Responses of *Lotus corniculatus* to environmental change. 4: Root carbohydrate levels at defoliation and regrowth climatic conditions are major drivers of phenolic content and forage quality

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Abstract:	The unpredictable nature of proanthocyanidin (condensed tannin) accumulation in regrowth vegetation of the perennial forage legume Lotus corniculatus represents a dilemma to the wider use of this species in agriculture, and a potential problem in the nutritional ecology of some terrestrial herbivores, as variable condensed tannin levels can result in either beneficial or detrimental effects on animal nutrition. However, the source of this variation has not been extensively explore. High levels of carbon allocation to roots during low temperature preconditioning of clonal plants were found to significantly increase condensed tannin and flavonol levels in regrowth foliage, while low levels of carbon allocation to roots during periods of high temperature preconditioning significantly decreased condensed tannin and flavonol levels . Phenolic accumulation and tissue digestibility were also differentially affected by regrowth of these defoliated plants at high CO 2 concentrations and by drought. Lower rates of digestion generally paralleled increases in tannin levels in regrowth leaves under the different environmental conditions, with rates of digestion falling in high tannin plants, despite correspondingly higher levels of leaf carbohydrates.Differential accumulation of root carbohydrates between seasons and years may therefore explain some of the variability found in the nutritional quality of the forage of this species.

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Responses of *Lotus corniculatus* to environmental change 4. Root carbohydrate levels at defoliation and regrowth climatic conditions are major drivers of phenolic content and forage quality

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Abbreviations: IVDMD - ln vitro dry matter digestibility: NSCs - Non-structural carbohydrates; TGA - Thioglycolic acid;

Keywords: Carbon allocation, Climate change, CO_2, Condensed tannins, Drought, Digestibility, Flavonoids, Growth temperature, Lignin,

Main Conclusion

Differential accumulation of root carbohydrates at defoliation have a higher impact than regrowth environmental conditions on the phenolic content and feed quality of the perennial forage legume *Lotus corniculatus*

Abstract

The unpredictable nature of proanthocyanidin (condensed tannin) accumulation in regrowth vegetation of the perennial forage legume *Lotus corniculatus* represents a dilemma to the wider use of this species in agriculture, and a potential problem in the nutritional ecology of some terrestrial herbivores, as variable condensed tannin levels can result in either beneficial or detrimental effects on animal nutrition. However, the source of this variation has not been extensively explored.

High levels of carbon allocation to roots during low temperature preconditioning of clonal plants were found to significantly increase condensed tannin and flavonol levels in regrowth foliage, while low levels of carbon allocation to roots during periods of high temperature preconditioning significantly decreased condensed tannin and flavonol levels. Phenolic accumulation and tissue digestibility were also differentially affected by regrowth of these defoliated plants at high CO_2 concentrations and by drought. Lower rates of digestion generally paralleled increases in tannin levels in regrowth leaves under the different environmental conditions, with rates of digestion falling in high tannin plants, despite correspondingly higher levels of leaf carbohydrates.

Differential accumulation of root carbohydrates between seasons and years may therefore explain some of the variability found in the nutritional quality of the forage of this species.

Introduction

Stress induced accumulation of plant secondary metabolites by both abiotic and biotic factors is well documented, as is the role of secondary metabolites in general herbivory and plant survival fitness (Swain 1977; Rosenthal and Berenbaum 1992; Bennett and Wallsgrove 1994; Dixon and Paiva 1995; Wink 2003; Iason 2005; Mithöfer and Bola 2012; Ramakrishna and Ravishankar 2013). Consequently, there is the potential for climate change to modify the levels of plant secondary metabolites resulting in either deleterious levels appearing in food or feed, or reduced levels compromising plant pathogen defence responses. However, not all anti-

nutritional metabolites may necessarily be required for plant survival and not all plant defence responses are likely to adversely affect plant nutritional quality. There is scope therefore for beneficial interventions in plant breeding programmes to improve crop performance under adverse environmental conditions. In order to formulate such strategies a more complete understanding of the consequences of likely climate change on the accumulation of compounds with potentially toxic, anti-nutritional or plant defence properties is required.

In relation to the perennial legume *Lotus corniculatus* (birdsfoot trefoil) both as a crop plant for ruminant feed and as a widely distributed species supplying herbage for wild species, our present understanding of the effects of environmental stress on secondary end product accumulation is incomplete and changes in the environment could affect the biosynthesis of secondary metabolites in these plants to a degree that may impact on forage intake, digestion and animal health, and reduce the nutritive value of the vegetation. This is particularly important when considering the accumulation of polyphenolic compounds such as proanthocyanidins (condensed tannins).

Condensed tannins are considered anti-nutritional at high concentrations (above 4-5% dry weight) because they reduce both digestibility and palatability (Barry and Duncan, 1984). In contrast, at low concentrations (2-3% dry weight) some condensed tannins are considered beneficial to ruminants as they bind to plant proteins and protect them from deamination by microbes present in the rumen, which reduces the possibility of bloat and increases the amount of protein available for post-ruminal digestion and absorption (Barry and Manley 1984; Waghorn et al. 1987), while some non ruminants use salivary proteins with high affinity with condensed tannins to cope with low concentrations of anti-nutritional dietary tannins (reviewed by Mueller Harvey 2006). Other mechanisms adopted by non-ruminant vertebrates to deal with condensed tannins and other plant secondary metabolites, have been extensively discussed by Dearing et al. (2005).

However, *Lotus corniculatus* is not currently extensively used as a forage crop as there are difficulties associated with plant establishment (Van Keuren, and Davis 1968; Taylor et al.

1973) and with the variability and unpredictability of the levels of condensed tannins in the harvestable tissues.

It is not surprising therefore to find a considerable interest in manipulating the biosynthesis of condensed tannins in an attempt to improve forage quality. Leaf tannin production in *Lotus corniculatus* is inherited as a monogenic dominant with tetrasomic inheritance, and a genetic polymorphism for tannin production in *Lotus corniculatus* results in some individuals that do not produce leaf tannins, while in others tannin production is constitutive (Ross and Jones 1983).

Several environmental factors implicated in climate change such as temperature, drought and atmospheric CO_2 concentrations affect the accumulation of plant phenolics in forage legumes. Increases in the accumulation of condensed tannins under controlled high temperature conditions were found in *Lotus pedunculatus* (Lees et al. 1994), but not in *L. corniculatus* (Ehike and LeGare 1993; Carter et al. 1999) and the optimum temperature and light intensity for *L. corniculatus* growth and phenolic accumulation has been determined in three clonal genotypes that varied in their tannin content (Morris et al. co-submitted). However, only a few studies have considered the effects of environmentally induced changes in condensed tannins biosynthesis on the nutritional quality of forage legumes. In one study the effects of elevated temperature, CO_2 and drought on the nutritive value of *L. corniculatus* in relation to condensed tannin accumulation showed that decreasing the growth temperature from 25ºC to 18ºC resulted in an increase in leaf, stem and root condensed tannins as well as in the levels of soluble and storage carbohydrates in these tissues, both at normal and at elevated CO_2 levels and under drought conditions (Carter et al. 1999). Analysis of tissue digestibility showed that the nutritive value of the leaves was inversely related to condensed tannin levels and that a negative relationship existed between condensed tannin of more than 30 g per kg dry weight and the initial rates of gas evolution when subjected to in-vitro fermentation by micro-organisms (Carter et al. 1999).

Herbaceous C3 plants grown in elevated CO_2 show increases in carbohydrate and starch accumulation within source leaves, with changes in C partitioning between root and shoot.

Root: shoot ratios tend to increase in CO_2-enriched plants but in contrast, increased temperatures tend to decrease both root:shoot ratios and carbohydrate accumulation in source and sink organs (Farrar and Williams 1991). However, increasing temperature with CO_2-enriched plants generally increases sucrose synthesis, and carbohydrate accumulation in the leaves (Farrar and Williams 1991). Metabolism of reserve carbohydrates, such as starch accumulated in taproots of *Lotus corniculatus*, is thought to be essential for regrowth following defoliation and for tolerance to environmental stress with taproot starch concentrations declined in defoliated plants, while increasing in taproots of undefoliated plants (Boyce et al. 1992). For example, *Lotus corniculatus* grown at 18^{o}C exhibited typical carbohydrate root reserve cycles but plants grown at 32^{o}C did not restore their root starch reserves (Nelson and Smith 1968).

A positive correlation has been found between spring herbage yields and the mean nonstructural carbohydrate level of the two preceding years, and it was suggested that there is a cumulative effect of carbohydrate levels on growth of *Lotus corniculatus*, and that management practises and breeding strategies should be designed to maintain high levels of root non-structural carbohydrates (Alison and Hoveland 1989). Limited information is available however regarding how root starch accumulation affects phenolic metabolism in shoots following regrowth after defoliation.

Previous studies have demonstrated that reduced nutritional quality in some forage legumes is not solely due to condensed tannins, and that the adverse effects of condensed tannins may be combined with the detrimental dietary effects of lignin and that both factors should be considered when determining nutritive value (Barry and Duncan, 1984; Miller and Ehlke 1996). Barry et al. (1986) established that in *L. pedunculatus* and *L. corniculatus* the reduced nutritive value under stress conditions was due to the simultaneous increase in the concentrations of lignin and condensed tannins, both of which depressed rumen carbohydrate fermentation and voluntary intake in sheep (Barry et al. 1986). Regardless of the concomitant effects condensed tannins and lignin levels may have on the digestibility of plant material, it is clear that at low concentrations both can result in improved voluntary feed intake.

In previous experiments (Morris et al. co-submitted) we noticed some anomalous results that suggested that the conditions under which plants were grown affected the levels of tannins accumulated in subsequent vegetative tissues, following harvesting and regrowth. It has also been found that in *Arabidopsis* the current and prior temperature experience of the mother plant controls seed dormancy through inhibition of proanthocyanidin biosynthesis in fruits, resulting in altered seed coat tannin content (Chen et al. 2014).

Although it may be predictable that future climate change will alter the levels of secondary metabolites in plants, our understanding of how this will affect plant digestibility and nutritional quality is limited. In particular how perennial forage species, normally subject to intense herbivory, or used as animal feed, may respond after defoliation or in the following year to unusually warm summers or cold winters, and how this affects forage quality has not been widely studied.

The aims of this work were firstly to establish how changes in growth temperature modify assimilate partitioning into roots, stems and leaves and secondly to determine the effect of different CO_2 concentrations and water availability during regrowth of plants preconditioned at different temperatures, on the relationships between tannin, flavonoid and lignin accumulation, and subsequent how these changes impact on tissue digestibility.

Materials and methods

Growth of plants

Replicate clonally propagated plants of a high tannin genotype S41 of *L corniculatus* cv Leo selected from seed obtained from The Genetic Resource Unit, IGER Aberystwyth SY23 3EB, UK. (Carron et al. 1994), were established by crown division of mature plants into 15 cm ramets containing well-developed roots and shoots and grown in low-N compost in 13-cm pots under ambient greenhouse conditions for 6 weeks and then shoots defoliated 5 cm above soil level. Plants were then grown under two pre-conditioning temperatures (10ºC or 25ºC) for 56 days at a light intensity of 450 µmol m^{-2} s^{1}, and then defoliated to 5 cm and regrown at 18ºC day/10ºC

night with combinations of two CO_2 concentrations (ambient ~350ppmv or 700ppmv) and water availability (60% or 100%) for 42 days (Table 1). Plants were maintained at 70–75% relative humidity with an 18-h day length (light intensity, 450 µmol m^{-2} s^{-1}). The positions of pots in the growth chambers were randomised daily and mutual shading avoided by staking plants during their growth. Replicated whole plants (three per environment), were harvested after 21 and 42 days and separated into leaf, stem and roots. Sub samples were stored at -80°C, and then freeze dried for further analysis.

Determination of condensed tannins

Total condensed tannins were determined as the sum of extractable (acetone soluble) and bound fractions on duplicate 20-30mg samples of freeze-dried powdered leaves, stems and roots using a modification of the butanol-HCl (BuOH/HCl) method outlined by Terrill et al. (1992), as described previously (Carter et al. 1999).

Determination of flavonoid and flavonoid glycosides.

Freeze-dried samples (200 mg) were re-hydrated in 2 ml H_2O for 1 hour, and then ground tissue extracted with 70% aqueous methanol. Extracts were concentrated on an activated C18 Sep-Pak column (500 mg) [Waters Inc] and bound flavonoids for HPLC analysis eluted with 4 ml 100% methanol, as described previously (Morris et al. co-submitted). Alkali hydrolysis was carried out on 1 ml of extract with 1 M NaOH at 100°C for 1 h. The sample was cooled, diluted to 5 ml with distilled water and adjusted to between pH6 and pH7 with 0.1 M HCl. The sample was concentrated on an activated C18 Sep-Pak column and the hydrolysed flavonoids eluted with 4 ml of MeOH. Flavonoid profiles were obtained by reverse phase HPLC on a µNovapak 8x10 C18 RCM cartridge (Waters Inc), and quantified as described previously (Morris et al. co-submitted).

Determination of non-structural carbohydrates (NSC)

Total sugars and sucrose were determined with anthrone reagent using a modified micro method based on that of Van Handell (1968) as described in detail in Carter et al. 1999. Starch determination was carried out on the dried pellets after sugar extraction using the glucose oxidase and peroxidase method (Trinder glucose analysis kit [Sigma]) after amyloglucosidase treatment. The resulting coloured quinoneimine dye complex was analysed on a spectrophotometer at 510nm and compared to a glucose standard curve, as described in detail in Carter et al. 1999).

Determination of thioglycolic acid lignin

Thioglycolic acid soluble lignin (TGA-lignin) levels were determined using a modified version of the method described by Whitmore (1978). Freeze-dried samples (200 mg) were re-hydrated in 2 ml distilled H_2O for 1hour. The samples were then extracted with methanol and acetone. The extracts were discarded and the pellets dried using a sample concentrator. The pellets were then treated with 10% thioglycolic acid in 2M HCl (3 ml/50 mg of sample) at 100°C for 4 h as described previously (Morris et al. co-submitted). The TGA-lignin content of the samples was determined using a PV8700 series UV/Vis spectrophotometer (Philips Scientific and Analytical Equipment) at 280 nm. The TGA-lignin concentration was calculated using a standard curve of known weights of TGA lignin extracted in bulk from transgenic root cultures of *L. corniculatus* (Morris and Robbins 1992).

Determination of tissue digestibility

End point in-vitro-dry-matter-digestibility (IVDMD) of leaves and stems was determined by the in-vitro pepsin-cellulase method (Jones and Hayward 1973), and the initial rate of digestion estimated from the rate of gas evolution in 8-h batch fermentations using the pressure transducer technique of Theodorou et al. (1994). This technique quantified the increase in head-space gas pressure (and thus the gas volume) in closed batch cultures inoculated with rumen micro-organisms. The batch cultures contained 0.5g freeze dried powered tissue in 90 ml of bicarbonate buffered medium and were inoculated with rumen micro-organisms in 10 ml of

clarified rumen fluid, and incubated at 30°C. Initial rates of digestion were calculated from the linear kinetics of gas evolution over the first 6h of incubation.

Results

Plant Growth

As would be anticipated, reducing the temperature from the optimum of 25°C to 10°C resulted in a 2-fold decrease in plant height and a 6-fold decrease in the growth rate over the following 8-week preconditioning period (Fig. 1a and b). When, following defoliation, these plants were regrown at 18°C, under different climatic conditions of normal or high CO_2 and with *ad libitum* or restricted water supply, the subsequent growth rates and final plant heights were also highly divergent with plants preconditioned at 10°C having a 3 to 4 fold higher growth rate than plants preconditioned at 25°C (Fig.1b), and with a 3-4 week lag period before normal growth was established. Increasing the CO_2 concentration from ambient ~350 ppmv to 700 ppmv for the regrowth period increased the growth rates of both 10°C and 25°C preconditioned plants (Fig. 1b). Drought stress however reduced growth rates in both high and low CO_2 environments on regrowth of both 10°C and 25°C preconditioned plants (Fig. 1b), with 10°C grown plants maintaining at least a 4-fold higher growth rate than plants preconditioned at 25°C, in all environments

Accumulation of non-structural carbohydrates (NSCs)

Plants grown at 10°C for 8 weeks had a two-fold higher level of total NSCs (starch, sucrose and reducing sugars) in leaves stems and roots, compared with plants grown for 8 weeks at 25°C. In particular, roots and leaves accumulated up to 4% dry weight of starch at 10°C but less than 0.5% at 25°C and roots contained approximately 60% more sucrose than leaves. Levels of reducing sugars were also lower in leaves, stems and roots at the higher growth temperature (Fig. 2). When these plants were defoliated to 5 cm stem height and regrown for 6 weeks at 18°C at ambient CO_2 (~350 ppmv) or 700 ppmv CO_2, with and without drought stress (Table

1), leaves stems and roots of plants preconditioned at 10ºC consistently maintained higher levels of NSCs than plants preconditioned at 25ºC and the total NSCs in leaves stems and roots increased in plants preconditioning at 25ºC but generally decreased in plants preconditioned at 10ºC across all environments (Fig. S1-S3). At the higher CO_2 concentration, levels of total NSCs transiently increased in leaves irrespective of preconditioning temperature but this was not present in plants under water stress. Leaf starch in particular rapidly declined under drought stress in plants preconditioned at 10ºC at both 350 and 700 ppmv CO_2 but increased in 25ºC conditioned plants (Fig. S1). In roots, starch levels rapidly declined in all environments in plants conditioned at 10ºC, but significantly increased in plants conditioned at 25ºC (Fig. S3). Neither the higher CO_2 concentration nor drought stress had a significant effect on the sucrose or reducing sugar content of leaves of regrown plants conditioned at either temperature (Fig. S1). Sucrose concentrations were similar, and the predominant sugar found in roots of plants conditioned at both temperatures, increasing during shoot regrowth, but declining on drought stress at both CO_2 concentrations (Fig. 2 and S3).

Accumulation of condensed tannins

Plants grown at 10ºC containing high levels of non-structural carbohydrates, particularly starch, had much higher concentrations of condensed tannins than plants grown at a temperature for optimal growth, with total leaf, stem and root tannin levels increasing from 1.2% to 4.8% dry weight in leaves, 0.8% to 1.8% dry weight in stems and 2.8% to 5.8% dry weight in roots in low starch and high starch plants respectively, with a two-fold increase in the ratio of soluble to insoluble tannins in leaves and stems (Fig. 3).

Following defoliation, plants regrown for 6 weeks at 18ºC with 350 or 700 ppmv CO_2, with and without drought stress (Table 1), maintained high levels of condensed tannins, up to 8% dry weight in leaves, 2% dry weight in stems, and up to 6% dry weight in roots of plants preconditioned at 10ºC and lower levels in plants preconditioned at 25ºC (Fig. 3). High CO_2 levels increased tannin accumulation in leaves, stems and roots of both low and high root starch plants while drought stress reduced tannin levels in all treatments (Fig.3). The kinetics of

soluble and insoluble condensed tannin accumulation in leaves, stems and roots over the 42-day growth period in the eight environments are shown in Fig. S4-S6 respectively.

Accumulation of flavonoids

Growing plants at 10ºC increased the total flavonoids of leaves from 5.5% to 16.4% dry weight and in stems from 0.5% to 3.3% dry weight, compared to plants grown at 25°C [p =0.001] (Fig. 4a).

After harvesting and plant re-growth in each of the eight environments (Table 1), total flavonol levels of leaves varied from 11.5% to 19% dry weight depending on the environment (Fig. 4a), and were significantly higher than in stems (Fig. 4b). The pre-conditioning temperature was a major factor affecting total flavonols in stem tissue of regrown plants but less so in leaves, although total flavonol levels were significantly increased in both leaves and stems of regrown plants by both pre-treatments [p<0.001].

A near doubling of the CO_2 concentration from ~350ppm to 700ppm significantly increased total flavonoid levels in leaves and stems of both 10°C and 25°C preconditioned plants [p<0.001], but drought stress resulted in a decrease in total flavonol levels in leaves and stems [p<0.001] and particularly in 25°C conditioned plants grown at the high CO_2 level (Fig. 4).

Kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside were the major flavonoid glycosides in S41 *Lotus* leaves with minor amounts of kaempferol-7-rhamnoside, and kaempferol-3-rhamnoside-7-glucoside as well as a number of minor unidentified kaempferol glycosides (Morris et al. co-submitted). The pre-conditioning growth temperature was found to be the major factor affecting the accumulation of individual flavonols. The concentrations of kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside were significantly increased [p<0.001] at a lower pre-conditioning temperature. Doubling the CO_2 concentration resulted in higher levels of kaempferol-3-glucoside [p<0.001] and kaempferol-3,7-dirhamnoside [p=0.001] in regrown leaves and stems in 10°C preconditioned plants whereas drought stress reduced the concentration of kaempferol-3-glucoside [p=0.001] (Fig. 4). Analysis of the HPLC

profiles and UV/visible spectra following alkali hydrolysis of extracts showed that the flavonoids detected in the leaves and stems of genotype S41 were all kaempferol glycosides (data not show).

Thioglycolic acid (TGA) lignin

Unlike condensed tannins and flavonoids, the levels of TGA lignin in leaves (Fig. 5a) and stems (Fig. 5b) decreased in plants grown at 10ºC compared with plants grown at 25ºC and this was particularly significant in stem tissue, suggesting that lignin was less soluble in TGA by growth at low temperatures. This may due to changes in lignin structure rather than a reduction in the total lignin content as thioglycolic acid derivatization involves the formation of thioethers of lignin benzyl alcohol groups, which enables it to be extracted from cell walls by alkali.

When these defoliated plants were regrown for 6 weeks at 18ºC, the concentration of TGA soluble lignin in the leaves varied between 2.9% and 4.7% dry weight, and in stems from 5.2% to 9.5% dry weight across the eight different growth environments (Fig. 5).

In plants regrown at 18ºC at ambient CO_2, without water stress TGA soluble lignin levels were similar to levels found in plants grown at 25ºC. However, water stress significantly increased TGA soluble lignin in all four environments in leaves [$p<0.001$], and particularly in plants grown at 10ºC and regrown at high CO_2. To a lesser extent the levels of TGA soluble lignin in leaves also increased in all other CO_2 enriched environments [$p=0.003$] (Fig. 5a). Drought stress also increased the levels of TGA extractable stem lignin [$p=0.005$] in all environments except in plants grown at 10ºC and regrown at high CO_2, but no other significant effects of high CO_2 levels on stem TGA soluble lignin were found (Fig. 5b).

Effects on tissue digestibility

Despite a two-fold increase in the levels of readily fermentable carbohydrates in leaves of plants grown at 10ºC (Fig. 2a), there was a 28% decrease in the initial rate of digestion compared with leaves of plants grown at 25ºC, (Fig. 6A), but a simultaneous six-fold increase in leaf tannins (Fig. 3A). In contrast, there was a 2.6-fold increase in the initial rate of digestion of stem tissue

in plants preconditioned at 10ºC compared with plants preconditioned at 25ºC (Fig. 6b), and a combined effect of a 1.6-fold increase in fermentable carbohydrates (Fig. 2b), a 28% decrease TGA soluble stem lignin (Fig. 5b), and a 2-fold increase in stem tannins (Fig. 3a).

In regrowth leaves under the different environmental conditions, rates of digestion also generally paralleled increases in condensed tannins levels with rates of digestion falling from approximately 45 ml g^{-1} h^{-1} in low tannin plants conditioned at 25ºC to 30 ml g^{-1} h^{-1} in high tannin plants conditioned at 10ºC, despite correspondingly higher levels of NSCs (Fig. 6a).

A negative correlation between leaf digestibility and leaf tannin levels and between leaf digestibility and leaf carbohydrate content was found, and a positive correlation between leaf carbohydrates and leaf condensed tannin content. Across all ten treatments there was also a positive correlation between the condensed tannin and flavonoid content of leaves [R^2=0.74] and stems [R^2=0.69]. However, there was no significant relationship between condensed tannin and lignin content or between lignin and flavonoid content of leaves or stems, despite these phenolics being part of the same metabolic network. The concentrations of total NSCs in leaves however were positively correlated with both condensed tannin [R^2=0.74] and flavonoid [R^2=0.88] levels, but not between levels of total NSCs and TGA soluble lignin. Analysis of stem material also indicated a positive relationship between total NSC and condensed tannin concentrations [R^2=0.72]. Unlike in leaf tissues, there did not appear to be a strong correlation between total NSC and flavonols [R^2=0.43]. TGA soluble lignin showed no relationship with total NSCs in *Lotus* stems. No correlation between total NSC levels in the roots with condensed tannin or with TGA lignin content was found.

Discussion

Generally previous studies have focused on the effects of environmental factors such as drought (Anuraga et al. 1993, Carter et al. 1999) or temperature (Anuraga et al. 1993; Lees et al. 1994, Carter et al. 1999) on plant growth and polymeric phenols, and the stimulating effects of high CO_2 has also been confirmed (Pe˜nuelas et al., 1997; Pe˜nuelas and Estiarte, 1998; Estiarte et

al., 1999). However, many of these studies have not considered the flux of carbon into other pathways and end products or the consequences of carbon reallocation for tissue digestibility. In *L. corniculatus*, carbohydrate reserves are low from spring to autumn even in plants that grow without defoliation (Smith 1962). Although the growth rate of *L corniculatus* va Leo, was maximal at 25ºC in all three genotypes studied, (Morris et al. co-submitted) maturity was delayed at lower temperatures as found previously (Smith 1970).

Previous reports on the effects of growth temperature on tannin accumulation in *Lotus corniculatus* showed that accumulation increased in response to low temperature (Carter et al., 1999; Morris et al. co-submitted), and decreased at high temperature (Ehike and LeGare 1993; Carter et al. 1999; Morris et al. co-submitted), while other studies have shown that low temperature alone had limited effect on condensed tannin accumulation over several seasons (Anuraga et al. 1993). However, the cost of defense chemical production cannot be predicted merely on the basis of the molecular weight, composition or concentration of condensed tannins (Briggs and Schultz 1990) when total flavonoid concentrations of up to 20% dry weight in the leaves and 3% in stems and TGA extractable lignin concentrations of 5% dry weight in leaves and 10% dry weight in stems, are major pools for carbon allocation. Growing *Lotus* at a temperature below that required for optimum growth resulted in higher concentrations of total flavonoids in both leaf and stem tissues, largely due to significant changes in kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside. To a lesser extent drought stress also appeared to alter the accumulation of flavonoids, and in both leaves and stems kaempferol-3-glucoside was significantly lower under reduced water availability and this particularly contributed to a reduction in the total flavonoids of stems. In contrast changes in CO_2 availability resulted in significant increases in the level of kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside in leaves but not in stems.

The first branch from the general phenylpropanoid pathway leads to the biosynthesis of lignin. In leaves and stems drought stress was found to be the major factor affecting lignin solubility. In both tissues, lignin solubility decreased in response to reduced water availability while doubling the CO_2 concentration increased lignin extractability of leaves, but high CO_2 had no

obvious affect on stem lignin solubility. Barry and Manley, (1986) showed that climatic stress caused a large and similar increase in the accumulation of condensed tannin and lignin in the shoots of Lotus species which depressed nutritive value.

During cold stress there is a reduction in growth rate and shoot biomass and as a result the majority of the carbon fixed in photosynthesis is stored as non-structural carbohydrates in the roots. When plants are then defoliated and re-grown at a higher temperature, they are able to remobilise carbohydrates and use them in the production of new shoot tissues. Although the majority of the carbohydrates may be utilised in primary metabolism some of it is clearly routed towards secondary metabolism where there is an increase in flux towards both condensed tannins and flavonoids.

In stems drought had very little effect on the condensed tannin content but digestibility was significantly reduced, suggesting that a reduction in the fermentation rates of stem material might be due to changes in the levels of structural components such as lignin, as evidenced by the reduction in lignin solubility.

It has been clearly demonstrated that different environmental factors, both alone and in combination, affect the accumulation of the secondary phenolic metabolites of Lotus. However, by far the greatest determinant of tannin accumulation in shoot tissue and hence forage digestibility was found to be the extent of carbon allocation to roots prior to regrowth. Low temperature treatment, which mimics the over-wintering of perennials, is characterized by a substantial increase in root carbon reserves and by a large-scale increase in tannin levels in regrowth tissues, when compared with plants with non-carbon loaded root systems such as would be found during warmer winters.

Current models that address developmental and environmental effects on whole-plant carbon partitioning to the phenylpropanoid biosynthetic pathway, such as the protein competition model (PCM) and the extended growth/differentiation balance model (GDB) (Mattson et al. 2005), give divergent predictions. The PCM model predicts that partitioning to phenolic compounds will decline, whereas the GDB model predicts that partitioning to phenolic

compounds will increase if CO_2 enrichment simultaneously stimulates both photosynthesis and growth (Mattson et al. 2005).

However, the results of this study do not appear to support either the PCM or the GDB model as a generally applicable model in *Lotus* as differential carbohydrate loading of roots and drought stress resulted in contradictory accumulation of phenolics in different tissues. For example, while high CO_2 levels increased growth rates in both low root carbohydrate and high root carbohydrate plants when defoliated and regrown at 18°C, increasing CO_2 resulted in an increase in both tannins (by 41% and 16% respectively), and flavonoids (by 6% and 25% respectively) in leaves and stems of low root carbohydrate plants, but a reduction in leaf and stem tannins (by 13% and 11% respectively) in plants with high root carbohydrate reserves. Under drought conditions however, regrowth of low root carbohydrates plants at high CO_2 resulted in a non-significant reduction in leaf tannins and a non-significant increase in stem tannins (by 3% in both cases) and a non-significant increase in leaf flavonoids (3%), but a significant decrease in stem flavonoids (21%). However, in plants with high root carbohydrate levels, drought plus high CO_2 resulted in a reduction in both leaf and stem tannins (by 18% and 13% respectively), a non-significant increase in leaf flavonoids (3%) but a significant decrease in stem flavonoids (10%).

It may also be considered that the increased accumulation of root storage carbohydrates in response to low temperature, resulting in differential growth and phenolic accumulation following defoliation and regrowth, could be an example of a recently developed theory of ecological stress memory, defined by Walter et al. (2012) as "any response of a single plant after a stress experience that improves the response of the plant towards future stress experience and which is assessed on a whole plant level". Possible mechanisms envisaged for this are the accumulation of proteins, transcription factors or protective metabolites, as well as epigenetic modifications or morphological change (Walter et al. 2013). In particular an emphasis has been placed on epigenetic responses in intergenerational stress memory as these are known to result in heritable responses to environmental stress (Bruce et al. 2007, Boyko and Kovalchul, 2011). However, ecological stress memory appears to require either a persisting "imprint" modifying

future stress response, or be mediated via intergenerational meiotic events, and it is not clear that these requirements are met in this case.

Differential carbohydrate accumulation in roots as a response to environmental stress should not therefore be considered to be either stress memory at the single plant level or intergenerational stress memory mediated via mitotic events as a result of vegetative propagation. Furthermore, this is not a newly observed response of perennial species, although the resulting major effect on subsequent condensed tannin and flavonoid accumulation in regrowth tissues has not been a previously reported response.

As previously found in *L. corniculatus* (Carter et al. 1999) there was a positive correlation between condensed tannin and flavonoid content of leaves and stems, which has rarely been demonstrated, and implies co-regulation of condensed tannin and flavonoid biosynthesis in shoot tissues. Similarly, the relationships established here, also suggest that the accumulation of total non-structural carbohydrates, condensed tannins and flavonols in *L. corniculatus* shoots may be regulated by a common mechanism.

The extent of carbon loading of roots in previous periods of plant growth therefore not only affects subsequent plant growth but also the levels of phenolic accumulation. This could partially explain the high levels of seasonal and annual variation in the tannin content and quality the forage of this perennial legume. As *L. corniculatus* is widely distributed in the wild, these effects would not be confined to the use of Lotus as a forage or feed for ruminants, but may well have wider implications for any herbivores which forage on this species.

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Author contributions

PM devised the project and wrote the manuscript, EBC produced the experimental material and carried out sugar, tannin and digestibility analysis, JWH and BH carried out the flavonoid and lignin analysis, and GA edited the manuscript.

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Figure Legends

Fig. 1 Effect of preconditioning temperature (25ºC or 10ºC), on plant height (**a**) and growth rate (**b**) of genotype S41 after defoliation and plant re-growth for six weeks in each of the eight environments as indicated in Table 1. Different letters in the graph represent significant differences between treatments ($P < 0.05$; $n = 3$).

Fig. 2 Effect of preconditioning temperature (25°C or 10°C), on total non-structural carbohydrates (NSC) starch, sucrose and reducing sugars, in leaves (**a**), stems (**b**) and roots (**c**) of genotype S41 after defoliation and plant re-growth for six weeks in each of the eight environments as indicated in Table 1. Bars represent mean values ± SE, (*n*=3 replicate plants). Different letters in the graph represent significant differences between treatments for total NSPs ($P < 0.05$).

Fig. 3 Effect of preconditioning temperature (25°C or 10°C), on soluble and insoluble tannins in leaves (**a**), stems (**b**) and roots (**c**) of genotype S41 after defoliation and plant re-growth for six weeks in each of the eight environments as indicated in Table 1. Bars represent mean values ± SE, (*n*=3 replicate plants). Different letters in the graph represent significant differences between treatments for total tannins ($P < 0.05$).

Fig. 4 Effect of preconditioning temperature (25ºC or 10ºC), on the major flavonoids in leaves (**a**) and stems (**b**) of genotype S41 and after defoliation and plant re-growth for six weeks in each of the eight environments as indicated in Table 1. Bars represent mean values ± SE, (*n*=3 replicate plants). K-3G = kaempferol-3-glucoside*, K-3,7dR = kaempferol-3,7-dirhamnoside*, K-7R = kaempferol-7-rhamnoside*, K-3R-7G = kaempferol-7-rhamnoside-3 glucoside*. Peaks identified by reference to authentic standards. Different letters in the graph represent significant differences between treatments for total flavonoids ($P < 0.05$).

Fig. 5 Effect of preconditioning temperature (25ºC or 10ºC), on total thioglycolic acid soluble lignin in leaves (**a**) and stems (**b**) of genotype S41 after harvesting and plant re-growth in each of the eight environments for six weeks as indicated in Table 1. Bars represent mean values ± SE, (*n*=3 replicate plants. Different letters in the graph represent significant differences between treatments ($P < 0.05$).

Fig. 6 Effect of preconditioning temperature (25ºC or 10ºC), on the initial rate of gas evolution in a simulated rumen environment of leaves (**a**) and stems (**b**) of genotype S41 after harvesting and plant re-growth for six weeks in each of the eight environments as indicated in Table 1. Bars represent mean values ± SE (*n*=3) replicate plants. Different letters in the graph represent significant differences between treatments ($P < 0.05$).

Figure 1

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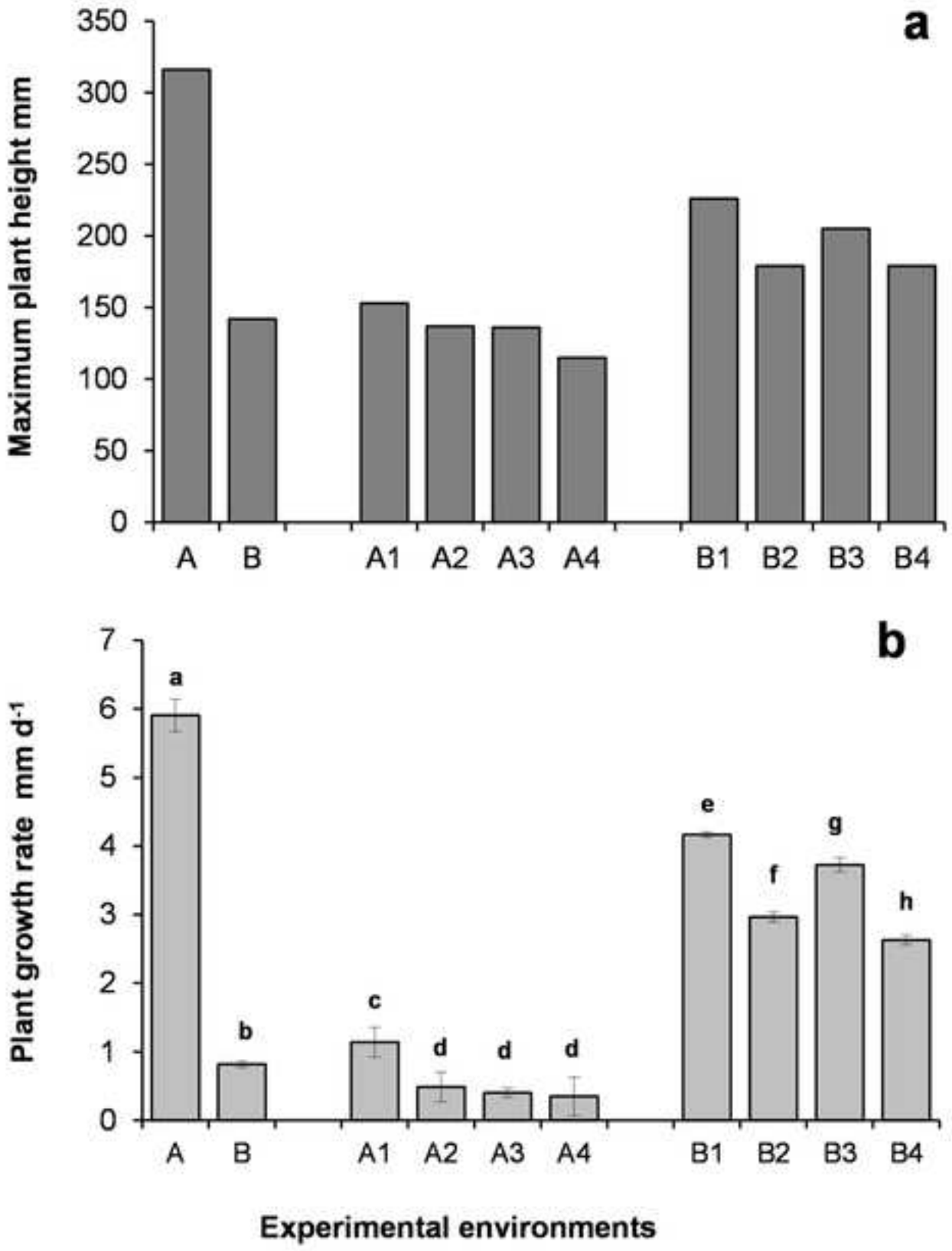

Figure 2

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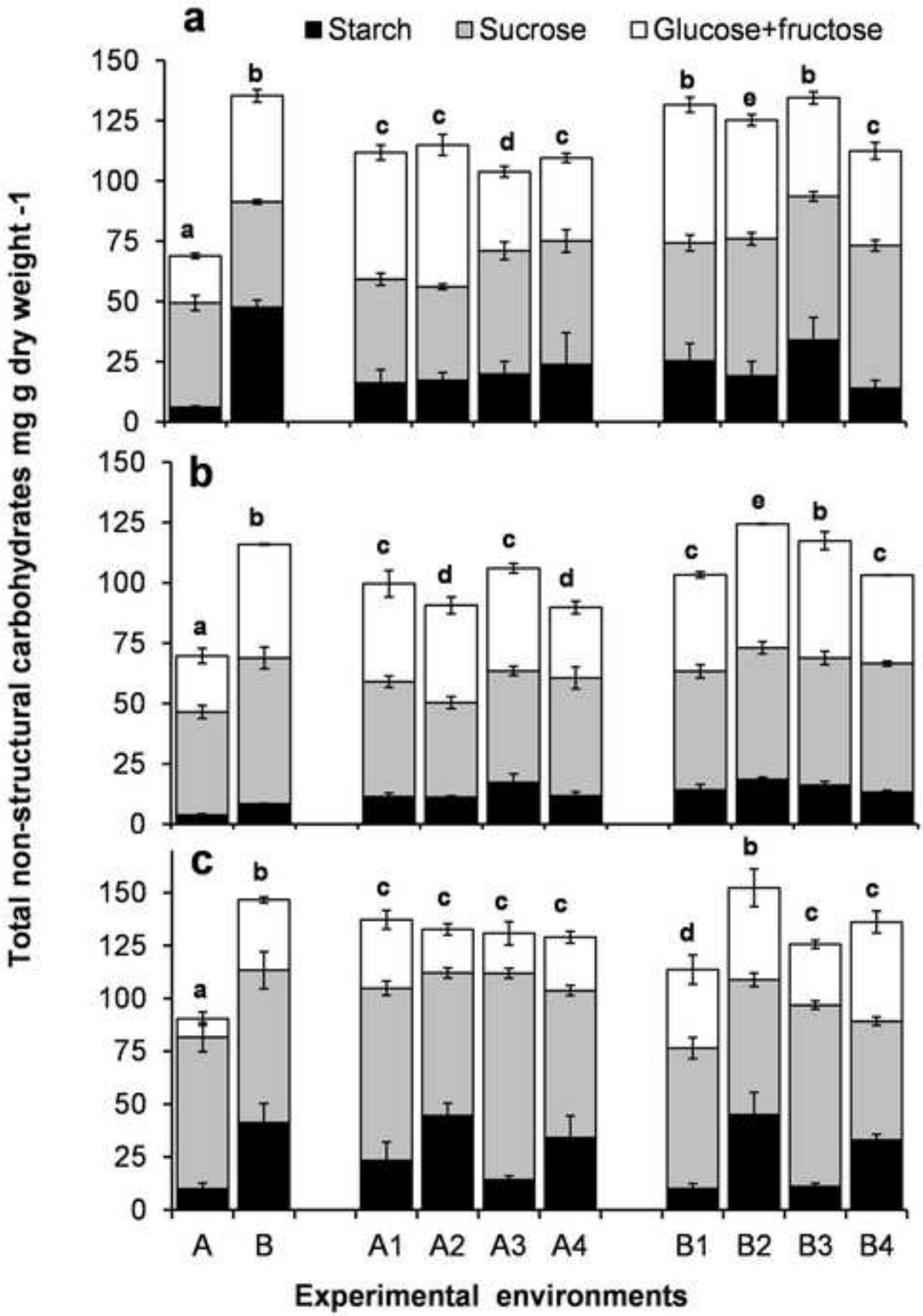

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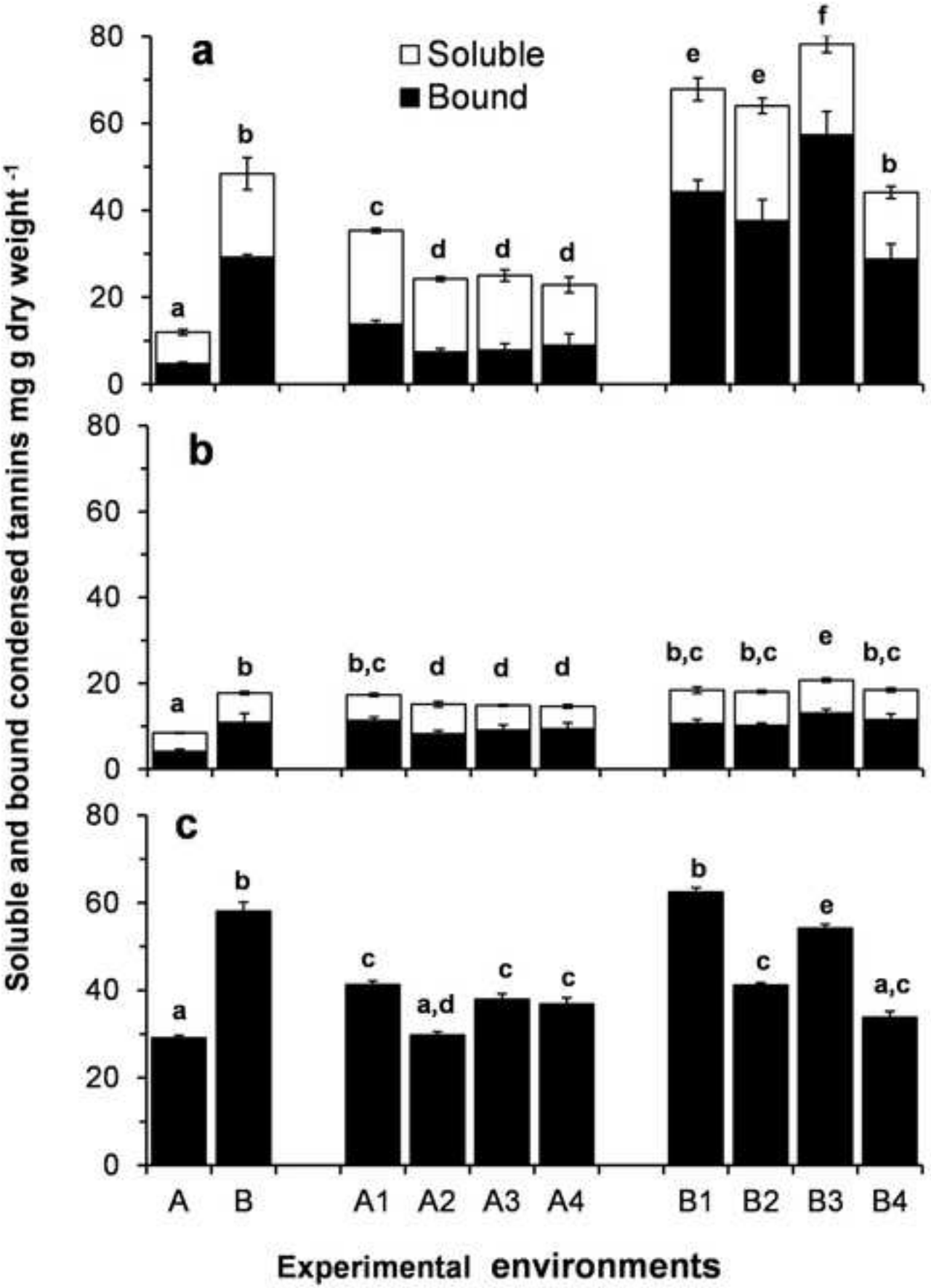

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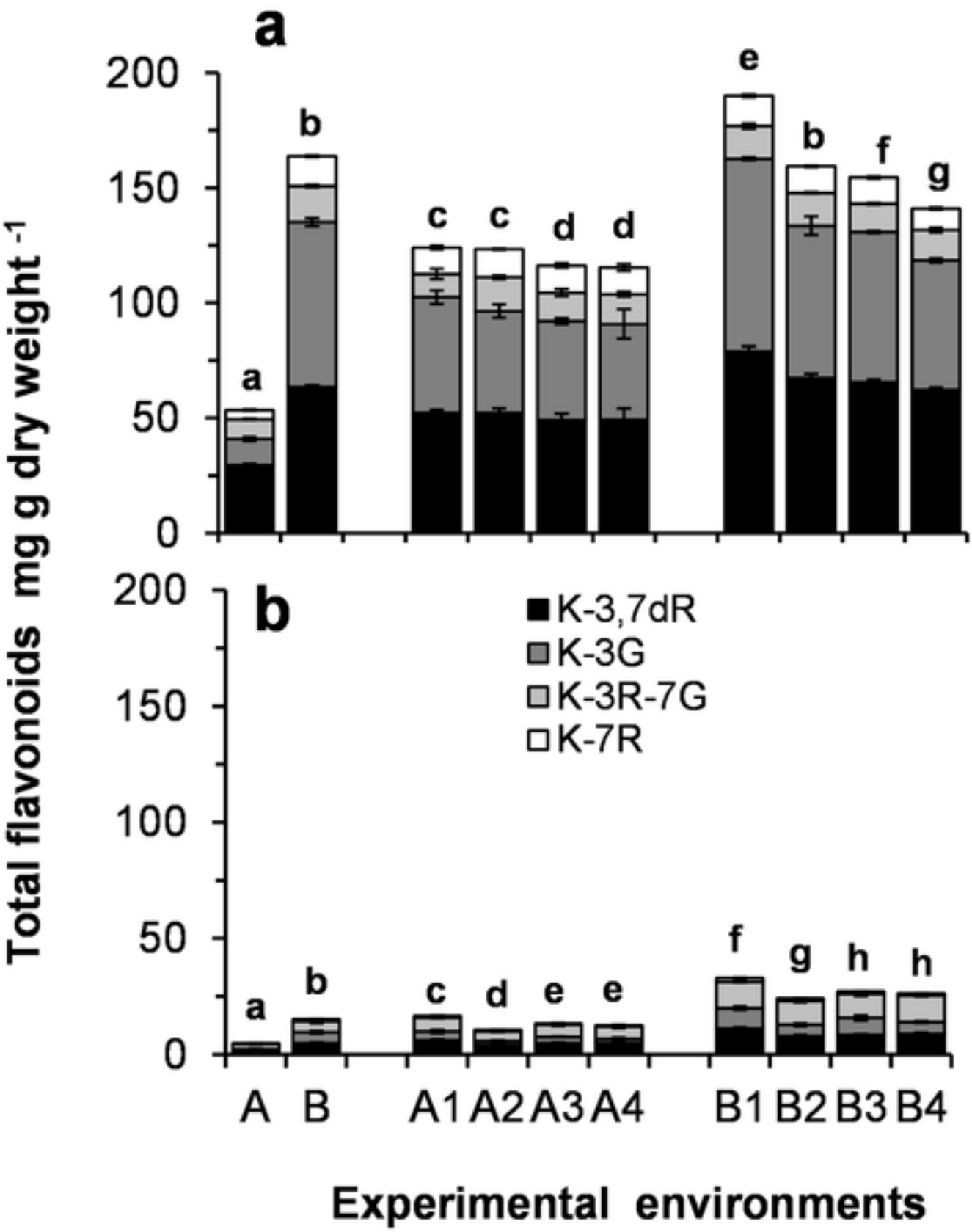

Figure 5

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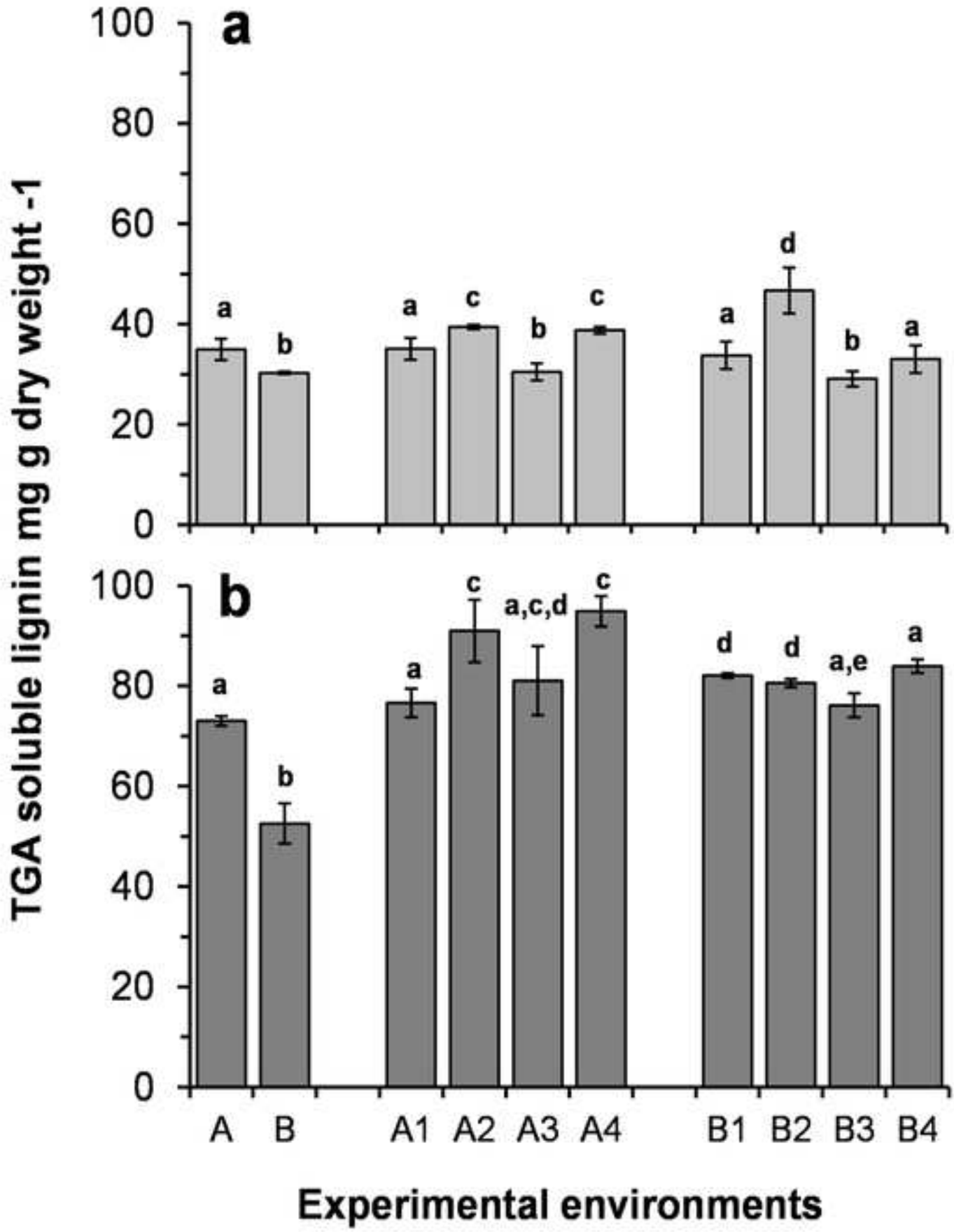

Figure 6

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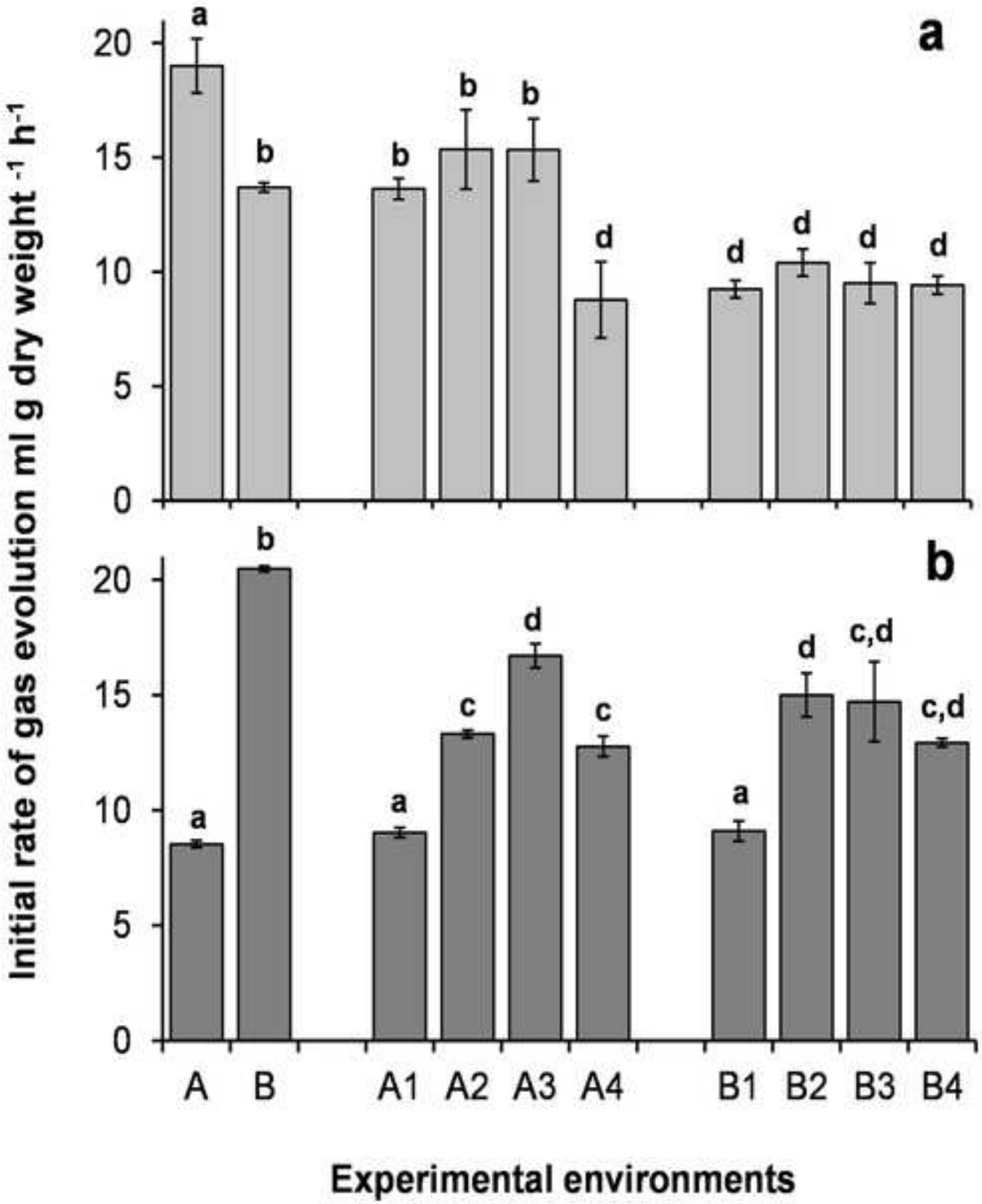

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Responses of Lotus corniculatus to environmental change. 4.
Root carbohydrate levels at defoliation and regrowth climatic conditions are important drivers of variation in phenolic content and forage quality.

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Tables

Table 1. Experimental environments. A and B indicate the preconditioning (light intensity, 450 µmol m^{-2} s^{-1}) received before plants of genotype S41 were placed into either experimental environments A 1-4 or B 1-4. Environment B3 was the control environment to reflect average midsummer conditions in the United Kingdom.

Growth Environment	Temperature (°C) (Day/Night)	CO_2 (ppmv)	Water (% of ad libitum)	Days Growth
Precondition A	25/25	350	100	56
Precondition B	10/10	350	100	56
A1	25/15	700	100	42
A2	25/15	700	60	42
A3	25/15	350	100	42
A4	25/15	350	60	42
B1	18/10	700	100	42
B2	18/10	700	60	42
B3	18/10	350	100	42
B4	18/10	350	60	42

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