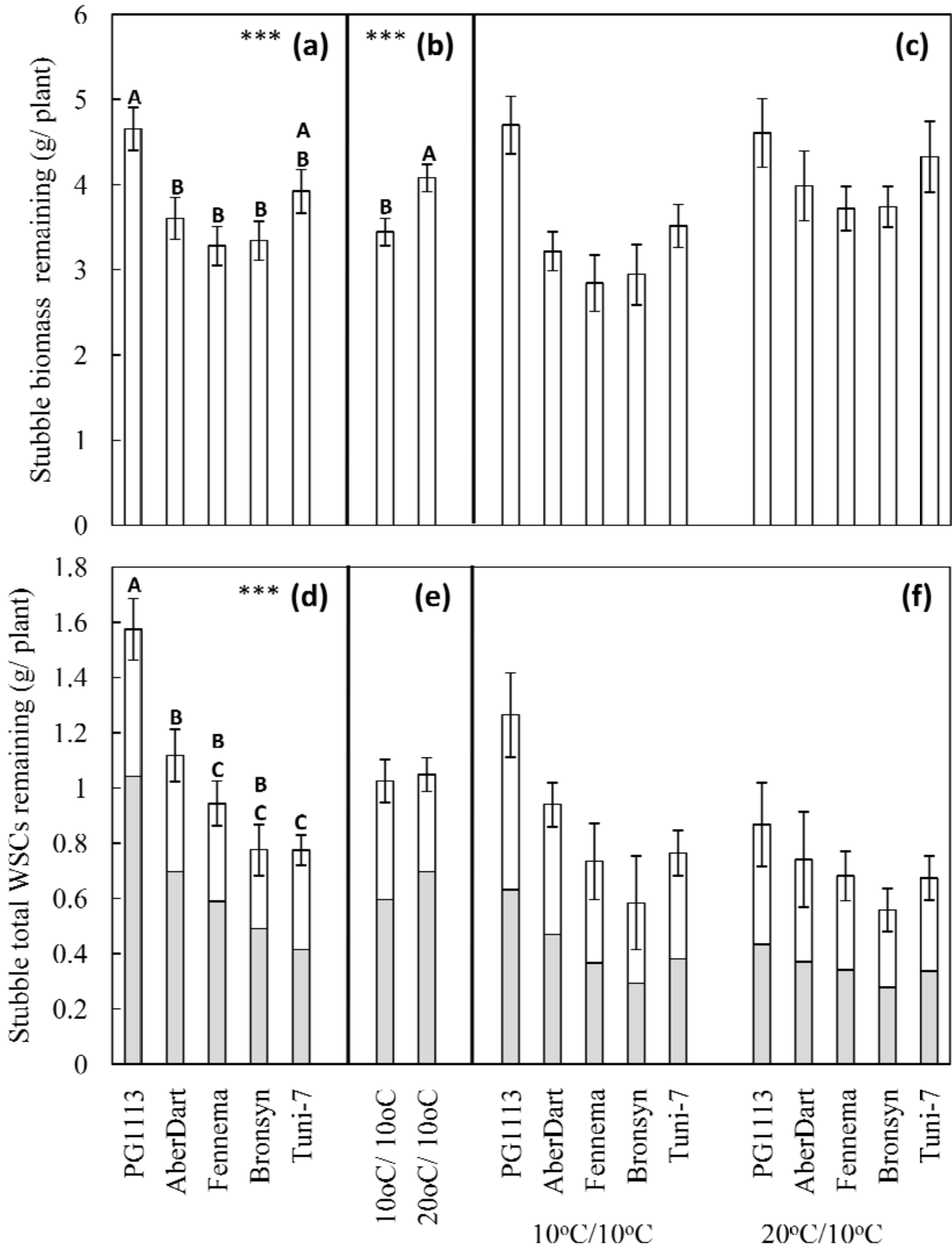
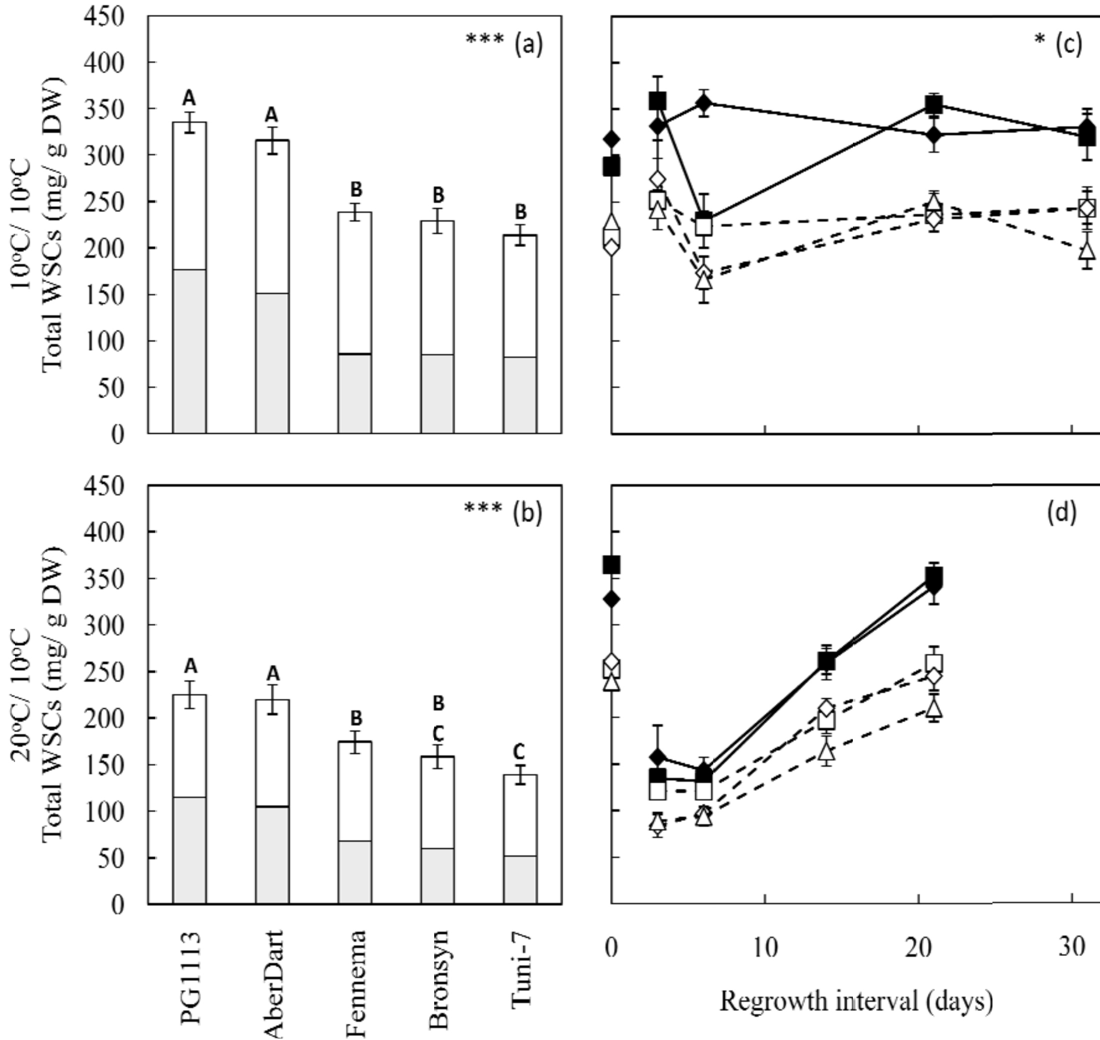


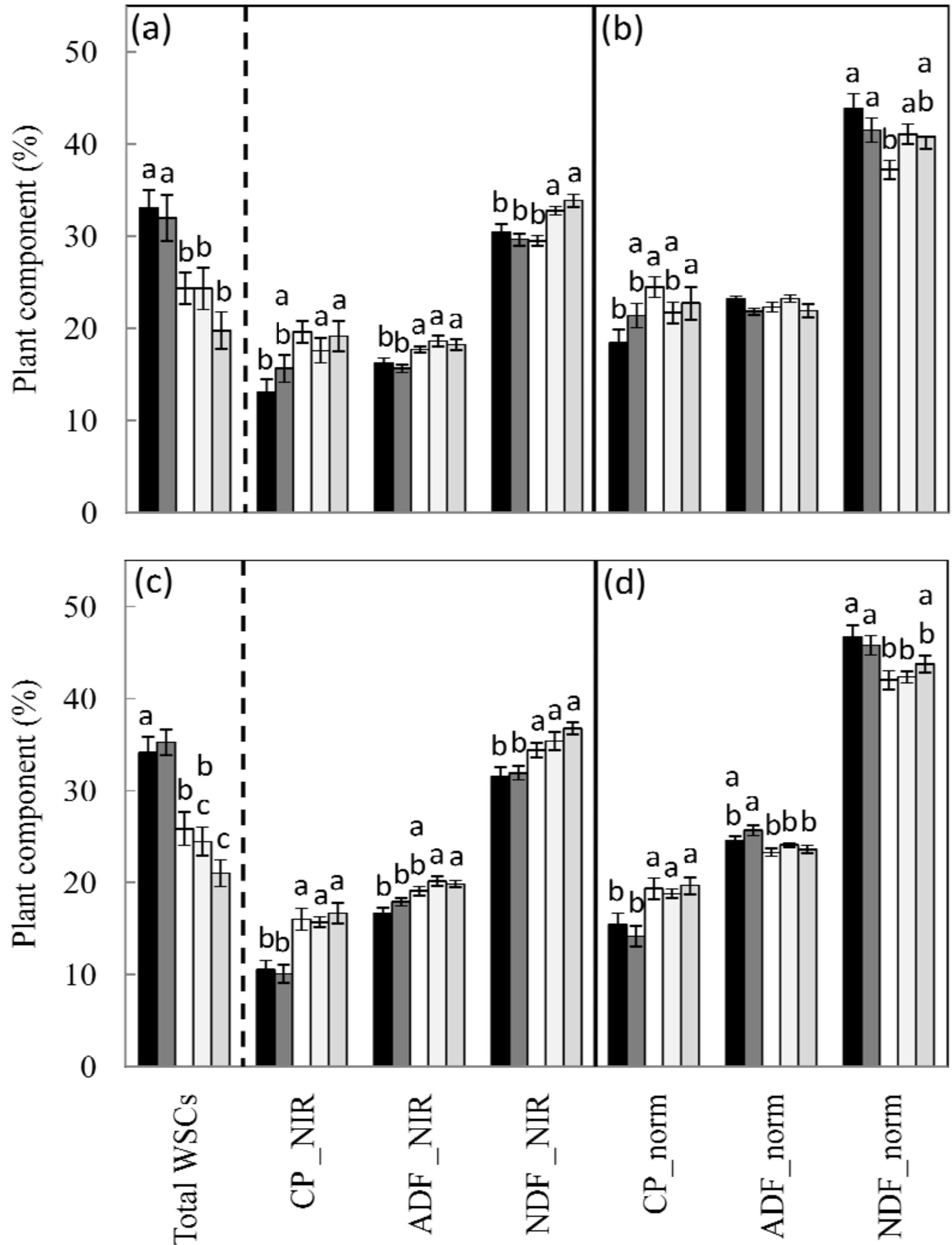
Figure 3 Analysis (by ANOVA) of the changes in concentrations of WSCs in leaf blades during regrowth. The main effects of cultivar: see (a) and (b), and the patterns of change (G x M interaction) in concentrations during regrowth: see (c) and (d) are shown for the 10°C/10°C (a), (c) and 20°C/10°C (b), (d) treatments. Grey bar areas in a, b indicate HMW WSCs and white bar areas LMW WSCs. *** indicate P< 0.001; * indicates P<0.05. Filled diamonds – 'PG1113' (P), filled squares – 'AberDart' (A), open squares – 'Fennema' (F), open diamonds – 'Bronsyn' (B), open triangles – 'Tuni-7' (T). Symbols on y-axis at 0 days (c, d) represent total WSC *concentrations* in the blades removed before the regrowth experiment (at t = 0). Different letters indicate significantly different means according to THD test of ANOVA; shown are untransformed means and standard errors (bars) of untransformed means.



to meristems to supply elongating blades with carbon skeletons and energy until these blades emerge from the sheaths and restore a positive net photosynthetic carbon/ energy gain. Fructan content in mature blades is usually lower than in sheaths, and only reaches higher levels, switching from supply to storage, as the blade matures.

This implies there will be an interplay between temperature (that affects meristem activity, growth rate and the balance of demand and supply of sugars) and regrowth duration. In the present study, we proposed that this exposes a possible conflict: a) low temperatures *per se* are recognised to lead to higher sugar concentrations in leaf blades (Edwards *et al*. 2007a, b) than are seen at higher temperatures (so low temperatures could result in higher sugars), but b) following defoliation, warmer temperatures may stimulate faster recovery of leaf area during regrowth (so in this case higher temperatures could result in higher sugars). Here we report on a study of how potential differences in WSCs between five cultivars (G) are affected by the duration of regrowth

Figure 4 Composition (g/100g DW) of blades after 31 days regrowth in 10/10 treatment: see (a) and (b) and 21 days regrowth in 20/10 treatment: see(c) and (d). Composition is first presented conventionally (see text for details) in (a) and (c). Then, in (b) and (d) values are normalised to a "WSC-free" plant to remove dilution effects of higher WSC (see text for details). Black bars – 'PG1113', dark grey – 'AberDart', white – 'Fennema', very light grey – 'Bronsyn', light grey – 'Tuni-7'. Shown are untransformed means and standard errors of untransformed means. Separate ANOVA of CV effects were performed for each component, and different letters indicate significantly different means.



(M), in two different temperature regimes (E) (20 °C /10 °C; 10 °C /10 °C, see Methods), following defoliation to a single residual stubble height consistent with good grassland management practice.

Methods

Plant material

We used two *Lolium perenne* cultivars from the UK (‘AberDart’, ‘Fennema’), one from NZ (‘Bronsyn’), one experimental breeding line from NZ (‘PG1113’), and one ecotype from Tunisia (‘Tuni-7’). ‘AberDart’ was bred at IGER, UK and is marketed worldwide as a HSG. ‘PG1113’ was produced by Grasslands Innovation Ltd in NZ and has been bred into the recently released cultivar ‘Expo’ by PGG Wrightson Ltd. ‘Fennema’ is a UK control while ‘Bronsyn’ is a NZ control. ‘Tuni-7’ was an ecotype with uncertain WSC status. We use ‘cultivar’ as a common term for all five populations, for brevity.

Seeds were germinated on nets floating on water, and individual seedlings planted after five to 10 days into pots (8 x 8 x 17 cm) containing nutrient rich potting mix. Plants (100/cultivar) were glasshouse grown at Palmerston North from December 2007, and defoliated to 6 cm above ground fortnightly. In March 2008, 50 plants of each cultivar were transferred to two controlled climate chambers (HortResearch, Palmerston North) and again defoliated to 6 cm. Growth conditions in one chamber were: 20°C, 14 h light; 10°C, 10 h dark (20/10); and in the other chamber: 10°C, 14 h light; 10°C, 10 h dark (10/10). Light intensity was 620 μmols/m/s, pots were watered through tap water saturated mats, and 50 ml of half-strength Hoagland solution was added weekly to each pot to ensure sustained high nutrient supply.

Regrowth experiment

Measurements were made immediately following a single defoliation, and at four times during regrowth (as detailed below) using separate plants, destructively harvested, on each occasion. We used 10 replicate plants per cultivar per temperature regime per occasion (so 10 x 2 x 5 = 100 plants per cultivar; 500 plants in total). In all cases, plant material was harvested at a set time of day (8 to 10 h after start of light) to avoid complications due to diurnal patterns of sugar metabolism.

In the 20/10 chamber, all plants were allowed to grow for 21 days unimpeded, and then all were defoliated again to 6 cm above ground. At this harvest (t = 0), on one subset of 10 plants/cultivar, the remaining ‘stubble’ was also cut at soil level. The fresh weight of blades and stubble was determined and a sub-sample of the tissues oven-dried to obtain a FW/DW ratio. The remaining material was immediately frozen in liquid nitrogen and stored at -20°C for further processing. For each cultivar, a further 10 replicates were harvested destructively (cut at soil level) after 3, 6, 14, and 21 days regrowth. On these subsequent harvests, tissues were separated into blades (at the ligule) and pseudostems (sheaths plus enclosed elongating blades). In the 10/10 chamber, the same procedures were followed, but the first harvest (t=0) was after 31 days of unimpeded regrowth, and the subsequent harvests were after 3, 6, 21, and 31 days regrowth. This pattern makes comparisons possible at either the same number of days (three examples), or at a similar (final) stage of regrowth.

Carbohydrate concentrations and plant composition

Note all material for analysis was harvested at a set time of day, and stored in liquid nitrogen. Plant material was ground to a fine powder, still in liquid nitrogen, in a mortar and then freeze-dried under reduced pressure. All WSC analyses were performed by ‘wet’ chemistry. Low and high molecular weight (LMW, HMW) WSCs were extracted separately and quantified using the colorimetric agent anthrone (Jermyn 1956; Hunt *et al*. 2005). Other plant components: crude protein, lignin, fibre (ADF, NDF), lipids and ash were determined separately by NIR (Corson *et al*. 1999).

As all components are presented as a percentage of the same total dry matter, an increase in one component will inevitably lead to a reduction in all other components, simply due to ‘dilution’. We therefore also present results normalised so that each non-WSC component is expressed as a “WSC-free” plant composition e.g. (CP / (CP+NDF+ADF+lignin+lipids+ash)*100).

Statistical analysis

Because we do not have space to present all data at all time points and in all tissues, we present results analysed as a single (G x E) ANOVA at the outset of regrowth (t = 0), and two further (G x M) ANOVA of changes in WSC in the new leaf tissues over the 4 durations of regrowth in each temperature regime. All data were Box-Cox transformed to homogenise the error variance. Tukey-Kramer’s Honestly Significant Difference (HSD) test was used to identify significant differences between means. We present plant composition data for CP, ADF, NDF analysed by ANOVA for the final harvest dates.

Results

Biomass and WSC at the start of regrowth

Biomass of blades harvested at the start of regrowth (t = 0) was significantly greater in the 20/10 treatment (after 21 days regrowth) than in the 10/10 treatment (after 31 days regrowth) (Fig. 1b). Blade biomass of ‘PG1113’ was significantly greater than ‘Fennema’ and ‘Bronsyn’, but only in the 10/10 treatment (Fig. 1c).

Using the measured WSC concentrations (those for

blades presented later) we present in Fig. 1 (d to f), the amounts of total WSCs per plant (concentration x biomass). The statistical analysis is presented for the sum of LMW and HMW WSCs, depicted as white and grey areas (respectively) within each bar. Generally, differences in HMW WSCs were larger compared to LMW WSCs. In blades, amounts of total WSCs were significantly higher in 'PG1113' and 'AberDart' compared to the three other cultivars (Fig. 1d). There was a significant G x E interaction between cultivars and temperatures (Fig. 1f). In the 10/10 treatment total WSCs were higher in 'PG1113' compared to 'Fennema', 'Bronsyn' and 'Tuni-7', while in the 20/10 treatment only 'AberDart' had significantly higher amounts of total WSCs compared to 'Fennema' and 'Tuni-7'. Amounts of total WSCs were considerably higher in blades in the 20/ 10 compared to the 10/10 treatment due to higher biomass production (Fig. 1e).

Biomass of stubble was significantly greater in 'PG1113'compared to 'AberDart', 'Fennema' and 'Bronsyn' (Fig. 2a). Amounts of total WSCs were also significantly higher in 'PG1113' compared to all other cultivars (Fig. 2d), which reflects its greater stubble biomass and (not shown here) also higher total WSC concentrations in this tissue.

Effects of duration of regrowth on carbohydrate concentrations

The concentrations of total (LMW+HMW) WSCs in blades, after 3, 6, 21, and 31 days of regrowth in the 10/10, and after 3, 6, 14 and 21 days of regrowth in the 20/10 are shown in Fig. 3 (a, c) and 3 (b, d), respectively. Overall, total WSC concentrations were higher in 'PG1113' and 'AberDart' compared to the other cultivars in both temperature treatments, and this effect was larger for HMW WSCs (Fig. 3a, b) (statistics for HMW, LMW separately, not shown).

There was a significant G x M (cultivar x regrowth duration) interaction in WSC concentrations in blades, but only in the 10/10 treatment (Fig. 3c). In this treatment, WSC concentrations were lower after six compared to 3 days regrowth in all cultivars except in 'PG1113', and this drop was particularly strong in 'AberDart'. However, with increased regrowth duration WSC levels were significantly higher in both 'AberDart' and 'PG1113' compared to 'Fennema', 'Bronsyn' and 'Tuni-7'. There was no significant G x M interaction in blades in the 20/10 treatment.

Plant composition

The differences in chemical composition between cultivars in leaf blades, at the time of the final harvest (21 days in 20/10; 31 days in 10/10) are shown in Fig. 4. The WSC concentrations shown are those measured by wet chemistry, whereas the remaining components are predictions by NIR. In Fig. 4 (a, c) we present the data conventionally. At 10/10 (Fig. 4a) there was a significant difference between cultivars in total WSCs ($P<0.001$); CP ($P=0.012$); ADF ($P< 0.001$); and NDF ($P< 0.001$), with the two cultivars which had significantly higher total WSCs at the time of this harvest tending to have lower concentrations of ADF and NDF compared to the other cultivars (for significance between cultivars see Fig. 4a). CP at 10/10 was significantly lower only in 'PG1113'. At 20/10 (Fig. 4c), a similar pattern was apparent with significant differences between cultivars in WSCs ($P<0.001$); CP ($P<0.001$); ADF ($P< 0.001$); and NDF ($P< 0.001$) and elevated WSCs in 'AberDart' and 'PG1113' again tending to have lower CP, ADF and NDF.

Normalising the results to remove the 'dilution' effect of the increase in sugars *per se* (Fig. 4b, d) critically alters the interpretation. At 10/10 (Fig. 4b) the difference between cultivars in CP has reduced ($P=0.046$) and in ADF has all but gone ($P=0.103$), and indeed while there was still a significant difference in NDF ($P=0.015$), the concentrations in 'AberDart' and 'PG1113'are now significantly greater than in 'Fennema'. At 20/10 (Fig. 4d) significant differences between cultivars were still evident in CP ($P< 0.001$); ADF ($P=0.004$) and NDF ($P=0.006$), and although CP was still significantly lower in 'AberDart' and 'PG1113', NDF was now significantly greater in these, than in 'Fennema' and 'Bronsyn'.

Discussion

Sugar recovery patterns following defoliation

In a previous paper (Parsons *et al*. 2004) we used a controlled environment study to achieve greater control over the multitude of factors affecting trait expression, than would be possible in the field. We tested the effects of temperature on the expression of the HS trait in two (then UK derived) HSGs ('AberDart', 'AberDove') compared to an EU control grass ('Fennema') under a single management (14 days regrowth interval). We found that at warm day and low night temperatures (20/10), these HSGs sustained significantly higher levels of WSCs, and that the difference between those and the European control cultivar 'Fennema' were larger when plants had gone through simulated (UK) winter conditions. No significant difference in WSCs, between HSGs and EU controls were seen at, for example, 20/20. This led to concern that the HS trait was affected by a significant G x E interaction.

Here, we report an expanded controlled environment study which included cultivars from NZ, and looked at how WSC concentrations and amounts, in five cultivars, may differ at the start of regrowth following a single common defoliation, and how WSCs change with regrowth duration, again in two temperature regimes.

Our results show that 'AberDart', and the new line 'PG1113', can sustain high concentrations of WSCs compared to the other cultivars tested. But the changes in WSC content during regrowth were not simple.

WSC concentrations, and differences between HSGs and control cultivars, showed a different pattern during regrowth depending on the temperature at which the plants were grown. At the warmer temperature WSC concentrations in leaf blades were substantially lower following defoliation, and recovered increasingly with the duration of regrowth (Fig. 3d). At the lower temperature WSC concentrations were depressed less markedly and later, before recovering. Moreover, in the colder temperature treatment, some individuals in one of the populations ('PG1113'), had an unusual strategy for accumulation of sugars and did not show the drop in blade WSCs after 6 days of regrowth as seen in all other cultivars.

The more substantial decline in WSC concentration following defoliation in the 20/10 treatment would appear to be due to the direct effect of temperature in stimulating growth rate (meristematic activity imposing a greater demand/sink for C skeletons and energy). We conclude this because the starting total WSC amounts per plant (in stubble) were similar in both temperature treatments (Fig. 2e) even though, at the time of initial harvest, far more (about x 2) WSCs had been removed as leaf blade (Fig. 1e). Differences in plant morphology, for example, more prostrate, smaller tillers at low temperatures, would help retain residual leaf area, though in this study defoliation was sufficiently severe to remove nearly all leaf in both temperature regimes. Regrowth was sufficiently stimulated by warmer temperatures to achieve a greater yield in 21 days at 20/10 as in 31 days at 10/10. It is possible, however, that the greater capacity to sustain WSC concentrations seen in some individuals in 'PG1113' (see Fig. 3c) was due to the greater amount of WSCs stored in stubble at the start of regrowth in this cultivar (Fig. 2d) leading to faster recovery of leaf area and net C supply. An alternative is that these individuals deploy different isoforms of putative sugar storage/consumption control genes, and this is being investigated currently in these same tissue samples.

Management to harness the benefits of high WSCs

Our study indicates that to follow accepted pasture management guidelines (Parsons & Chapman 2000) is probably sufficient to harness higher WSC levels whether from HSGs or 'controls'. Longer regrowth periods are seen here to increase the prospect of harvesting high WSCs, notably in warmer environments, without depressing stubble biomass or WSC content. However, we must recognise that extended periods of grazing at high stocking rates (more days 'on paddock' in rotation, or under continuous grazing) can lead to mean defoliation intervals (at the patch scale) of only 7-10 days (Fig. 3.12 in Parsons & Chapman 2000; Parsons & Dumont 2003), which our study suggests (Fig. 3c, d) could suppress WSCs and the smaller absolute differences in WSCs between cultivars could lead to difficulties in detecting the HSG trait (if using lower replication, for example, than in this study). The lack of a significant G x M interaction in the 20/10 regime can be shown to be because the difference between cultivars was significant, and rankings of cultivars similar, at each time point. If a lower level of replication were used in a field trial, the capacity to distinguish significantly between cultivars when the absolute differences between them were small, for example, after only a short period of regrowth, would arguably be reduced. We conclude that the identification of germplasm suitable for the production of HS ryegrass lines, and its evaluation, should be accompanied by phenotyping under controlled conditions, as presented here, to resolve the complication of G x E and G x M interactions.

Targets for HSGs

It is of concern that a simple change in how plant composition is calculated can have a critical effect on the interpretation of results (see Fig. 4). Because WSCs are a large and variable component of the total DM, increases in WSCs of the order seen between HSGs and controls, has a simple arithmetic effect in 'diluting' (reducing) the concentrations of all other components *pro-rata*. Removing this 'dilution' effect reveals that reductions in fibre content, for example, in HSGs may not be a fundamental change in plant structural chemistry (nor do they explain the increase in WSCs), but that the HSG trait was, here, associated with either no change, or even a small increase, in NDF in several circumstances (see Results and Fig. 4 for details). Only CP appeared to be reduced, in general, in HSGs. Certainly, the standard analysis (Fig. 4a, c) represents what, for example, an animal eats, and so is of ultimate importance. The recalculation to a sugar-free composition is important only to highlight what associated plant traits may need to be considered in breeding programmes, and also is important to reconsider any proposed causality between WSC gain and changes in other plant components.

Even though we can confirm that some current HSGs can accumulate more WSCs compared to standard cultivars, under a range of environment and management conditions, we have previously calculated that WSC content would need to be increased to some 36% to achieve the WSC:CP ratio of c. 1.2-1.5 in high N (and so high CP) pastures that would be necessary to provide the same low proportional loss of ingested N seen otherwise only in very low N content, unproductive,

pastures (Edwards *et al*. 2007a, b). Our analyses in that review demonstrated that progressive, incremental gains in WSC:CP ratios would be beneficial, but we add here it would take decades of incremental gains to match the desired 'step' change in benefits. Such a WSC concentration is clearly eminently achievable, as the plants in this controlled environment study have close to 36% WSCs by the end of each full regrowth (21 and 31 days). Our previous work (Parsons *et al*. 2004) suggests the low night temperatures (c. 10°C) may have contributed to that. To get the same levels of WSCs consistently expressed under NZ field conditions, in the near future, depends on increasing our understanding of the mechanisms controlling sugar content in pasture grasses. Despite the agronomic and environmental importance of fructan accumulation, its biosynthesis in *L. perenne* and other temperate grasses is still poorly understood. Only three out of potentially six biosynthetic genes have been fully characterised for ryegrass. Moreover, almost nothing is known about molecular regulators driving the expression of these genes (Chalmers *et al*. 2005). This lack of knowledge hampers both conventional breeding of improved HSGs as well as strategies based on genetic modification.

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