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Seasonal and species variation in raffinose, short-chain fructan, and long-chain fructan accumulation in tall fescue (*Festuca arundinacea* Schreb.) and timothy (*Phleum pratense* L.) grown in Central Kentucky

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Abstract

Fructans in cool-season grasses may have some negative effects on equine health. However, they may have positive effects on ruminant performance, and fructans of different lengths appear to be metabolized differently in the rumen. Hence, seasonal variation in fructan concentrations may impact equine and ruminant performance. Long-chain fructan with degree of polymerization (DP) > 8, short-chain fructan (DP 4 to 8), raffinose, and three fructan trisaccharides were profiled and quantified in timothy (*Phleum pratense* L.) cultivar 'Clair' and tall fescue (*Festuca arundinacea* Schreb.) cultivar 'Cajun II' harvested in April, June, August, and October of two consecutive years in central Kentucky. Harvest year influenced concentrations of long-chain fructan ($p = .0017$). Harvest date influenced species differences in raffinose ($p = .0035$), which was most abundant in timothy in June, and in 1-kestose and neokestose ($p < .0001$), which were most abundant in tall fescue in April. Harvest date influenced species differences in short- and long-chain fructan ($p < .0001$). Tall fescue had two- to three-fold more short-chain fructan than timothy in April, August, and October. Timothy had two- to five-fold more long-chain fructan than tall fescue in April, June, and October. Species choice and weather patterns may have contributed to relatively low concentrations of all the carbohydrates measured in this study. Fermentation or feeding studies could help to determine if the concentrations present could affect equine health or ruminant performance.

KEYWORDS
cool-season grasses, fructan, high-performance anion-exchange chromatography (HPAEC), raffinose, tall fescue, timothy

1 | INTRODUCTION

The water-soluble carbohydrates (WSCs) of cool-season grasses include mono- and disaccharides, as well as raffinose (Chatterton et al., 1990) and fructans. The latter are linear or branched polymers of fructose, usually with a glucose moiety at the end of or within the polymer (Benkeblia, 2013). Fructans with a chain length, or degree of

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polymerization (DP) less than 10 tend to be referred to as fructooligosaccharides (FOS), while longer polymers are referred to as fructans (Benkeblia, 2013). Among the FOS are tri- and tetrasaccharides. The fructose moieties in FOS and fructans can be β-1,2-linked or β-2,6-linked, and they can be linear or branched (Benkeblia, 2013). Inulin, found in plants such as chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.), is an example of the linear β-1,2 linkage type; and levan, synthesized in bacteria and some cool-season grasses, is an example of the linear β-2,6 linkage type (Vijn & Smeekens, 1999). Beta-2,6-linked fructan is also called phlein (Suzuki, 1968). Fructan produced by timothy (*Phleum pratense* L.) is β-2,6-linked (Cairns et al., 1999). Besides these linear fructans, branched fructans with both linkage types exist (Benkeblia, 2013). Branched FOS, such as bifurcose, have been identified in cool-season grasses such as cocksfoot (*Dactylis glomerata* L.; Chatterton et al., 1993). In this study, FOS and fructans are referred to as short-chain and long-chain fructan, respectively.

In the rumen of grazing animals, fermentation of carbohydrates from grasses provides energy for rumen bacteria to convert amino acids into microbial protein, whereas an absence of carbohydrates results in amino acids being converted into ammonia and subsequently being excreted as urea (Marley et al., 2007; Miller et al., 2001). In vitro, fructans (chicory inulin and bacterial levan) decreased ammonia accumulation in rumen fluid from cattle and sheep (Biggs & Hancock, 1998). Dairy cows fed perennial ryegrass (*Lolium perenne* L.) of differing WSC concentrations excreted about 30% less nitrogen in the urine when fed a high-WSC cultivar than when fed a standard cultivar (Miller et al., 2001). Grazing lambs had a higher liveweight gain on high-WSC perennial ryegrass than on the standard perennial ryegrass cultivar (Lee et al., 2001). Hence, the presence of WSCs can be beneficial in terms of both environmental management and ruminant performance.

An understanding of the fructan composition of forage grasses may aid in managing ruminant performance because polymers like fructans are fermented more slowly than glucose, and the fructan fermentations yield different organic acids for the animal to utilize, compared to the organic acids profile arising from glucose fermentations (reviewed by Klevenhusen & Zebeli, 2021). Glucose fermentation by mixed ruminal microbiota has been found to yield greater amounts of lactate than fructan fermentations (Hall & Weimer, 2016).

Those results suggest that the presence of fructans in cool-season grasses, like the presence of WSC as a whole, may affect rumen fermentation and ruminant performance. In addition, fructan chain length may affect fermentation patterns, because in vitro studies with mixed bovine ruminal microbiota resulted in long-chain fructans being metabolized prior to short-chain fructans (Kagan et al., 2022). An understanding of seasonal and species variation in fructan concentration and DP may complement assessment of forage WSCs when managing ruminant performance.

In contrast to the results with ruminants, studies with horses indicate potential negative effects of fructans. Feeding haylage higher in nonstructural carbohydrates (WSCs and starch), and with higher fructan concentrations, was positively correlated with insulin response variables in horses (Lindåse et al., 2018). This correlation may represent an increased risk for laminitis because large increases in serum insulin have been observed in laminitis-prone ponies fed grass high in fructans or hay supplemented with inulin (Bailey et al., 2007).

Horses fed large amounts of short-chain inulin (7.5 to 12.5 g · kg body weight^{-1}) developed laminitis within 48 h (Van Eps & Pollitt, 2006). Short-chain inulin (Raftilose P95, which has a DP of about 12 (Twomey et al., 2003)) may not be the best model for grass fructans, which can have a DP over 100 (Suzuki, 1989). However, when horses were fed inulin of DP 2 to 60 (3 g · kg body weight^{-1}), faecal pH decreased, and faecal concentrations of a few amines linked to digital blood flow increased (Crawford et al., 2007). The pH decline suggested that fermentation of longer fructans might increase the production of lactic acid by bacteria within the gastrointestinal tract, and the increase in amine concentrations suggested that digital blood flow could be affected as well, with a risk of decreased blood flow to the feet (Crawford et al., 2007). Lactate, if in excess of amounts metabolisable by lactate-utilizing bacteria, may lead to a decline in pH, which may lead to epithelial damage, a subsequent influx of toxins, and eventually a laminitic episode (Ince et al., 2013). An in vitro study with timothy demonstrated that incubation of equine digesta with timothy high in long-chain fructan resulted in greater total lactate than did incubation of equine digesta with timothy lower in long-chain fructan (Ince et al., 2013). Whether differences in glucose or fructose concentrations (1.5- and 10-fold higher, respectively, in the timothy highest in long-chain fructan) might have contributed to observed differences between fermentations with the different grasses was not discussed.

Both species and environment affect fructan characteristics. Fructan concentrations vary with season (Jensen et al., 2014;Pollock & Jones, 1979; Suzuki, 1989), precipitation (Volaire & Lelièvre, 1997), and temperature (Suzuki, 1989). Fructan DP is affected by season (Pollock & Jones, 1979; Suzuki, 1989) and temperature (Suzuki, 1989; Thorsteinsson et al., 2002). Species also differ in the relative sizes of fructans present (Suzuki, 1989). Seasonal changes in fructan DP and/or concentration have been followed in various grasses in Britain (Pollock & Jones, 1979), Canada (Suzuki, 1989), and the High Plains region of the United States (Jensen et al., 2014), but only briefly and for one species (Brown & Blaser, 1965) in the transition zone of the United States, where a substantial number of beef cattle are raised.

When fructan tri- and tetrasaccharides were profiled in eight cool-season grass species grown under controlled low-temperature conditions, some trisaccharides were common to all species, but some differed (Chatterton et al., 1990). Raffinose was also present in *Festuca novae-zelandiae* exposed to drought stress (Clark et al., 2004), and it has been quantified in cold- and drought-stressed *Deschampsia antarctica* (Cui et al., 2020). However, seasonal effects on these trisaccharides have not been determined in grasses of the transition zone of the United States. The purpose of this study was to examine seasonal changes in the concentration and composition of short-chain and long-chain fructan, as well as changes in raffinose and three fructan trisaccharides, from timothy and tall fescue (*Festuca arundinacea* Schreb.) harvested from April to October in the transition zone.

2 | MATERIALS AND METHODS

2.1 | Forage establishment and sampling

Timothy cultivar (cv.) 'Clair' and tall fescue cv. 'Cajun II' were among five cool-season grasses and one warm-season grass planted on 10 September 2019. The fructan profiling of timothy and tall fescue was part of a larger study. The field plots were planted at the Spindletop Research Farm of the University of Kentucky, Lexington, KY, USA (38°07′39″ N, 84°30′04″ W, coordinates determined from Google Earth map). Four replicate plots of each grass (1.5 × 4.6 m per plot) were planted in a randomized complete block design. Plots were irrigated on 24 September 2019 and 2 October 2019.

Grasses were harvested in April, June, August, and October of 2020 and 2021. In 2020, tall fescue was harvested on 15 April, and timothy was harvested on 21 April because it was slightly delayed in growth. Plots were mowed after each harvest. Harvest dates, and approximate weeks of regrowth prior to each harvest, are in Table 1.

TABLE 1 Harvest dates and approximate weeks of regrowth prior to each harvest, 2020 and 2021.

Harvest date	Approximate weeks of regrowth represented
15 April 2020 (tall fescue) 21 April 2020 (timothy)	First growth of season
16 June 2020	5 weeks (mowed in May)
18 August 2020	8 weeks[a]
8 October 2020	6 weeks[a]
19 April 2021	First growth of season
15 June 2021	5 weeks (mowed in May)
20 August 2021	8 weeks
20 October 2021	8 weeks

[a]Plots were mowed 8 days after the harvest date.

Grasses were harvested between noon and 4:00 PM, and duration of harvesting varied with the amount of field assistance. Afternoon harvesting was chosen to minimize diurnal variation in WSC concentrations (Kagan et al., 2018).

Weather data (maximum and minimum temperature, and precipitation) were acquired from weather stations located at the Spindletop Research Farm. Data from 2020 were acquired from a weather station operated by the University of Kentucky Ag Weather Centre, and data from 2021 were acquired from a weather station operated by the University of Kentucky Soil Physics Lab. Cumulative precipitation between harvest dates is in Table 2, as are mean maximum and minimum temperatures between harvest dates. Daily maximum and minimum temperatures and total precipitation, from 5 days before the first harvest of the season until the final harvest date in October, are displayed in Figure S1.

Samples (200 to 1000 g, depending on forage availability and moisture content of forage when cut) were collected by cutting 5 cm above the ground with hand shears (April 2020 and June 2020), with an electric hedge trimmer in October 2020 (Black and Decker, Towson, MD, USA), or on other dates with a sickle bar mower (BCS America, Oregon City, OR, USA). Seedheads and dead leaves were manually sorted out of the samples. Insufficient tissue was available from two timothy plots in 2020 (one in August, and one in October) due to a large amount of dead material, leading to three replicates instead of four for those two harvest dates. In October of 2021, insufficient tissue was available from two timothy plots due to poor growth and a large proportion of dead material, leading to two replicates for the October harvest date.

Samples were transported on ice to storage at −20°C and subsequently lyophilised (model 18DX48SA, Botanique Preservation Equipment, Phoenix, AZ, USA). The entire sample from each field plot was ground through a 4-mm mesh in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). A 2- to 5-g subsample was

TABLE 2 Total precipitation, and mean maximum and minimum temperatures between harvests in 2020 and 2021. Intervals do not include harvest days.

Harvest interval	Total precipitation, mm	Mean maximum temperature, °C (± SD)[b]	Mean minimum temperature, °C (± SD)[b]
10 through 14 April 2020 (tall fescue)[a]	61.2	14.2 ± 4.4	2.3 ± 4.6
16 through 20 April 2020 (timothy)[a]	10.2	15.6 ± 2.9	2.6 ± 3.3
14 through 18 April 2021	10.2	14.4 ± 1.7	3.2 ± 2.6
22 April through 15 June 2020	432	23.6 ± 6.2	12.2 ± 6.1
20 April through 14 June 2021	236	22.9 ± 6.1	11.3 ± 6.4
16 June through 17 August 2020	295	31.9 ± 2.7	19.9 ± 2.4
16 June through 19 August 2021	253	28.8 ± 2.6	18.3 ± 3.2
19 August through 7 October 2020	185	27.1 ± 4.3	14.6 ± 5.8
21 August through 19 October 2021	231	26.7 ± 3.8	14.9 ± 4.2

[a]In April 2020, tall fescue and timothy were harvested on separate days (15 April 2020 for tall fescue and 21 April 2020 for timothy).
[b]SD, standard deviation.

subsequently ground through a 1-mm mesh in a cyclone mill (model CT293 Cyclotec, FOSS North America Inc., Eden Prairie, MN, USA). The proportion of dry matter (DM) in each freeze-dried, ground subsample was determined by drying a 0.5-g portion for 24 h (95 to 100°C).

2.2 | Reagents and chemicals

Raffinose and 1-kestose were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6-kestose, neokestose, 1-nystose, bifurcose, and 6-nystose were a kind gift from Dr. P.A. Harrison of the USDA-ARS Forage and Range Research Lab (Logan, UT, USA), as were purified β-2,6-linked cocksfoot (*Dactylis glomerata* L.) fructan (DP 7 to >60) and β-2,6-linked fructan of DP 4 to 12 from Sandberg bluegrass (*Poa secunda* J. Presl). Orafti inulins (trade name OPS, short-chain; and trade name HP, long-chain) were a kind gift from Beneo Inc. (Parsippany, NJ, USA). Sodium hydroxide solution (50% w/w) was purchased from Fisher Scientific (Waltham, MA, USA), and anhydrous sodium acetate was purchased from Sigma-Aldrich. Methanol was purchased from VWR (Radnor, PA, USA). Millipore water (18 MΩ, Millipore Sigma, Burlington, MA, USA) was used for all extractions and separations.

2.3 | WSC extraction and preparation for analysis

Samples were replicated by plot (four plots per timepoint, except for two missing timothy samples in 2020 and two missing timothy samples in 2021, as mentioned in Section 2.1). Samples (100 mg) were extracted by shaking in water at ambient temperature for 1 h, as described by Kagan et al. (2018). Extracts were filtered through #4 filter paper (Whatman, GE Healthcare, Buckinghamshire, England) and brought to 25 mL with water. These extracts were put through end-capped C_{18} solid-phase extraction columns (Clean-Up, United Chemical Technologies, Bristol, PA, USA) containing 100 mg sorbent. Columns were conditioned with methanol and water as described by Kagan et al. (2022), after which 0.9 mL of sample was vacuum-filtered through the column and collected. SPE-cleaned filtrates were stored at −20°C until analysis. On the day of analysis, filtrates were thawed and sonicated 4 min at 40 to 45°C in a sonicating water bath (model 5510, Branson Ultrasonics Corporation, Brookfield, CT, USA) to dissolve fructans that might have precipitated while frozen (Chatterton & Harrison, 1997). Sonicated filtrates were vortexed and diluted 8-fold in water for high-performance anion-exchange chromatography (HPAEC) analysis.

2.4 | HPAEC analysis

Samples, replicated by plot, were separated on an ICS5000 HPAEC system (Thermo Fisher Scientific, Waltham, MA, USA). Injections (25 µL) were separated on a Thermo Fisher CarboPac PA200 anion-exchange column (3 mm i.d. × 250 mm length) equipped with a guard column (3 mm i.d. × 50 mm length). Column particle size was 5.5 µm. Solvent A was 0.1 M sodium hydroxide, and solvent B was 1 M anhydrous sodium acetate in 0.1 M sodium hydroxide. The programme used for separation (at 28°C) was that of Kagan et al. (2011), except that in steps employing 5% A/95% B, 100% B was used. Pulsed amperometric detection, with a gold electrode, a silver chloride pH electrode, and a quadruple potential waveform, was employed to detect WSCs. Peaks were integrated with Chromeleon software (version 7.2.10). The gold electrode was polished at the start of analyses and once during the study, when a series of small peaks within the fructan region of the chromatogram was observed in water blanks, suggesting electrode contamination.

2.5 | Detection and quantification of long- and short-chain fructan, raffinose, and fructan trisaccharides

Raffinose, 1-kestose, 6-kestose, and neokestose, as well as glucose, fructose, sucrose, 1-nystose, bifurcose, and 6-nystose were identified by comparing peak retention times to those in a mixture of standards (Figure 1a). Peaks were quantified as a function of peak area, using linear calibration curves from 0 to 20 or 25 µM oligosaccharide (Table 3). New calibration curves were run after each time that the gold electrode was polished (two sets of calibration curves total). Although peaks with the retention times of 1-nystose, bifurcose, and 6-nystose were present in some samples, based on a mixture of standards, they were not quantified because the presence of those sugars had not been reported in those species previously (Chatterton et al., 1990), and there was no method besides retention time to confirm their presence.

The peak ranges in the long- and short-chain inulin standards complemented each other relatively well, with long-chain inulin beginning approximately where short-chain inulin ended (Figure 1b). In WSC extracts of grass samples, peaks eluting within the range of the short-chain inulin (retention time 11.8 to 19.4 min, Figure 1a) were treated as short-chain fructan. The DP of the short-chain inulin peaks within those retention times was 4 to 8 when compared to *Poa secunda* fructan (β-2,6-linked FOS) of DP 4 to 12 (Figure 1b) and to 1-nystose (β-2,1-linked tetrasaccharide), which elutes before its β-2,6-linked counterpart, 6-nystose (Figure 1a). In extracts, areas of individual peaks eluting between 1-nystose (11.67 to 12.02 min) and 19.9 min were summed, and total short-chain fructan was estimated based on the calibration curve for short-chain inulin in Table 3. The latter was calculated by summing the areas of all peaks eluting from 1-nystose onward (see overlaid chromatogram on Figure 1a). Although this quantification method overlooked the variation in slopes among different oligosaccharides (Pöhnl et al., 2017), it gave an approximation that permitted monitoring changes in this region.

Peaks eluting after 20 min and hence after the peak of DP 8 in the *P. secunda* fructan (Figure 1b) were treated as long-chain fructan. Areas of individual peaks were summed, and total long-chain fructan

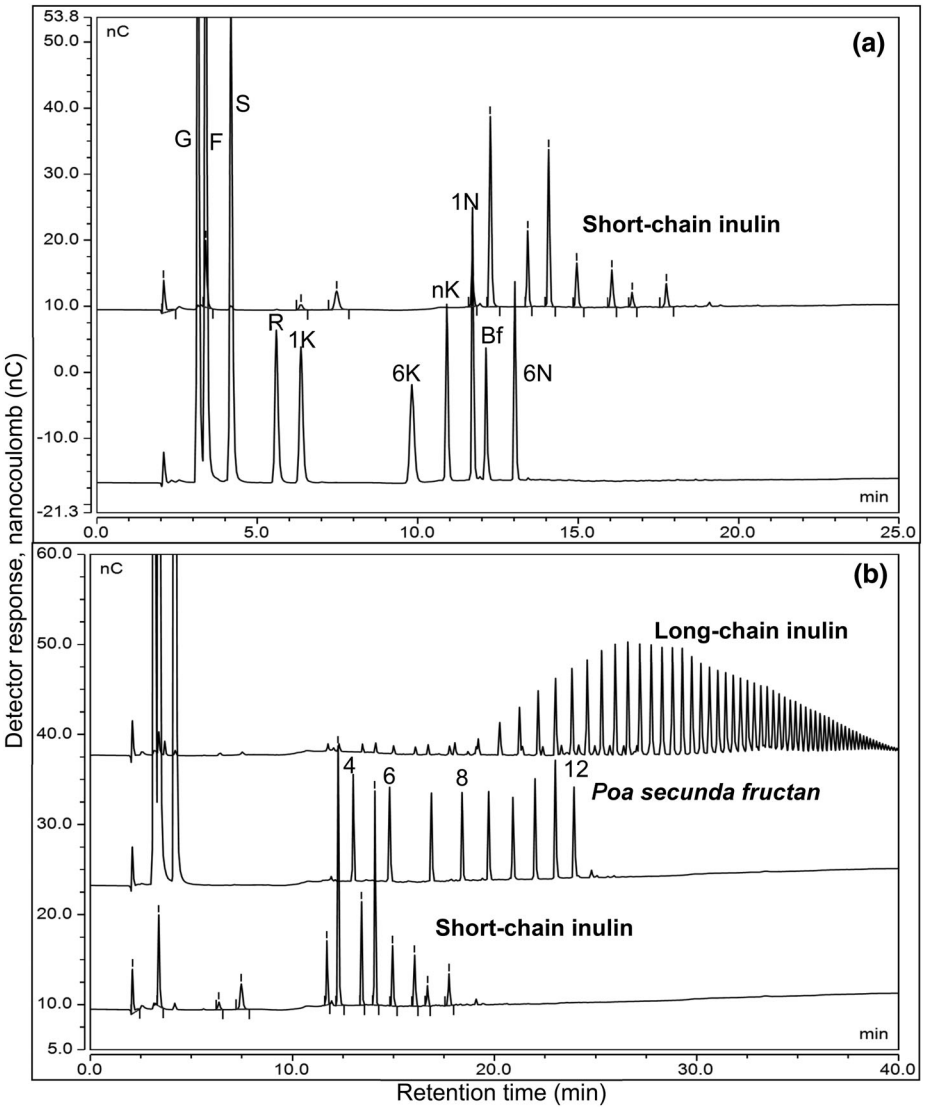

FIGURE 1 High-performance anion-exchange chromatography (HPAEC) profiles of (a) various sugars overlaid with short-chain chicory inulin and (b) long- and short-chain chicory inulin and fructans of degree of polymerization (DP) 4 to 12 from *Poa secunda* (Sandberg bluegrass). Abbreviations in Figure 1a: G, glucose; F, fructose; S, sucrose; R, raffinose; 1K, 1-kestose; 6K, 6-kestose; nK, neokestose; 1N, 1-nystose; Bf, bifurcose; 6N, 6-nystose. Numbers in Figure 1b correspond to the degree of polymerization (DP) of the β-2,6-linked fructans in *P. secunda*.

TABLE 3 Slope, *y*-intercept, and R^2 value of standard curves for short-chain inulin of degree of polymerization (DP) 4 to 8, long-chain inulin of DP 8 to 70, and oligosaccharides raffinose, 1-kestose, 6-kestose, and neokestose.[a,b]

Standard	Concentration range	Slope (m)	*y*-intercept (b)	R^2
Raffinose	0 to 20 μM	2.173	0.2921	0.9979
		2.1634	0.3099	0.9979
1-kestose	0 to 20 μM	2.429	0.3524	0.9971
		1.9477	0.3245	0.9972
6-kestose	0 to 20 μM	2.192	0.219	0.9987
		2.1227	0.2479	0.9987
Neokestose	0 to 20 μM	1.658	0.466	0.9919
		1.4858	0.4068	0.9932
Short-Chain Inulin, DP 4 To 8	0.25 to 20 μg/mL	3.3634	0.6055	0.9978
		2.898	0.3789	0.9987
Long-Chain Inulin, DP 8 To 70	1 to 50 μg/mL	1.3933	−1.2666	0.9999
	1.25 to 50 μg/mL	1.4684	−1.3708	0.9999

[a]Standard curves are based on peak areas, with short-chain and long-chain fructans representing a summation of peaks at each concentration.
[b]Second row of values represents slope, *y*-intercept, R^2, and range obtained after the gold electrode was polished partway through the analyses.

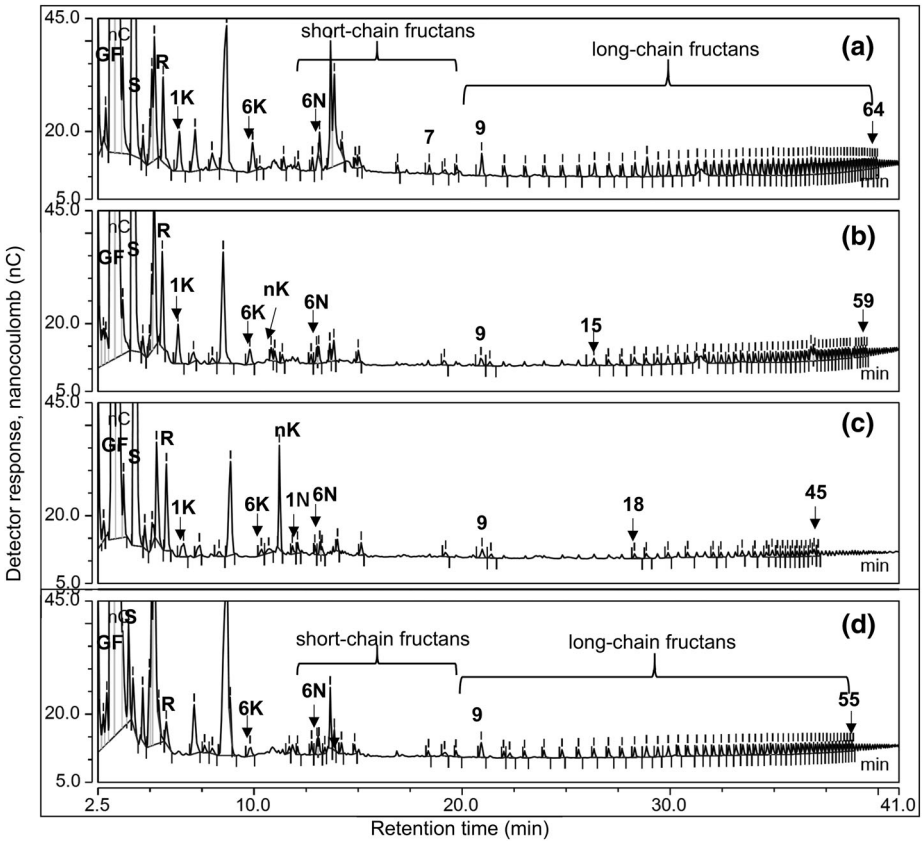

FIGURE 2 High-performance anion-exchange chromatography (HPAEC) profiles of timothy sampled in April (a), June (b), August (c), and October (d) of 2021. Poorly-resolved glucose (G) and fructose (F) peaks are designated, along with sucrose (S), raffinose (R), 1-kestose (1K), 6-kestose (6K), neokestose (nK), 1-nystose (1N) and 6-nystose (6N). Numbers indicate the degree of polymerization (DP) of fructans, based on alignment with fructan of DP 4 to 12 from *Poa secunda* (Sandberg bluegrass).

was estimated based on the calibration curve for long-chain inulin in Table 3. Total long-chain fructan was counted as zero if the summed peak areas were less than 90% of the lowest value in the standard curve, because it seemed uncertain that the fructan could be quantified accurately when below the lower limit of the standard curve. In constructing the standard curve, some peaks with a DP below 8 were also detected in the standard of long-chain inulin at higher concentrations (Figure 1b). These, which contributed 0.4% to 2.6% of the total peak area (data not shown), were omitted from the peak summation for the standard.

2.6 | Statistical model

All data were analysed using the MIXED procedure with repeated measures of SAS (SAS version 9.3; SAS Inst. Inc., Cary, NC, USA). The model included plot as the experimental unit and the variable of interest (e.g., raffinose, kestose, etc.) as the dependent variable. Species (tall fescue or timothy), harvest date, year, and the interactions of species × year and species × harvest date were included as fixed effects in the model. The Kenward-Roger method was used to compute the denominator degrees of freedom for each fixed effect. When a main effect was detected, means were separated using the pdiff option. Statistical significance was set at $p \leq .05$ and trends were defined at $p \leq .10$. The p-values and standard error of the mean (SEM) for main effects and interactions are shown in Table 4.

3 | RESULTS

3.1 | Weather patterns and general growth patterns

Lowest mean temperatures were in April, followed by October, in both years (Table 2). The total precipitation between the April and the June harvests of 2020 was about twice that of the total precipitation between the April and the June harvests of 2021 (Table 2).

In April of both harvest years, growth was quite abundant, resulting in relative ease of obtaining 1 kg fresh material per plot. In June of both years, timothy plots had many seedheads (which were discarded), despite the plots having been mowed in mid-May. In October of both years and August of 2021, considerable dead material was present in plots, and less tissue could be harvested per plot. Grass blades were harvested if the blade below a brown tip was still green, so the milled tissue samples contained a mixture of living and dead material.

3.2 | Effects of harvest and species on trisaccharide concentrations

An interaction of harvest date and species influenced concentrations of raffinose ($p = .0035$), 1-kestose ($p < .0001$), and neokestose ($p < .0001$). A trend for an interaction of species and harvest date ($p = .0572$) was present for 6-kestose. Raffinose in timothy (0.7 to

TABLE 4 *p*-values and standard error of the mean (SEM) for main effects of species, harvest, and year on concentrations of trisaccharides, short-chain fructan of degree of polymerization (DP) 4 to 8, and long-chain fructan of DP >8, as well as for species by year interactions on those carbohydrates.[a,b]

	Raffinose	1-Kestose	6-Kestose	Neokestose	Short-chain fructan	Long-chain fructan
Species	$p = .0096$ SEM 0.13	$p = .0005$ SEM 0.14	$p = .0007$ SEM 0.08	$p < .0001$ SEM 0.14	$p < .0001$ SEM 0.28	$p < .0001$ SEM 0.76
Harvest	$p = .0002$ SEM 0.15	$p < .0001$ SEM 0.17	$p = .0228$ SEM 0.10	$p < .0001$ SEM 0.19	$p < .00001$ SEM 0.40	$p < .0001$ SEM 1.12
Year	$p = .299$, SEM 0.13	$p = .309$, SEM 0.14	$p = .199$ SEM 0.08	$p = .856$ SEM 0.14	$p = .525$ SEM 0.28	$p = .0017$ SEM 0.78
Species × year	$p = .703$, SEM 0.16	$p = .568$, SEM 0.18	$p = .341$, SEM 0.10	$p = .837$, SEM 0.19	$p = .964$, SEM 0.42	$p = .518$, SEM 1.16

[a]*p*-values and SEM for interactions of species by harvest date on the above-listed carbohydrates are shown in Table 4.
[b]Means for each harvest date are determined from four field replicates, except for timothy in August 2020 ($N = 3$), October 2020 ($N = 3$), and October 2021 ($N = 2$).

TABLE 5 Interaction of species by harvest date effects on concentrations (mg · g DM^{-1}) of raffinose, 1-kestose, 6-kestose, neokestose, short-chain fructan with degree of polymerization (DP) of 4 to 8, and long-chain fructan (DP >8) from timothy or tall fescue.[1,2]

		Harvest				Statistics	
Component (mg g DM^{-1})	Species	April	June	August	October	*p*-value	SEM
Raffinose	Timothy	0.71^{b}	$1.71^{a,\alpha}$	0.67^{b}	$0.94^{b,\alpha}$	$p = .0035$	0.20
	Tall Fescue	1.17^{a}	$0.88^{a,\beta}$	0.34^{b}	$0.29^{b,\beta}$		
1-kestose	Timothy	0.39^{β}	0.55	0.22	0.78	$p < .0001$	0.23
	Tall Fescue	$3.18^{a,\alpha}$	0.55^{b}	0.14^{b}	0.41^{b}		
6-kestose	Timothy	$0.56^{a,\alpha}$	0.19^{b}	0.13^{b}	$0.73^{a,\alpha}$	$p = .0572$	0.13
	Tall Fescue	0.20^{β}	0.13	0.015	0.034^{β}		
Neokestose	Timothy	0.13^{β}	0.013^{β}	0.25	0.67	$p < .0001$	0.26
	Tall Fescue	$4.85^{a,\alpha}$	$0.66^{b,\alpha}$	0.12^{b}	0.70^{b}		
Short-chain Fructan	Timothy	$4.41^{a,\beta}$	1.51^{b}	$1.15^{c,\beta}$	$2.81^{b,\beta}$	$p < .0001$	0.58
	Tall Fescue	$12.0^{a,\alpha}$	1.05^{d}	$2.63^{c,\alpha}$	$5.53^{b,\alpha}$		
Long-chain fructan	Timothy	$36.4^{a,\alpha}$	$15.5^{b,\alpha}$	1.69^{d}	$10.1^{c,\alpha}$	$p < .0001$	1.62
	Tall Fescue	$6.74^{a,\beta}$	$3.16^{ab,\beta}$	0.00^{b}	$5.08^{a,\beta}$		
Total fructans and raffinose	Timothy	42.6	19.5	4.1	16.0		
	Tall fescue	28.1	5.6	3.2	12.0		

[1]Values with different Latin letters (^{a,b,c}) are statistically different within species between harvests ($p < .05$); values with different Greek letters ($^{\alpha,\beta}$) are statistically different within harvest between species ($p < .05$).
[2]Means for each harvest date are determined from four field replicates, except for timothy in August 2020 ($N = 3$), October 2020 ($N = 3$), and October 2021 ($N = 2$).

1.7 mg · g DM^{-1}) was highest in June, while raffinose in tall fescue (0.3 to 1.2 mg · g DM^{-1}) was highest in April and June. The raffinose concentration of timothy in June was about twice that of tall fescue in June (Table 5, Figures 2b and 3b).

Mean concentrations of 1-kestose were low in timothy (0.4 to 0.8 mg · g DM^{-1}) and did not differ among harvests, whereas in tall fescue, mean 1-kestose concentrations were eight times those of timothy in April (3.2 mg · g DM^{-1}) and then returned to similarly low concentrations (0.1 to 0.6 mg · g DM^{-1}) for the remaining harvests (Table 5). In tall fescue, 6-kestose was consistently low across all harvests (0.02 to 0.2 mg · g DM^{-1}), while in timothy, 6-kestose was higher in April and October, when it was at least double the tall fescue 6-kestose concentrations. Neokestose was consistently low in timothy, whereas in tall fescue, the April concentrations of neokestose were at least five times greater than in subsequent harvests. Neokestose in tall fescue was 37-fold greater than in timothy in April, and 50-fold greater than in timothy in June (Table 5).

3.3 | Effects of harvest and species on short-chain fructan concentrations

Harvest date influenced the effect of species on short-chain fructan concentrations ($p < .0001$). Short-chain fructan was 2- to 3-fold

higher in tall fescue than in timothy in April, August, and October (Table 5). In tall fescue, the highest concentrations were detected in April, followed by October. In timothy, short-chain fructan concentrations were higher in June than in August, while the reverse was the case in tall fescue. Species differences in the abundance of short-chain fructan can be seen by comparing timothy profiles in Figure 2 to tall fescue profiles in Figure 3. In April, short-chain fructan in tall fescue included several peaks that are not shown in their entirety on the chromatogram of Figure 3a, due to a desire to maintain the same scale on all chromatograms. Peaks in timothy short-chain fructan were not as large or numerous as those in tall fescue on any date (Figure 2).

3.4 | Effects of year, harvest and species on long-chain fructan concentrations

Harvest year influenced long-chain fructan concentrations ($p = .0017$). Long-chain fructan in year 1, averaged across both species and four sampling dates, was about 50% greater than long-chain fructan in year 2 (12.8 ± 2.7 mg · g DM^{-1} vs. 8.0 ± 1.7 mg · g DM^{-1}, true mean ± standard error of mean). Year effects are not presented in Table 5 because this was the only instance in which there was a year effect.

Harvest date influenced the effect of species on long-chain fructan concentrations ($p < .0001$). Timothy and tall fescue did not differ in long-chain fructan in August, when both species reached their lowest concentrations (Table 5). Timothy had the highest concentrations of long-chain fructan in April, followed by June and then October (Table 5). Tall fescue, in contrast, had similar concentrations of long-chain fructan at those three harvests, and all were one-half to one-fifth those of timothy on the same harvest dates (Table 5). Species differences in the abundance of long-chain fructan are apparent when comparing Figures 2 and 3, particularly the profiles of the April harvest in Figures 2a and 3a. The maximum DP of fructans at each harvest ranged from 34 to 72 in timothy, and from 10 to 30 in tall fescue (Table 6).

TABLE 6 Maximum degree of polymerization (DP) for timothy and tall fescue at each harvest.[a]

	Timothy maximum DP		Tall fescue maximum DP	
Harvest date	Mean	SD	Mean	SD
April 2020	72	3	20	1
June 2020	63	2	30	2
August 2020	38	6	10	0
October 2020	61	4	26	2
April 2021	64	1	22	5
June 2021	59	0	22	2
August 2021	34	15	13	6
October 2021	56	1	23	2

[a]Mean and standard deviation (SD) based on results of four field plots, except for timothy in August 2020 ($N = 3$), October 2020 ($N = 3$), and October 2021 ($N = 2$).

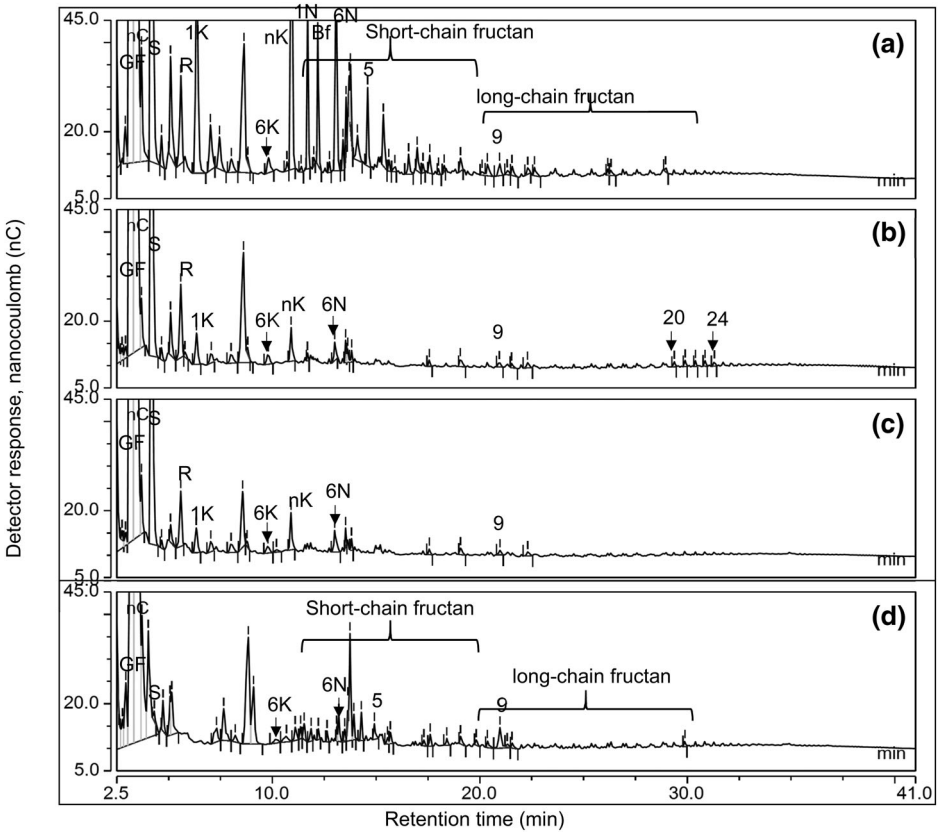

FIGURE 3 High-performance anion-exchange chromatography (HPAEC) profiles of tall fescue sampled in April (a), June (b), August (c), and October (d) of 2021. Poorly-resolved glucose (G) and fructose (F) are designated, as are sucrose (S), raffinose (R), 1-kestose (1K), 6-kestose (6K), neokestose (nK), 1-nystose (1N), bifurcose (Bf), and 6-nystose (6N). Numbers indicate degree of polymerization (DP) of fructans, based on alignment with fructan of DP 4 to 12 from *Poa secunda* (Sandberg bluegrass) and with fructan of DP 7 to >60 from *Dactylis glomerata* (cocksfoot).

4 | DISCUSSION

Regardless of species, raffinose and fructan concentrations (including fructan trisaccharides, long-chain fructan, and short-chain fructan) tended to reach a minimum in the August harvests (Table 5). The results are similar to those of Jensen et al. (2014), who found that cool-season grasses in Utah had the lowest mean fructan concentrations in July, August, and September. The decrease in the present study may have been due to the higher temperatures during that time (Table 2), because Thorsteinsson et al. (2002) found that timothy accumulated greater amounts of long-chain fructan at a day/night temperature regimen of 10/5°C than at 20°C. Presumably, higher temperatures might lead to similar or greater decreases in fructan accumulation. In addition, because blades were often brown at the tip, harvested samples contained a mixture of living and dead material. Fructan concentrations may have been diluted by dead tissue.

In timothy, apart from raffinose in June, trisaccharides were in consistently low concentrations, and few fructans of DP <9 were present. These results are similar to those of Thorsteinsson et al. (2002), who found few fructans of DP < 12 in the 'Vega' and 'Climax' cultivars of timothy grown at a day/night temperature regimen of 10/5°C. The difference in the lowest DP measured may reflect uses of different standards to measure DP, or cultivar differences. In addition, Suzuki and Pollock (1986) found that the enzyme phlein sucrase, isolated from timothy, synthesized mostly long-chain fructans and few oligosaccharides. The fructan polymerase isolated from timothy by Cairns et al. (1999) produced trisaccharides but at low concentrations of the enzyme.

Suzuki (1989) identified four classes of fructan among eight cool-season grass species and grouped timothy and tall fescue fructan into different classes, partly due to size. The maximum DP of 10 to 30 measured in tall fescue fructans in this study agrees with the findings of Suzuki (1989) for tall fescue, whereas the maximum DP of 34 to 72 measured in timothy in this study is lower than that determined for timothy by Suzuki (1989). Possibly timothy fructans of a higher DP would have been detected in timothy extracts injected at a higher concentration, which was avoided in order to minimize the risk of electrode fouling.

The year effect on long-chain fructan concentrations may have been due to variations in the April and June harvests of the two years. In April, timothy long-chain fructan concentrations decreased 40% in year 2 (data not shown). In June of year 1, tall fescue long-chain fructan ranged from 5 to 9 mg · g DM^{-1}, while in June of year 2, long-chain fructan concentrations were too low to be quantified, as evidenced by the small number of long-chain fructan peaks in Figure 3b. These differences between years 1 and 2 may have been related to the nearly 2-fold greater precipitation in year 1 (Table 2), which may have led to greater growth and accumulation of more WSCs. In addition, timothy, being harvested a week later than other grasses in April 2020 due to slower growth, may have been less mature in April 2020 than in April 2021. Fructans may be more abundant in less mature grass, as seen by greater abundance of fructan in cocksfoot harvested in mid-April relative to early May (Kagan et al., 2011).

Although patterns of fructan accumulation were discernible in this study, it should be noted that apart from the averaged April harvests, the sum of fructan and raffinose concentrations did not exceed 20 mg · g DM^{-1} (Table 5). Whether the concentrations determined would be enough to affect ruminal fermentation, or equine hindgut fermentation, is uncertain. Higher fructan concentrations (30 to 80 mg · g DM^{-1}) were found between April and June in cocksfoot cv. 'Prairie' grown in the Piedmont region of Virginia (Kagan et al., 2011), suggesting that a different choice of cultivar or species might have led to higher fructan concentrations. Weather differences may have contributed to the observed differences in these two studies. Minimum temperatures in the Virginia Piedmont study did not exceed 17°C between April and June, whereas minimum temperatures in the current study reached 20°C in early June (Figure S1). As discussed above, lower temperatures tend to lead to greater accumulation of fructans (Thorsteinsson et al., 2002). Photosynthetically active radiation (PAR) was not measured in the current study, but fructan concentrations increase in the presence of light (Ciavarella et al., 2000). Hence, comparison of PAR between the Virginia location and our Kentucky location might have revealed other factors contributing to differences in fructan concentrations. In spring-to-fall harvests of cool-season grasses grown in the High Plains region of Utah, timothy (cv. 'Climax') fructan concentrations ranged from 19 to 140 mg · g DM^{-1}, and tall fescue (cv. 'Fawn') fructan concentrations ranged from 30 to 80 mg · g DM^{-1} (Jensen et al., 2014). In that study, minimum temperatures did not exceed 15°C and were below 10°C during most months. It thus seems possible that a different choice of cultivars of timothy and tall fescue, and/or lower minimum temperatures during the harvest months, might have led to higher fructan concentrations in our study.

To determine if the observed concentrations of fructans, raffinose, or fructan trisaccharides could affect ruminal fermentation, changes in ammonia (Biggs & Hancock, 1998) and microbial protein (Hall & Weimer, 2016) could be monitored in studies with mixed ruminal microbiota. If no effect on ammonia production or microbial protein accumulation were seen at the observed fructan concentrations in fermentations with ruminal microbiota, the fructan concentrations could be too low to have an effect, and higher concentrations might be possible through cultivar choice or harvest location (e.g. choice of a location with lower temperatures).

To determine if the observed concentrations of fructans, raffinose, or fructan trisaccharides could affect equine health, monitoring changes in the hindgut, such as pH (Crawford et al., 2007), lactate concentrations (Ince et al., 2013), and bacterial populations (reviewed by Milinovich et al., 2010) might provide some insights obtained more readily than a feeding study. Should no effect on those factors be seen at the observed fructan concentrations in fermentations with equine hindgut microbiota, the grasses might be considered suitable for equine grazing.

5 | CONCLUSIONS

Short-chain fructan and the fructan trisaccharides were highest in April harvests of tall fescue, and long-chain fructan was highest in April

harvests of timothy. Lowest concentrations of short-chain fructan were in August for timothy and in June for tall fescue. Lowest concentrations of long-chain fructan were in August for both species. The results of this study suggest some options for feeding horses or ruminants, depending on the desirability of the different components. If lower fructan concentrations are desirable, grazing in August or June may be preferred. If higher fructan concentrations are desirable, grazing in April may be preferred. Whether the fructan concentrations determined in this study would affect animal performance is uncertain, as are benefits of raffinose or the fructan trisaccharides examined in this study. While feeding studies would ultimately be needed to document beneficial or detrimental effects, in vitro fermentation studies with the grasses obtained for this study might provide information on whether the concentrations of fructan, raffinose, or fructan trisaccharides could affect equine hindgut or ruminal fermentation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Bailey, S. R., Menzies-Gow, N. J., Harris, P. A., Habershon-Butcher, J. L., Crawford, C., Berhane, Y., Boston, R. C., & Elliott, J. (2007). Effect of dietary fructans and dexamethasone administration on the insulin response of ponies predisposed to laminitis. *Journal of the American Veterinary Medical Association*, *231*, 1365–1373.

Benkeblia, N. (2013). Fructooligosaccharides and fructans analysis in plants and food crops. *Journal of Chromatography A*, *1313*, 54–61.

Biggs, D. R., & Hancock, K. R. (1998). In vitro digestion of bacterial and plant fructans and effects on ammonia accumulation in cow and sheep rumen fluids. *Journal of General and Applied Microbiology*, *44*, 167–171.

Brown, R. H., & Blaser, R. E. (1965). Relationships between reserve carbohydrate accumulation and growth rate in orchardgrass and tall fescue. *Crop Science*, *5*, 577–582.

Cairns, A. J., Nash, R., Machado de Carvalho, M.-A., & Sims, I. M. (1999). Characterization of the enzymatic polymerization of 2,6-linked fructan by leaf extracts from timothy grass (*Phleum pratense*). *New Phytologist*, *142*, 79–91.

Chatterton, N. J., & Harrison, P. A. (1997). Fructan oligomers in *Poa ampla*. *New Phytologist*, *136*, 3–10.

Chatterton, N. J., Harrison, P. A., Thornley, W. R., & Bennett, J. H. (1993). Structures of fructan oligomers in orchardgrass (*Dactylis glomerata* L.). *Journal of Plant Physiology*, *142*, 552–556.

Chatterton, N. J., Harrison, P. A., Thornley, W. R., & Draper, E. A. (1990). Oligosaccharides in foliage of *Agropyron*, *Bromus*, *Dactylis*, *Festuca*, *Lolium* and *Phleum*. *New Phytologist*, *114*, 167–171.

Ciavarella, T. A., Simpson, R. J., Dove, H., Leury, B. J., & Sims, I. M. (2000). Diurnal changes in the concentration of water-soluble carbohydrates in *Phalaris aquatica* L. pasture in spring, and the effect of short-term shading. *Australian Journal of Agricultural Research*, *51*, 749–756.

Clark, G. T., Zuther, E., Outred, H. A., McManus, M. T., & Heyer, A. G. (2004). Tissue-specific changes in remobilisation of fructan in the xerophytic tussock species *Festuca novae-zelandiae* in response to a water deficit. *Functional Plant Biology*, *31*, 377–389.

Crawford, C., Sepulveda, M. F., Elliott, J., Harris, P. A., & Bailey, S. R. (2007). Dietary fructan carbohydrate increases amine production in the equine large intestine: Implications for pasture-associated laminitis. *Journal of Animal Science*, *85*, 2949–2958.

Cui, L. H., Byun, M. Y., Oh, H. G., Kim, S. J., Lee, J., Park, H., Lee, H., & Kim, W. T. (2020). Poaceae type II galactinol synthase 2 from Antarctic flowering plant *Deschampsia antarctica* and rice improves cold and drought tolerance by accumulation of raffinose family oligosaccharides in transgenic rice plants. *Plant Cell Physiology*, *61*, 88–104.

Hall, M. B., & Weimer, P. J. (2016). Divergent utilization patterns of grass fructan, inulin, and other nonfiber carbohydrates by ruminal microbes. *Journal of Dairy Science*, *99*, 245–257.

Ince, J. C., Longland, A. C., Moore-Colyer, M. J. S., & Harris, P. A. (2013). *In vitro* degradation of grass fructan by equid gastrointestinal digesta. *Grass and Forage Science*, *69*, 514–523.

Jensen, K. B., Harrison, P., Chatterton, N. J., Bushman, B. S., & Creech, J. E. (2014). Seasonal trends in nonstructural carbohydrates in cool- and warm-season grasses. *Crop Science*, *54*, 2328–2340.

Kagan, I. A., Harlow, B. E., & Flythe, M. D. (2022). A chromatographic method to monitor fructan catabolism in two cool-season grasses fermented by mixed bovine ruminal microbiota. *Journal of the Science of Food and Agriculture Reports*, *2*, 264–271.

Kagan, I. A., Kirch, B. H., Thatcher, C. D., Strickland, J. R., Elvinger, F., Teutsch, C. D., & Pleasant, S. R. (2011). Seasonal and diurnal variation in simple sugar and fructan composition of orchardgrass pasture and hay in the Piedmont region of the United States. *Journal of Equine Veterinary Science*, *31*, 488–497.

Kagan, I. A., Lawrence, L. M., Seman, D. H., Prince, K. J., Fowler, A. L., & Smith, S. R. (2018). Effects of sampling time, cultivar, and methodology on water- and ethanol-soluble carbohydrate profiles of three cool-season grasses. *Journal of Equine Veterinary Science*, *61*, 99–107.

Klevenhusen, F., & Zebeli, Q. (2021). A review on the potentials of using feeds rich in water-soluble carbohydrates to enhance rumen health and sustainability of dairy cattle. *Journal of the Science of Food and Agriculture*, *101*, 5737–5746.

Lee, M. R. F., Jones, E. L., Moorby, J. M., Humphreys, M. O., Theodorou, M. K., MacRae, J. C., & Scollan, N. D. (2001). Production responses from lambs grazed on *Lolium perenne* selected for an elevated water-soluble carbohydrate concentration. *Animal Research*, *50*, 441–449.

Lindåse, S., Müller, C., Nostell, K., & Bröjer, J. (2018). Evaluation of glucose and insulin response to haylage diets with different content of

nonstructural carbohydrates in 2 breeds of horses. *Domestic Animal Endocrinology*, *64*, 49–58.

Marley, C. L., Fraser, M. D., Fisher, W. J., Forbes, A. B., Jones, R., Moorby, J. M., MacRae, J. C., & Theodorou, M. K. (2007). Effects of continuous or rotational grazing of two perennial ryegrass varieties on the chemical composition of the herbage and the performance of finishing lambs. *Grass and Forage Science*, *62*, 255–264.

Milinovich, G. J., Klieve, A. V., Pollitt, C. C., & Trott, D. J. (2010). Microbial events in the hindgut during carbohydrate-induced equine laminitis. *Veterinary Clinics of North America Equine Practice*, *26*, 79–94.

Miller, L. A., Moorby, J. M., Davies, D. R., Humphreys, M. O., Scollan, N. D., MacRae, J. C., & Theodorou, M. K. (2001). Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.): Milk production from late-lactation dairy cows. *Grass and Forage Science*, *56*, 383–394.

Pöhnl, T., Böttcher, C., Schulz, H., Stürtz, M., Widder, S., Carle, R., & Schweiggert, R. M. (2017). Comparison of high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and ultra-high performance liquid chromatography with evaporative light scattering (UHPLC-ELSD) for the analyses of fructooligosaccharides in onion (*Allium cepa* L.). *Journal of Food Composition and Analysis*, *63*, 148–156.

Pollock, C. J., & Jones, T. (1979). Seasonal patterns of fructan metabolism in forage grasses. *New Phytologist*, *83*, 9–15.

Suzuki, M. (1968). Fructosan in the timothy haplocorm. *Canadian Journal of Botany*, *46*, 1201–1206.

Suzuki, M. (1989). Fructans in forage grasses with varying degrees of cold-hardiness. *Journal of Plant Physiology*, *134*, 224–231.

Suzuki, M., & Pollock, C. J. (1986). Extraction and characterization of the enzymes of fructan biosynthesis in timothy (*Phleum pratense*). *Canadian Journal of Botany*, *64*, 1884–1887.

Thorsteinsson, B., Harrison, P. A., & Chatterton, N. J. (2002). Fructan and total carbohydrate accumulation in leaves of two cultivars of timothy (*Phleum pratense* Vega and Climax) as affected by temperature. *Journal of Plant Physiology*, *159*, 999–1003.

Twomey, L. N., Pluske, J. R., Rowe, J. B., Choct, M., Brown, W., & Pethick, D. W. (2003). The effects of added fructooligosaccharide (Raftilose P95) and inulinase on faecal quality and digestibility in dogs. *Animal Feed Science and Technology*, *108*, 83–93.

Van Eps, A. W., & Pollitt, C. C. (2006). Equine laminitis induced with oligofructose. *Equine Veterinary Journal*, *38*, 203–208.

Vijn, I., & Smeekens, S. (1999). Fructan: More than a reserve carbohydrate? *Plant Physiology*, *120*, 351–359.

Volaire, F., & Lelièvre, F. (1997). Production, persistence, and water-soluble carbohydrate accumulation in 21 contrasting populations of *Dactylis glomerata* L. subjected to severe drought in the south of France. *Australian Journal of Agricultural Research*, *48*, 933–944.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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