

## Some 'high sugar grasses' don't like it hot

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### Abstract

Cultivars of *Lolium perenne* with high concentrations of water soluble carbohydrates (WSC) are seen as desirable for the reduction of nitrogen losses and greenhouse gases (notably N<sub>2</sub>O) produced from grazing by livestock, as well as offering some opportunities for increasing meat and milk production. These benefits have been shown consistently in the UK, but here we report a series of investigations which suggest the high sugar grass (HSG) trait may not be so consistently or readily expressed in field conditions in New Zealand.

First, the cultivars AberDart (HSG) and Fennema (control) were grown in paddocks in the Manawatu (Aorangi) and studied from July 2001 to October 2002. Total WSC levels in the harvestable component (leaf snips) increased during spring in both cultivars, but the differences between the HSG and control were smaller than seen in the UK and were only marginally significant ( $P = 0.063$ ). Likewise, no consistent differences in WSC in leaf blades were found in a second trial, grown this time in pots outdoors, where water and nutrients were more controlled. This second trial included not just AberDart, but the original HSG, AberDove, which had been the focus of many successful trials in the UK.

An analysis of the environmental factors that might be relevant to the expression of the quality trait 'high sugar', and of possible differences between UK and NZ climates and trials, led us to a third series of experiments, conducted in NZ, in controlled environment chambers. Total WSC became substantially greater ( $> 2$ fold) in all three cultivars when grown at 10°C (day and night) than at 20°C but only at 10°C did one HSG, AberDove, show a small, but significantly greater WSC, than Fennema ( $P < 0.05$ ). However, significantly higher levels of WSC ( $P < 0.05$ ) were expressed in leaf blades of both AberDart and AberDove, compared to Fennema, when grown at temperatures of 20°C day / 10°C night (14h day), and especially (68% and 46% respectively) when this followed a period of cold (10 weeks at 5°C) and short days. Our findings suggest that low temperatures, either low night temperatures, or previous periods of sustained cold ( $< 10^\circ\text{C}$ ), may be necessary for expressing a sustained higher WSC trait in the harvestable components of *Lolium perenne*.

**Keywords:** High Sugar Grasses, greenhouse gases, perennial ryegrass, water soluble carbohydrates

### Introduction

New cultivars of *Lolium perenne* with high concentrations of water soluble carbohydrates (WSC) are presently being advocated in New Zealand for having a number of valuable attributes. High WSC help capture nitrogen released from forage proteins degraded in the rumen. This potentially reduces the proportion of N ingested (in the diet) lost in urine (Miller *et al.* 2001) and offers environmental benefits. A reduced proportion of N returned in a labile form at high concentrations to the soil would help reduce N leaching and emissions of the potent greenhouse gas nitrous oxide (N<sub>2</sub>O) (see MAF 2004). There is also evidence (from the UK) of production benefits, with reports of increases in meat (Lee *et al.* 2000, 2001) and milk production (Miller *et al.* 1999, 2001). Several high WSC *Lolium perenne* cultivars have been developed in the UK, initially 'AberDove', but later 'AberDart', where in trials using both sheep and dairy systems, these high sugar grasses (HSG) expressed 25-50% higher total WSC compared to other UK standard cultivars such as Fennema (e.g. Orr *et al.* 2000, 2003; Lee *et al.* 2001; Miller *et al.* 2001).

In *Lolium perenne*, in common with many cool-temperate perennial grasses, there is a marked seasonal pattern of accumulation of WSC, notably of high molecular weight (HMW) sugars, fructans. Total WSC accumulate throughout summer and autumn, leading to the highest total accumulated during winter (Pollock & Jones 1979; Pollock 1986). In early spring, HMW sugar levels fall, co-incident with flowering. The bulk of long term accumulation is in sheath bases (near the ground), though in the high sugar cultivars, higher sugar levels are sustained also in the harvestable component, the leaves, during successive regrowths. In UK trials in South England and Wales, the substantial differences in WSC between the high sugar cultivars and the standard controls were expressed in leaves produced throughout spring and early summer. We report here findings from a series of investigations, conducted in NZ, on WSC contents in AberDove and AberDart = HSG and Fennema = control, where the original goal was to explore some ecosystem wide implications of the high sugar trait, but an unexpected spin-off was the observation that the trait might not be so readily or consistently expressed (sugars not higher) when these cultivars are grown under field conditions in NZ.

## Material and methods

### i) Initial field and pot trial

AberDart (HSG) and Fennema (control) were sown as monocultures in 0.25 ha plots (2 replicates per cultivar) at Aorangi (Manawatu) in December 2000 and established and grown under grazing during a 12 month sampling period from October 2001 to October 2002. These plants received natural rainfall and 100 kg fertiliser N/ha (urea) was applied in December 2001. Leaf 'snips' (10 samples per cultivar) were taken at random every 2 to 4 weeks to analyse the material available to grazing animals.

Later, in November 2001, seedlings of AberDart, Fennema, and a second HSG AberDove, were transplanted into pots (18 x 18 x 25 cm, 4 plants per pot, 20 replicate pots per cultivar) in nutrient rich potting mix and grown outdoors from November 2001 to March 2002. These plants were watered daily and were cut back weekly (to 6 cm above ground) and analyzed fortnightly for WSC of blades.

### ii) Climate chamber trial

Here, seedlings of all three cultivars were transplanted into pots (also 18 x 18 x 25cm, 4 plants per pot, 20 replicate pots per cultivar per temperature treatment) in potting mix, and transferred to two controlled climate chambers (HortResearch, NZ). These received daily water and a modified Hoagland nutrient solution, containing 9 mmol/l N. The light intensity in the growth rooms was high (620  $\mu\text{mol}/\text{m}^2/\text{s}$ ). Details of the temperature and daylength regimes are given in the Results.

### Sample preparation

In all cases the plant material was harvested at a set time of day (1pm) to avoid complications due to diurnal patterns of sugar metabolism, and frozen immediately in liquid N<sub>2</sub> on site, stored at -20°C until freeze dried, and ground (shattered under liquid N<sub>2</sub>) to a fine powder. Sugars were differentially extracted in 80% ethanol, and subsequently water (Pollock & Jones 1979) and WSC were determined in the low molecular weight (LMW) and high molecular weight (HMW) extracts with the colour reagent anthrone. Anthrone reaction conditions were optimised for fructose containing carbohydrates (Jermyn *et al.* 1956).

### Statistical analysis

For the field trial, we used the mean values from each plot and performed a split-unit analysis of a repeated measures design (Mead 1988), with cultivar (two levels) as the main-unit treatment and time (18 occasions) as the repeated measure. Likewise for the controlled environment studies we used pots (mean of 4 plants) as the unit of replication with cultivar (three levels) and environment

(two levels) as the whole unit factors, and time (three levels, see Results) as the repeated measure. Analyses were performed using the data for each pot at the end of each of the three temperature comparisons. We used the natural log of the total WSC as our dependent variable. The transformation was necessary to homogenise the error variance.

## Results

### i) Initial field and pot trials

In the field trial, total WSC levels in leaf 'snips' increased during spring in both cultivars, but the concentrations found in AberDart (HSG) were only slightly and inconsistently different (between -7% and +20%) from Fennema, (see Figure 1 and associated statistical analysis). There was no significant time x cultivar interaction, so the difference between cultivars is based on the whole period of study. The graph shows total WSC, but the lack of consistent differences between AberDart and Fennema was true in both LMW and HMW sugar components.

In the outdoor pot trial, likewise, total WSC levels in the leaves of the now two HSG cultivars (AberDove and AberDart) varied between -12% and +29% those of Fennema, but were not significantly different from Fennema over the whole period of the trial (data not shown).

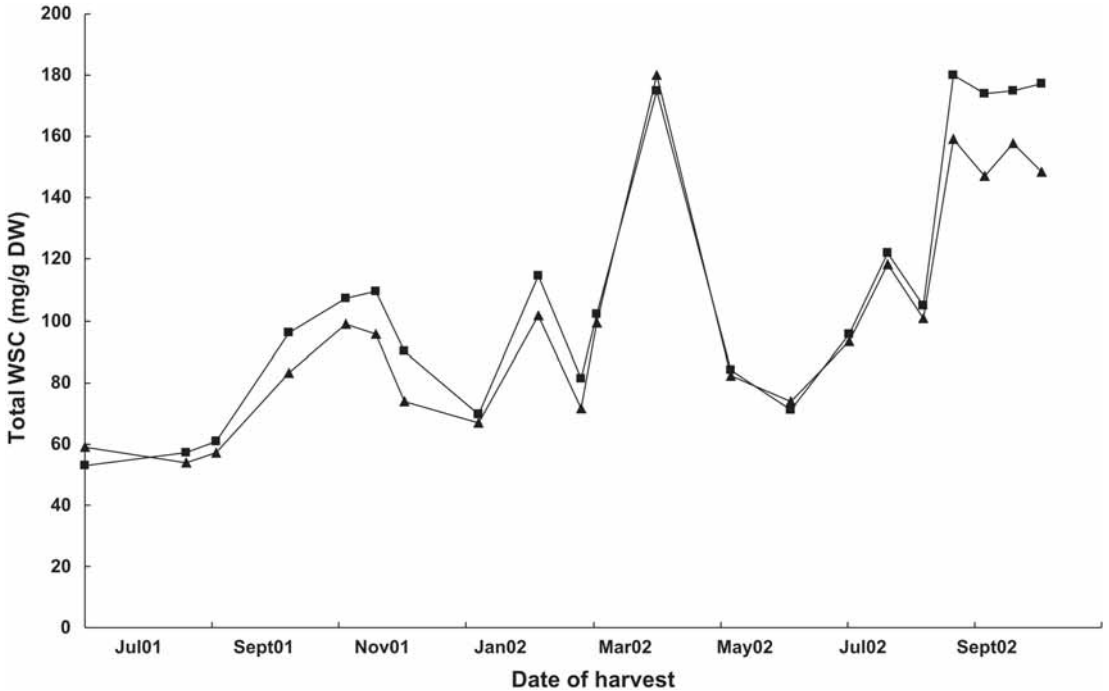
### ii) Climate chamber trial

A review of environmental factors leading to the accumulation of high WSC levels and a comparison of UK and NZ conditions suggested that one major factor important for the expression of the quality trait "HSG" could be generally lower temperatures in the UK, especially in winter and early spring. This was explored in three parts:

A comparison was first made of WSC levels in leaf blades of AberDove, AberDart and Fennema between plants grown at 20°C and plants grown at 10°C (constant day/night temperature, 14h day, 75% RH) over a period of 10 weeks. We did not detect any significant differences in sugar levels between the three cultivars when grown at 20/20°C (Figure 2a) and concentrations of WSC remained low, e.g. total WSC between 40 and 50 mg/g DW. However, in plants grown at 10/10°C, WSC levels increased up to 3-fold in all cultivars (Figure 2a), the largest relative increase being in HMW fructans. By the end of the 10 week period, the WSC level was significantly higher than in Fennema in only one HSG, AberDove (see summary and statistical contrasts, Figure 3).

Having seen an effect of temperature *per se* we next induced conditions closer to a UK winter by reducing the temperature in the colder room from 10°C to 5°C (day/night) and imposing short days (SD, 7h) for 10

**Figure 1** Field: total WSC concentration in leaf snips of *L. perenne* cultivars AberDart (HSG) and Fennema (control) sampled from paddocks from July 2001 to October 2002 AberDart (■), Fennema (▲). (The graph contains three extra, hence 21 not 18, values where analyses were made for more details of sugar metabolism).



**Table 1** Statistical analysis (ANOVA table) of field data shown in Figure 1.

Source	df	MS	F	P
Cultivar (CV)	1	0.06489	14.331	0.0632
Whole unit error	2	0.00453		
Time	17	0.56403	64.309	< 0.00001
Time x CV	17	0.00581	0.6621	0.8160

weeks. The warmer room remained at 20°C and long days (LD, 14h). All grasses in the cold treatment sustained high levels of total (and HMW) sugars, with AberDove remaining the highest (but no longer significantly different).

To simulate the transition to a UK spring (warmer days with still cool nights), both growth rooms (all plants) were finally switched to the same conditions of 20°C day/10°C night with 14h days for a further 14 weeks. This highlights whether *previous* conditions *per se* (cold, and SD induction of flowering) have an influence on the expression of high sugars between cultivars, as the current conditions were identical (20/10°C LD) for all cultivars.

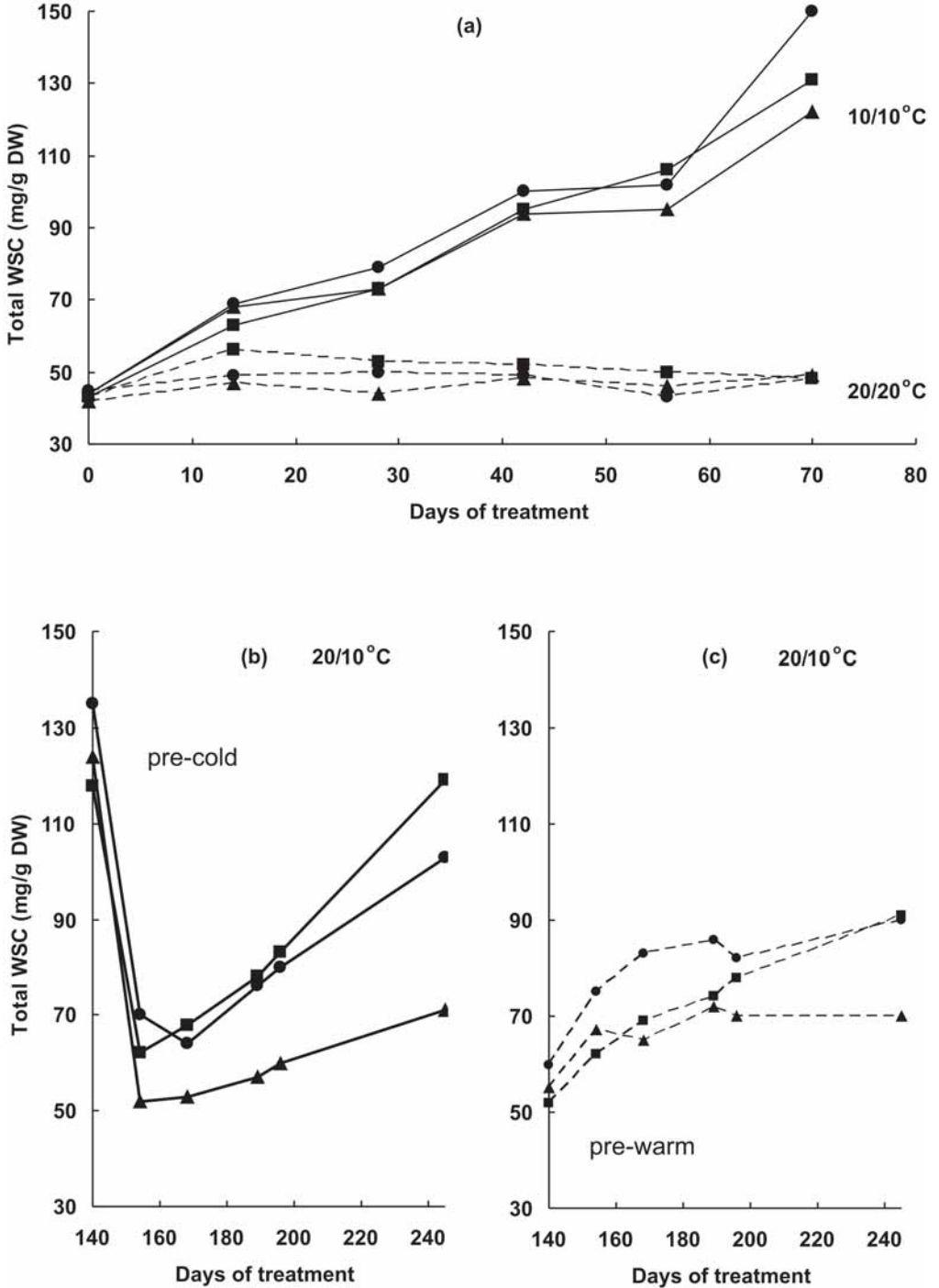
Total WSC concentrations dropped substantially initially in those plants previously grown at 5/5°C SD, but then built up again (Figure 2b), mainly due to a strong increase in HMW fructans. There were now significant differences between both AberDart (+68%)

and AberDove (+46%) WSC levels compared to Fennema in the plants previously grown at sustained 5/5°C SD (winter) conditions (Figure 2b). In plants grown previously at warmer 20°C day/night and long day (LD) conditions, sugar concentrations also increased when night temperatures dropped to 10°C and there were now significant differences in total WSC for both AberDove, AberDart (*c.* 30% higher) compared to Fennema (control) by the end of the 14 week period (Figure 2c).

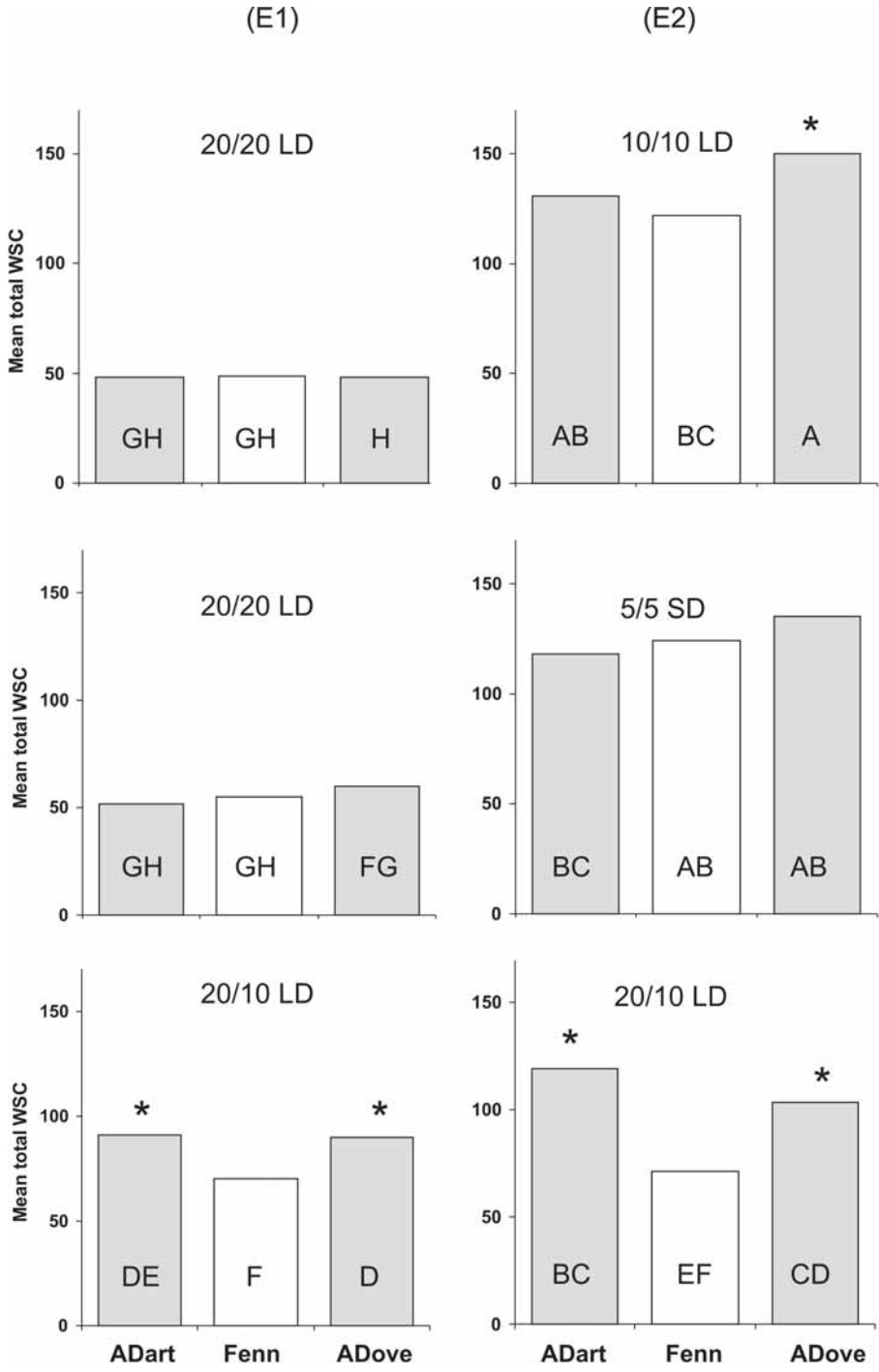
## Discussion

There is no question that the high sugar grass cultivars of *L. perenne* studied recently in the UK can sustain a greater level of total WSC sugars (which it is understood arises predominantly from greater levels of HMW fructan) in the harvested component, notably the leaves. Initial studies in the UK were conducted using AberDove. This showed mean increases of 40% and 26% greater

**Figure 2** Climate chamber: the effects of (a) current temperature and (b,c) previous temperature, on total WSC concentration in leaf blades of *L. perenne* cultivars AberDart (■), AberDove (●), and Fennema (▲). In (a) plants were grown at 10/10°C day/night (—) or 20/20°C day/ night (---), both long (14h) days. In (b,c) all plants are growing under the same 20°C day / 10°C night long days (14 h), but where in (b) (—) the plants had a low temperature (simulated winter) pre-treatment (10/10°C LD for 10 weeks followed by 5 / 5°C SD for 10 weeks), whereas in (c) (---) plants were previously grown for 20 weeks at 20/20 °C LD. For statistical analysis see Figure 3.



**Figure 3** Climate chamber: summary and statistical analysis of the effects of current and previous temperature and daylength regimes on WSC. There were two environment sequences (E1, E2). In one case (E1) plants went from 20/20 °C LD to (same) 20/20 °C LD before moving to 20/10 °C LD. In the other (E2) plants went through 10/10 °C LD, 5/5 °C SD, before the same 20/10 °C LD (simulated UK winter and spring). Histograms show WSC at the end of each of the three stages in each sequence. Bars marked with a star (\*) are significantly different from the Fennema control. Bars which do not share the same letter are significantly different from each other (Tukey-Kramer HSD test).



total WSC over two successive seasons (April–November) under continuous grazing by sheep (Orr *et al.* 2003). Those trials involved high N inputs, were well watered/irrigated and the defoliation regime (sustained 6 cm sward height) will have involved a wide range, but of relatively short, defoliation intervals (Parsons & Dumont 2003). Other trials, using AberDove showed improvements in liveweight gain of sheep, over a control grass (AberElan). The HSG had greater digestibility and lower fibre content, and it was concluded it was not possible to distinguish if the benefits were due to sugar content *per se*, or to low fibre (Lee *et al.* 2000, 2001). A more extensive series of studies were conducted using dairy cattle across a range of stages of lactation (Miller *et al.* 2001). These revealed sustained greater total WSC in a HSG variety (early papers named AberDove) than in AberElan during herbage regrowths, grown over periods of around 6 weeks. Dairy cattle fed this grass showed increased milk yields, for the same dry matter intake. This was shown to arise from an increased digestibility and so increased *digestible* matter intake. Milk yield per kg *digestible* DM intake was reported to be unchanged (Miller *et al.* 1999). Detailed analyses of rumen metabolism revealed a substantial shift in the fate of N, with more N captured as microbial N, leading to 0.30 vs. 0.23 g milk N / g feed N in the HSG relative to the control (Miller *et al.* 2001). The cultivar AberDart is now available in NZ.

Our current study was not set up initially to field trial the HSG in NZ, but on the presumption the trait (being a genetic characteristic) would be consistently expressed, our goal was to study the impact of a sustained higher sugar level on diet preference; resistance to insects (the UK grasses contain no endophyte); and the way sustained differences in grass metabolite levels might affect the grass/endophyte (and so alkaloid) interaction. Persistence of (and the value of) new grasses in NZ may depend on this.

Our findings clearly suggest that low temperatures, either low night temperatures, or previous periods of sustained low temperature, may be necessary for expressing a sustained higher WSC in the harvestable components (mostly leaf blades) of *Lolium perenne*.

Our initial difficulties in getting the trait to express, led us to analyse a range of differences between the UK (and the trials there) and NZ. During the initial field trial, additional tests confirmed the no-endophyte status (none present in the seed stock or the field samples) of these cultivars. We also ran gels (looking for distinct isozyme 'bands') to confirm the seed material matched that in the UK and standards in the Margot Forde Germplasm Centre, and so had not been mislabelled. On occasions we harvested both sheath as well as lamina, and compared the ratio of these, to confirm that the differences between

the UK and NZ results did not depend solely on the harvest regime (and the % sheath in the sample).

Temperature, by modifying growth and respiration relative to photosynthesis, is long recognised as a major determinant of sugar accumulation in plant tissues. An analysis of UK vs NZ conditions reveals that although the UK is on average only 2°C cooler, temperatures in spring are a surprising 5–7°C lower in the UK around the equinox, and this follows a much colder and longer winter. Having ruled out nutrient status (the outdoor pot experiment had ample water and nutrient supply relative to the field trial), the growth chamber trial confirms that a UK-type temperature regime may be important in expressing fully the high sugar trait. The results suggest that the HSG not only express more sugars in their leaves under appropriate current conditions, but that in conditions similar to early spring, when HMW sugars in all cases fall, the levels of HMW sugars expressed in Fennema may fall lower than those in AberDart and AberDove, accentuating differences between the cultivars.

One key feature of the present study is that it uses exclusively imported UK /European varieties, including Fennema as the control. Differences in total WSC between these and NZ varieties may reveal different results. In one preliminary trial (unpublished results) we found some evidence that even Fennema had greater total WSC (notably HMW) than NZ cultivars (Pacific, Samson and Impact). If this were to be confirmed, then an imported cold region HSG may have higher WSC than NZ varieties, even if the HSG trait was not expressed in NZ.

Certainly, more extensive field based trials, preferably at a range of latitudes/altitude, in NZ are required to confirm/refute the present study. The combination of field trials (to reveal an effect) and a controlled environment/experimental approach (to analyse causality) is surely to be recommended for the testing of imported or other new germplasm opportunities. It is imperative that full field trials include both UK and NZ controls.

## Conclusions

The HSG cultivar AberDart did not express significantly more WSC than the control cultivar Fennema under the outdoor environmental conditions of NZ during our trials. The expression of higher total WSC was evident in AberDove under sustained low temperatures and in both HSG under low night temperatures, and/or following extended periods of cold. These findings alert plant breeders to some potential pitfalls of selection procedures for sugar accumulating traits across dissimilar environments, that will require both field trials and controlled experimentation to resolve. Work is underway with these same cultivars to test their interaction with

'wild type' and 'safe/low toxicity' endophytes and the associated metabolic (eg. alkaloid) profiles, necessary for persistence in NZ.

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