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Seasonal and Diurnal Variation in Water-Soluble Carbohydrate Concentrations of Repeatedly Defoliated Red and White Clovers in Central Kentucky

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ABSTRACT

Nonstructural carbohydrates of pasture plants, comprising water-soluble carbohydrates (WSCs) and starch, may contribute to excessive consumption of rapidly fermentable carbohydrates by grazing horses. Seasonal and diurnal variation in WSCs were studied in red (*Trifolium pratense* L.) and white clovers (*Trifolium repens* L.) subjected to a typical management regime of rotationally grazed horse pastures. Two red and two white clover cultivars from monoculture plots were harvested after 4 weeks of growth from April to October of 2015, in the morning and afternoon of each harvest date. Water-soluble carbohydrates were quantified for each harvest, and starch was quantified for two harvests. Mean monthly WSC concentrations ranged from 80 to 99 mg/g (freeze-dried weight basis), whereas mean starch concentrations were 31 and 40 mg/g. In September, white clover had 14% more WSCs than red clover ($P < .0001$). Water-soluble carbohydrate concentrations were 10% higher in the afternoon than in the morning ($P < .0001$). Starch concentrations were 290% higher in the afternoon than in the morning ($P < .0001$), and nonstructural carbohydrate concentrations in the afternoon averaged 150 mg/g. Further studies are needed to determine whether the mixed grass-legume pastures of central Kentucky accumulate enough nonstructural carbohydrates to present risk factors for equine metabolic or digestive dysfunction.

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1. Introduction

Pastures are an important source of nutrients for grazing animals. In central Kentucky, cool-season grasses often dominate in horse pastures, but legumes are present as well [1]. Legumes, such as red (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.), are usually higher in crude protein and lower in neutral detergent fiber (a measure of cell wall concentration) than cool-season grasses [2]. Hence, the presence of legumes can enhance a pasture's nutritional value. Mixtures of legumes and grasses tend to have greater dry matter yields and to be higher in crude protein than grass monocultures [3,4].

In addition to protein, grasses and legumes contain nonstructural carbohydrates, which accumulate during the daytime as products of photosynthesis and are used for growth and respiration during the nighttime, in the absence of photosynthesis [5,6]. Nonstructural carbohydrates comprise water-soluble carbohydrates (WSCs) and starch [5,7]. Water-soluble carbohydrates in cool-season grasses consist mainly of monosaccharides and disaccharides and fructans (fructose polymers) [8,9]. Monosaccharides and disaccharides and short-chain fructans are soluble in ethanol-water mixtures as well as in water [10] and are sometimes classified as ethanol-soluble carbohydrates (ESCs). In legumes, fructans are absent [11,12]. Legume WSC and ESC include monosaccharides and disaccharides [11]. Legumes also accumulate

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starch, usually at higher concentrations than found in cool-season grasses [13–16], although starch concentrations in cool-season grasses can vary substantially with temperature [17].

High intakes of nonstructural carbohydrates from cool-season grasses have been implicated in the onset of equine pasture-associated laminitis, an inflammatory disease that leads to lameness (reviewed - by Geor [18]). An excess of nonstructural carbohydrates may be fermented in the hindgut and lead to proliferation of certain bacteria, resulting in decreased hindgut pH and various inflammatory responses [18]. Concerns exist about the potential role of fructan, most of which is thought to be fermented in the hindgut, in triggering laminitis [18]. Purified short-chain fructan can induce laminitis in large doses [19]. Starch, as well as WSC, is a concern in equine nutrition because feeding studies [20] have demonstrated that starch intakes in excess of 0.4% of body weight are not completely digested in the small intestine and hence are more likely to lead to digestive dysfunction in the large intestine. Others [21] have recommended a maximum starch intake of 0.2% of body weight. Consumption of monosaccharides [22] or ESC components in general [23], may increase blood insulin concentrations, which could be a risk factor for laminitis [23]. When starch is digested to glucose in the small intestine, there is potential to increase insulin concentrations.

Because clovers do not contain fructans, they have received less scrutiny from equine nutritionists and researchers in regard to their contribution to the total nonstructural carbohydrate intake of grazing horses. However, the contribution of clover starch to total nonstructural carbohydrate intake may be a concern [24].

The proportion of starch in clover nonstructural carbohydrate varies. WSCs constituted the majority of the nonstructural carbohydrate in some studies of red clover [15,25] and white clover [15]. Starch constituted the majority of the nonstructural carbohydrate in lines of white clover derived from the variety “Munida” [5], as well as in ladino clover [26].

WSC [27,28] and starch [28] concentrations of legumes have been found to vary seasonally in different geographic regions. Both WSC and starch concentrations varied diurnally in red clover [25], and white clover [5]. Understanding seasonal and diurnal trends can aid in making grazing management decisions that minimize the risk of consuming excessive amounts of nonstructural carbohydrates. Seasonal and diurnal variations in clover WSC or starch concentrations have not been assessed in central Kentucky, or in pastures managed like rotationally grazed horse pastures (with monthly defoliation).

The goal of this study was to gain a better understanding of seasonal and diurnal variation in WSCs of repeatedly defoliated red and white clovers in central Kentucky, and of their potential contributions to the WSC content of rotationally grazed horse pastures. To assess the relative contribution of WSCs to nonstructural carbohydrates under these conditions, starch concentrations were determined for two harvests as well.

2. Materials and Methods

2.1. Forage Planting and Sampling

The research was conducted at the University of Kentucky Spindletop Research Farm (Lexington, KY). In a preliminary study done in 2014, plots from a variety trial [29] were used. Plots of red clover (cultivars “Kenland” and “GA-Bulldog-S”) and white clover (cultivars “Will” and “Durana”) were seeded on August 21, 2013. Based on soil test recommendations, no fertilizer applications were made. Sampling was done from three replicate plots, which were 1.5×4.5 m^2. In 2015, plots were on the same farm but in a different pasture. The same clover cultivars were used, except that GA-Bulldog-S was replaced by the “Cinnamon Plus” cultivar. Plots for the 2015 study were seeded on August 20, 2014. The two white clover cultivars were chosen to represent the range of white clover cultivars grown in central Kentucky. Durana is an intermediate cultivar, or a cross between low-growing Dutch white clover and taller Ladino white clover, while Will is a Ladino cultivar, which, in addition to reaching a greater height, has bigger leaves than Dutch clover [30].

Four replicate plots (1.5×9 m) of each cultivar were planted, and cultivar plots were arranged in a randomized complete block design. The plots were sprayed on September 29, 2014 with Butyrac 200 (Albaugh, Ankeny, IA), Basagran (BASF, Florham Park, NJ), and Poast (BASF) for weed control. Plots were sprayed with Headline (BASF) on November 10, 2014 for prophylactic control of *Sclerotinia trifoliorum*. Based on soil test recommendations, no fertilizers were applied. Beginning on April 29, 2015, plots were mowed to a 10–13 cm height approximately every four weeks, owing to concern that more frequent mowing would be detrimental to the clover stands [31]. Mowed material was not put back onto plots. Consequently, every harvest represented about four weeks of growth. Monthly harvests have been used to assess seasonal variation in WSC of cool-season grasses [32].

Plots were sampled in the morning (0900 hours–1100 hours) and afternoon (1500 hours–1600 hours) of May 30 and October 17, 2014. In 2015, plots were sampled in the morning (0800 hours–0900 hours) and in the afternoon (1500 hours–1600 hours) of seven harvest dates: (April 15, May 27, June 24, July 22, August 19, September 15, and October 13).

Forage sampling was done by several workers positioned at different locations within a plot and selecting plants at random. Plants were cut to 5 cm above the soil level to simulate typical equine grazing heights. Cut tissue (about 100 g) was diced to 2–5 cm length, placed in an aluminum pan, and flash-frozen in liquid nitrogen. Samples were stored at −20°C until they were freeze-dried. The freeze-dried tissue was ground through a 1 mm mesh in a cyclone mill (UDY Corporation, Fort Collins, CO).

2.2. Environmental Monitoring and Height Measurements

Between mid-May and mid-October of 2015, temperature, precipitation, and photosynthetically active radiation (PAR) were obtained from a weather station (Campbell Scientific, Logan, UT), located next to the plots. Maximum and minimum temperatures, precipitation, and mean PAR were recorded every 15 minutes. The station was not operational during 2014 or the April 2015 harvest. In 2014, and for April 2015, temperature and precipitation data were obtained from a separate weather station at the Spindletop Research Farm that did not monitor PAR.

2.3. WSC Extraction and Colorimetric Analysis

Clover tissue (125 mg) was extracted by shaking in 25 mL water for 3 hours on a rocking shaker (model CR300t, FinePCR, South Korea) at 50–55 rpm. Samples were shaken in 50-mL Oak Ridge polypropylene centrifuge tubes (Thermo Scientific, Rochester, NY). After the 3-h extraction, samples were centrifuged (5 minutes, $3000\times g$, 25°C), and the supernatant was filtered through a #4 filter (Whatman, GE Healthcare, Buckinghamshire, United Kingdom). Volume was adjusted with water to 25 mL. Samples were stored at 4°C for up to 1 week or at −20°C for long-term storage before analysis.

WSC extracts were diluted 15-fold in water and quantified by the phenol-sulfuric acid assay of Dubois et al. [33], using 5% phenol and decreasing volumes by half. A standard curve of sucrose (0–60 μg/mL) was included daily in the analysis. Absorbance at 490 nm was measured with a spectrophotometer (model DU-800,

Beckman-Coulter, Brea, CA), and WSCs were quantified based on a linear regression obtained with the sucrose dilutions. Because dry matter content was not determined for the freeze-dried samples, concentrations are given in mg/g freeze-dried matter instead of mg/g dry matter (DM) and probably underestimate total WSC by 5%–8%, based on DM values of grasses sampled in a related study from 2014 [10]. Studies of grass WSC have also expressed WSC in terms of freeze-dried weight instead of dry weight [8].

2.4. Starch Extraction and Analysis

Starch analyses were determined with methods modified from Herrera-Saldana and Huber [34] and AOAC official method 2014.10 [35]. Samples (200 mg) were weighed into 50 mL glass centrifuge tubes with Teflon-lined caps in quadruplicate. One pair of tubes was used for determination of total glucose after enzyme addition and the other pair for free glucose determination. An additional pair of tubes without sample but containing other reagents (Enzyme blank) was used to determine the enzyme-free glucose.

Samples for total glucose determinations were incubated in 0.1 M sodium acetate buffer (pH = 5.0) with 100 μL of alpha-amylase (Spezyme Fred-L, Genencor International, Palo Alto, CA). Tubes were capped tightly and vortexed to mix. Tubes were placed in a preheated 100°C oven, removed every 10 minutes for mixing, and then returned to the oven. Tubes were removed after 50 minutes and placed into a preheated 60°C water bath to cool, and then 100 μL of amyloglucosidase (Optidex-L400, Genencor International) was added. After incubation at 60°C, samples were centrifuged 200× *g* for 10 minutes. Enzyme blank and free glucose tubes were handled as mentioned previously except for the difference in presence of sample or enzyme.

Glucose concentrations were analyzed electrochemically with an automated analyzer (YSI Inc., Yellow Springs, OH). Appropriate glucose standards were used to ensure linearity in the sample range. Total starch was calculated as the difference between total glucose and free glucose per sample with adjustment for the enzyme blank. A correction factor of 1.11 for glucose recovery from starch was used to convert mg of glucose to mg of starch.

2.5. Statistical Analysis

The Fit Model option of JMP 14.0 (The SAS Institute, Cary, NC), with the Restricted Maximum Likelihood method, was used for statistical analyses. Data were analyzed as a randomized complete block, split-split-plot design with factorial arrangement of treatments. Block, block*harvest, and cultivar (species) were considered to be random effects, whereas fixed effects included all combinations of species, harvest date, and time of sampling. The main plot consisted of species; subplot was harvest date; and split-split plot was time of sampling. Fixed effects were tested with their proper error terms. For interactions of main effects with $P < .05$, paired comparisons were made using the Slice command.

3. Results

3.1. Environmental Conditions

In 2014, during a preliminary study, maximum and minimum temperatures were 29°C and 18°C on May 30, 2014, and 23°C and 10°C on October 17, 2014. No precipitation occurred on either date. On April 15, 2015, maximum and minimum temperatures were 16°C and 12°C, respectively, and no precipitation occurred. Cumulative mean PAR from mid-May 2015 to mid-October 2015 is presented in Fig. 1A. Photosynthetically active radiation data were not obtained for the April 2015 harvest or for the 2014 preliminary study (see Section 2.2). On harvest dates, cumulative mean PAR from midnight to 0800 hours is shown (Fig. 1A), as is cumulative mean PAR from 0800 hours to 1500 hours, to indicate the cumulative mean PAR before both the morning and the afternoon harvests. Maximum and minimum daily temperatures, and precipitation, are depicted in Fig. 1B.

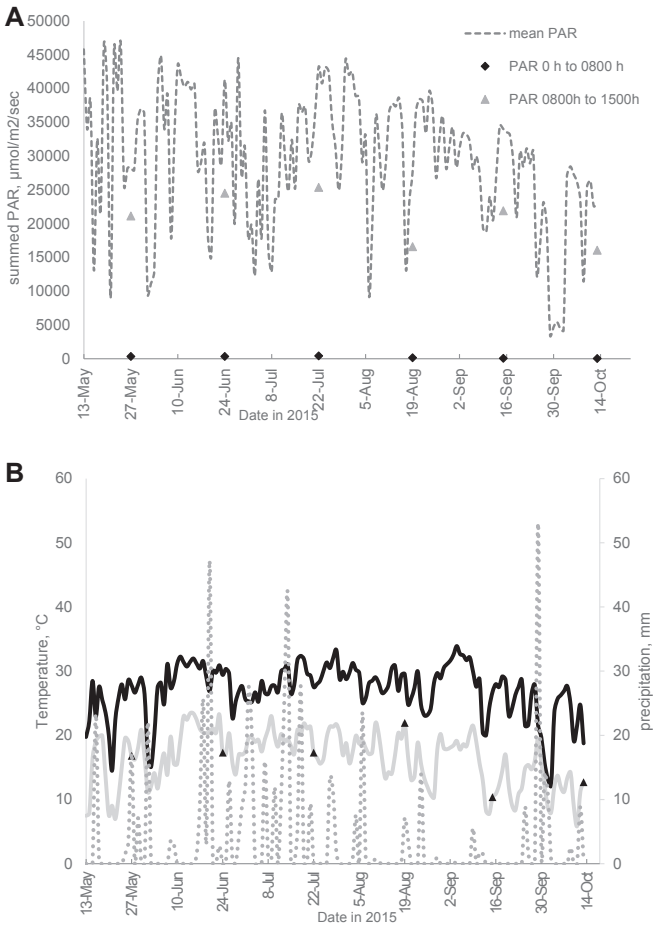

Fig. 1. Daily light, temperature, and precipitation conditions from mid-May to mid-October of 2015. (A) Cumulative mean photosynthetically active radiation (PAR). On harvest days, cumulative mean PAR before the morning harvests (black diamonds) and afternoon harvests (gray triangles) is shown. (B) Temperature (maximum, black solid line; minimum, gray solid line) and precipitation (gray dotted line). Black triangles indicate minimum temperature on each harvest date.

3.2. Main Effects of Harvest Date, Sampling Time, and Species on Clover WSC Concentrations in 2014

Harvest date, sampling time, and species influenced WSC concentrations in a preliminary study conducted in May and October of 2014. On the basis of freeze-dried weight, WSC concentrations (least square mean ± standard error) were about 10% higher (102 ± 2 vs. 94 ± 2 mg/g,) on May 30 than on October 17 ($P = .020$). Afternoon samples contained about 10% more WSC than morning samples (104 ± 2 vs. 93 ± 2 mg/g; $P = .0037$). WSC concentrations were about 10% higher in white than in red clover (104 ± 1 vs. 93 ± 1 mg/g; $P = .014$). No interactions of harvest date, sampling time, or species influenced WSC concentrations in 2014.

3.3. Main Effects of Harvest Date, Sampling Time, and Species on Clover WSC Concentrations in 2015

Harvest date influenced WSC concentrations when averaged across species and sampling times in 2015 ($P < .0001$). Concentrations (least squares means ± standard error) ranged from 80 ± 2.7 to 99 ± 2.7 mg/g (freeze-dried weight basis), with the lowest and highest concentrations in April and August, respectively (Table 1).

When averaged across harvest dates and species, WSC concentrations (least squares mean ± standard error) were 10% higher in the afternoon (97 ± 2 mg/g) than in the morning (88 ± 2 mg/g; $P < .0001$). Red and white clover did not differ ($P = .52$) in WSC concentrations averaged across all harvest dates and sampling times (91 ± 3 and 94 ± 3 mg/g, for red and white clover, respectively).

3.4. Main Effects of Harvest Date, Species, and Sampling Time on Starch and Nonstructural Carbohydrate Concentrations in July and October 2015

Sampling time influenced starch ($P < .0001$) and nonstructural carbohydrate ($P < .0001$; WSC plus starch) concentrations averaged across species and two harvest dates. Morning starch concentrations were 18 ± 3 mg/g and increased nearly 300% in the afternoon to 52 ± 3 mg/g, making the afternoon increase 30-fold greater than the mean afternoon increase in WSC. Morning nonstructural carbohydrate concentrations were 100 ± 5 mg/g and increased 50% to 150 ± 5 mg/g, making the afternoon increase 15-fold greater than the mean afternoon increase in WSC. Neither starch nor nonstructural carbohydrate concentrations, averaged across species and sampling times, differed between the two harvest dates (Table 1). Neither starch nor nonstructural carbohydrate concentrations, averaged across sampling times and the two harvest dates, differed between species ($P = .15$ for starch and 0.31 for nonstructural carbohydrate). Mean starch concentrations were 32 ± 3 and 38 ± 3 mg/g for red and white clover, respectively. Mean nonstructural carbohydrate concentrations were 124 ± 6 and 135 ± 6 mg/g for red and white clover, respectively.

3.5. Interactions of Harvest Date, Sampling Time, or Species Effects on WSC Concentrations

Harvest date influenced the effect of species on WSC concentrations ($P < .0001$). Species differed in WSC concentrations in September (Fig. 2A), when WSC concentrations were about 14% higher in white clover than in red clover (104 ± 4 and 88 ± 4 mg/g, respectively).

Harvest date also influenced the effect of sampling time on WSC concentrations ($P < .0001$; Fig. 2B). In June, July, September, and October, WSC concentrations were 15%–30% higher in the afternoon than in the morning (78–91 mg/g in the morning, and 94–110 mg/g in the afternoon; Fig. 2B). WSC concentrations in May were 10% higher in the morning than in the afternoon, and in April and August, no differences were observed between morning and afternoon WSC (Fig. 2B). The May and August harvests were accompanied by relatively low cumulative mean PAR in the afternoon (Fig. 1A) and by precipitation (Fig. 1B).

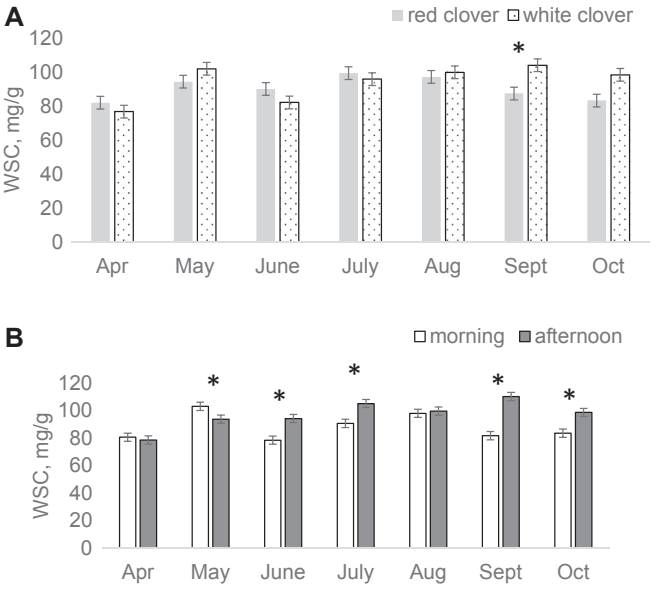

Fig. 2. Interactions ($P < .0001$) of effects of species and harvest month (A), or sampling time and harvest month (B) on clover water-soluble carbohydrate (WSC) concentrations. In panel A, least squares means of red and white clover WSC concentrations (light gray and stippled bars, respectively), with standard errors, are shown for each harvest date. The asterisk indicates that red and white clover differed in WSC concentrations in September, based on paired comparisons ($P = .046$). In panel B, least squares means of morning (white bars) and afternoon (dark gray bars) WSC concentrations, with standard errors, are shown for each harvest date. Asterisks indicate differences between morning and afternoon WSC concentrations, based on paired comparisons (*P*-values: 0.0005 in May, <0.0001 in June and July, and <0.0001 in September and October).

Species did not influence the effects of sampling time on WSC concentrations ($P = .24$). WSC of both red and white clover increased 10% between morning and afternoon when averaged across all harvests. Mean red clover WSC concentrations increased from 87 ± 3 to 95 ± 3 mg/g, and mean white clover WSC concentrations increased from 89 ± 3 to 100 ± 3 mg/g.

3.6. Interactions of Harvest Date, Species, or Sampling Time on Clover Starch and Nonstructural Carbohydrate Concentrations

Harvest date influenced the effects of sampling time on starch ($P < .0001$) and nonstructural carbohydrate ($P = .0004$) concentrations when averaged across species. Afternoon starch concentrations were about 200% higher than morning starch concentrations in July, and about 300% higher than morning starch concentrations in October (Table 2). Afternoon nonstructural carbohydrate concentrations were about 40% higher than morning nonstructural carbohydrate concentrations in July, and about 60% higher than

Table 1
Concentrations (mg/g freeze-dried weight) of water-soluble carbohydrate (WSC) at each harvest date, averaged across sampling times and species.

Date	15 April	27 May	24 June	22 July	19 August	15 September	13 October	SE	*P*-value
Carbohydrate class	mg/g carbohydrate (freeze-dried weight)								
WSC	80	98	86	98	99	96	91	2.7	<.0001
Starch	—	—	—	31	—	—	40	3.9	.058
NSC	—	—	—	128	—	—	131	5.3	.42

Least squares means are shown, and standard errors (SE) and *P*-values are listed to the right of the means. Starch and nonstructural carbohydrate (NSC; WSC plus starch) concentrations were obtained for the July and October harvests.

Table 2
Interaction of effects of sampling time and harvest date on starch and nonstructural carbohydrate (NSC) concentrations.

Harvest Month and Sampling Time	Starch, mg/g	NSC, mg/g
July, morning	19 c	110 c
July, afternoon	41 b	146 b
October, morning	18 c	102 c
October, afternoon	62 a	161 a
SE	4	6
P-value	<.0001	.0004

Least squares means and standard errors (SEs) are shown, and concentrations are in mg/g freeze-dried weight. Within a column, for $P < .05$, means with different lowercase letters differ, based on a Tukey's test.

Table 3
Interaction of effects of sampling time and species on starch and nonstructural carbohydrate (NSC) concentrations.

Species and Sampling Time	Starch, mg/g	NSC, mg/g
Red clover, morning	13 c	98
Red clover, afternoon	51 a	150
White clover, morning	24 b	113
White clover, afternoon	52 a	158
SE	5	7
P-value	.046	.25

Least squares means and standard errors (SE) are shown, and concentrations are in mg/g freeze-dried weight. Within a column, for $P < .05$, means with different lowercase letters differ, based on a Tukey's test.

morning nonstructural carbohydrate concentrations in October (Table 2).

Harvest date influenced the effect of species on nonstructural carbohydrate concentrations when averaged across sampling times ($P = .0033$). White clover nonstructural carbohydrate concentrations were higher in October than in July, whereas red clover nonstructural carbohydrate concentrations did not differ between July and October (Fig. 3). Harvest date did not influence the effect of species on starch concentrations averaged across sampling times ($P = .87$).

Species influenced the effects of sampling time on starch ($P = .046$) but not on nonstructural carbohydrate ($P = .25$) concentrations when averaged across harvest dates. Red clover starch concentrations increased 400% in the afternoon, and white clover starch concentrations increased 200% in the afternoon (Table 3), resulting in afternoon starch concentrations that were similar in the two species. Hence, afternoon increases in starch concentrations were 20–40 times greater than afternoon increases in WSC concentrations (see Section 3.5). Nonstructural carbohydrate concentrations increased about 50% in the afternoon in both species (Table 3).

4. Discussion

Clover WSC concentrations, averaged across species and sampling times, ranged from a low of 80 ± 2.7 mg/g freeze-dried weight in April to a high of 99 ± 2.7 mg/g in August. The range of mean clover WSC concentrations (about 20% variation) on the different harvest dates is similar to that determined for red clover harvested from May to July in Britain [27], suggesting that red clover, at least,

Fig. 3. Interaction ($P = .0033$) of harvest date and species effects on clover nonstructural carbohydrate (NSC) concentrations. Least squares means plus or minus standard errors are shown. The asterisk shows the difference ($P = .027$) between white clover concentrations in July (light gray bars) and October (stippled bars), based on paired comparisons.

may not exhibit much seasonal variation in WSC concentrations compared to some cool-season grasses [36]. Also, monthly defoliation may have minimized seasonal variation. Defoliation of ladino clover [26] temporarily decreased the concentrations of some nonstructural carbohydrates, possibly due to allocation of carbon to replacement tissue [37]. Concentrations increased to predefoliation amounts after about 4 weeks [26]. In the present study, the regimen of mowing every 4 weeks may have resulted in WSC reaching a fairly constant pattern of decreasing and then increasing to similar starting concentrations. Hence, management may have a strong effect on red and white clover WSC concentrations when clover is defoliated monthly, as would be the case in rotational grazing.

Greater WSC concentrations in white than in red clover, as observed in the preliminary 2014 study and in September 2015 (Fig. 2A), have been observed previously [27]. The difference may reflect leafier growth, with less stem, in white than in red clover. Leafy, relatively small alfalfa plants were found to accumulate more sucrose and hexoses in September than taller alfalfa plants, possibly because less carbon was directed toward structural carbohydrate synthesis in the smaller plants [28].

WSC concentrations increased in the afternoon on four harvest dates. Higher afternoon concentrations of WSC have been observed previously in clovers [5,25,38]. In the present study, the lack of difference between morning and afternoon WSC on August 19, 2015 may have been due to relatively low cumulative mean PAR by the time of the afternoon harvest (Fig. 1A), which, in turn, may have been due to precipitation (Fig. 1B) and presumably cloudy weather on that day. Light availability can greatly affect WSC concentrations in grasses [39]. On May 27, 2015, when afternoon WSC concentrations were lower than morning WSC concentrations, precipitation also occurred (Fig. 1B), and cumulative mean PAR was somewhat lower than it was on June 24 or July 22 (Fig. 1A), presumably due to cloudiness accompanying precipitation. Low PAR, and subsequent decreases in photosynthesis, may have led to daytime utilization of WSC in amounts greater than could be restored by photosynthesis.

Afternoon increases in WSC were smaller than the afternoon increases in starch observed in July and October (Table 2), although WSCs still comprised the majority of the nonstructural carbohydrate (Tables 2 and 3). Higher concentrations of starch and nonstructural carbohydrate in October than in July (Table 2, Fig. 3) may have been due to lower temperatures in October (Fig. 1B) because starch concentrations in cool-season grasses have been found to increase at lower temperatures [17]. Greater afternoon increases in starch than in monosaccharide and disaccharide concentrations have been observed previously in legumes [5,16,25,40] and reflect the importance of starch as a reserve carbohydrate in legumes.

The relative risk of nonstructural carbohydrates in clovers for pasture-associated laminitis is difficult to determine. On two harvest dates, starch concentrations in the afternoon (averaged across

both clover species) were 41 and 62 mg/g on a freeze-dried weight basis (Table 2). For a 500-kg horse to ingest more than the suggested maximum recommended intake of 0.2% of body weight (2 g starch per kg body weight in a meal) [21], it would have to consume 14–23 kg of dried clover. However, the nonstructural carbohydrate (approximately 150 mg/g freeze-dried weight) in the afternoon clover samples exceeded the concentration (100 mg/g DM) recommended for horses at risk for endocrinopathic laminitis [41].

Consuming enough clover to ingest these upper limits of starch or nonstructural carbohydrate might be difficult in central Kentucky, where horse pastures tend to be a mixture of tall fescue (*Lolium arundinaceum*), Kentucky bluegrass (*Poa pratensis*), orchardgrass (*Dactylis glomerata*), and white clover [1]. Average white clover abundance in selected pastures was 8%, whereas average Kentucky bluegrass and tall fescue abundances were 28 and 22%, respectively [1]. A study of cool-season grasses and legumes in Canada [16] found that red clover had afternoon nonstructural carbohydrate concentrations intermediate between those of Kentucky bluegrass and tall fescue. If that trend were to hold in rotationally grazed central Kentucky grass-legume pastures, nonstructural carbohydrate concentrations might exceed the amounts recommended for horses at risk for endocrinopathic laminitis. Hence, nonstructural carbohydrate concentrations need to be determined for pasture grasses as well.

5. Conclusions

This study revealed seasonal and diurnal variation in concentrations of WSC, starch, and nonstructural carbohydrates of red and white clovers in central Kentucky. However, further research is needed to understand why WSC might decrease in the afternoon as was observed in May, and to understand the relative contribution of clover to the seasonal and diurnal nonstructural carbohydrate concentrations of mixed grass-legume pastures in central Kentucky.

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