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Genetic Diversity of Diurnal Carbohydrate Accumulation in White Clover (*Trifolium repens* L.)

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Abstract: White clover (*Trifolium repens* L.) is one of the most important legumes for fodder production in temperate climates, particularly in intensive pasture systems. Like many other forage legumes, it lacks the energy content to maximize productivity of modern ruminant livestock breeds. White clover produces water-soluble carbohydrates and starch in its leaves as a diurnal product of photosynthesis. However, little is known about the genetically encoded variability of diel changes in carbohydrate content. We assessed the amount of glucose, fructose, sucrose, and starch in the leaves of 185 plants of a genetically diverse white clover population. Water-soluble carbohydrates only provided on average 10.6% of dry weight (DW) of the total analyzed non-structural carbohydrate (NSC) content at the end of the day (ED), while starch supplied 89.4% of the NSC content. The top 5% of individuals accumulated over 25% of their DW as starch at ED. The leaf starch content at ED showed up to a threefold difference between genotypes, with a repeatability value of 0.95. Our experiments illustrate both the physical potential of white clover to serve as a competitive energy source to meet the demand of modern ruminant livestock production and the genetic potential to improve this trait by breeding.

Keywords: legumes; white clover (*Trifolium repens* L.); non-structural carbohydrates; water-soluble carbohydrates; starch; photosynthesis; pasture energy content; breeding

1. Introduction

For centuries, ruminant livestock have provided meat and dairy from animals that were traditionally fed on pasture and grassland swards containing forage legumes. When well-managed, these perennial agroecosystems maintain carbon, water, and nutrient cycles [1–3]. Although animals fed on diets containing grasses and forage legumes are more productive than animals fed on grass alone, these pasture and grassland systems generally do not provide the energy content needed to maximize the productivity potential of modern animal breeds and meet the consumer demand for low-cost meat and dairy. Therefore, modern ruminant livestock production has increasingly shifted towards confined feeding operations (CFOs), where supplemented cereal grains increase the dietary-energy content and augment animal productivity. Cereal grains are often transported across large geographic ranges, disrupting natural carbon and nutrient cycles [3]. Therefore, there is a growing interest to maximize the protein and energy content of forage grasses and legumes in order to deliver a high proportion of the feed intake from locally produced roughage.

Although returning to traditional perennial pastures and locally produced roughage is increasingly being viewed as a way to improve the overall sustainability of ruminant-livestock production, currently

these systems are economically less competitive with CFOs in terms of energy supply [4]. For most animal diets, energy is primarily derived from non-structural carbohydrates (NSCs), which are differentiated from structural carbohydrates that form dietary fiber. NSCs can be further distinguished into water-soluble carbohydrates (WSCs) and starch. In maize and other cereal grains, 70–80% of the grain dry weight (DW) is starch [5].

In pasture and grassland systems, NSC content is determined by seasonal and diurnal accumulation of metabolites. Starch and many WSCs are products of photosynthesis that accumulate in the leaves of plants during the day [6]. These NSCs are mobilized at night to facilitate growth and respiration [7]. In perennial ryegrass (*Lolium perenne* L.), the WSC content is primarily derived from soluble fructans stored in the vacuoles of stems and leaves, which can reach 20–25% of the dry matter [8,9]. In forage legumes, the major WSCs are glucose, fructose, and sucrose, but osmolytes such as pinitol can become a major fraction during stress [10–12]. Starch can make up 25–35% of the DW biomass in some forage legumes such as red clover (*Trifolium pretense* L.) and alfalfa (*Medicago sativa* L.). Because starch is a product closely linked to photosynthesis, this accumulation is highly dependent on environmental factors such as light intensity, harvest time, temperature, and day length [13,14]. At night, starch mobilization in white clover (*T. repens* L.) is reduced under low temperatures and starch accumulates [15].

Because of the high degree at which both WSC and starch contents are influenced by the environment, such contents have been elusive for breeders of forage crops to select. The WSC content in perennial ryegrass is influenced by diel and seasonal changes, but genotypes with contrasting WSC accumulation potential could be reliably identified under low genotype-by-environment interactions [16]. Based on these studies, varieties of perennial ryegrass have been selected with as much as 40 g/kg DM more WSC content than conventional varieties. High-WSC varieties, such as AberMagic®, have been commercially successful [17]. In alfalfa, a similar approach was taken for NSCs, and genotypes with reproducible high and low NSC contents were identified, but the reliability of selection was less than that observed for WSCs in perennial ryegrass [14]. This observation is consistent with the NSC content in alfalfa being more strongly influenced by diel environmental factors than the NSC content in perennial ryegrass, which seasonally accumulates fructans as an osmoprotectant [18].

White clover is a perennial forage legume and one of the principal forage crops in grazing systems of temperate zones worldwide [19]. Because the majority of NSCs are readily fermented or respired post-harvest in silage and hay production systems [20,21], the improvement of NSCs in white clover is more promising, as it is primarily used in direct grazing systems. In pasture systems, breeding advances that increase the NSC content can be directly realized by grazing animals and would not need to overcome substantial post-harvest losses. White clover has been shown to have the potential to accumulate 10–40% of its biomass as NSCs in its leaves [15,22]. Improved energy content would add to the major nutritional benefits of white clover, which are its high protein and low structural fiber content. These nutritive properties complement those of perennial grasses, grown in mixed legume-grass swards [23]. In addition, white clover is able to furnish atmospheric nitrogen to companion grasses through N-fixation [24,25].

Evidence that increased NSC accumulation can improve the animal production potential in grazing systems comes from experiments where animal grazing times were restricted to the evening. These animals were repeatedly shown to have increases in milk or meat production, depending on the agriculture system [26]. This increase in animal productivity correlates with the higher overall NSC content that is observed in the forage herbage grazed in the evening. The upper end of the NSC content corresponds to the energy content of high energy diets typically observed in CFO systems [27]. Therefore, understanding the basis for the large variation in energy content of forage in grazing systems could be useful in making grazing systems more competitive with CFO systems.

Although economically attractive, improving NSC content has been challenging, because of the influence of diel cycles, light environments, and abiotic stress. In forage legumes, the predominant NSC is starch, although reported starch contents are highly variable and generally range from 1 to

30%, depending on environment, harvesting time, post-harvest treatment, genotypic potential, and precision of quantitation [13]. Environmental factors that influence NSC content in model systems have been intensively studied, but little is known with respect to forage legumes [6,28]. The genetics and physiology of diel leaf starch metabolism is best understood in the model plant Arabidopsis (*Arabidopsis thaliana* L.), but much of the current knowledge of carbohydrate content in white clover is difficult to compare due to inconsistent sampling during the diel cycle, post-harvest treatment, lack of standardized quantitation, and developmental and morphological differences [29,30]. Therefore, this study adapts strategies for sampling and quantitation of NSC content from model systems to a diverse set of white clover genotypes, with the aim to better understand the physiology and genetic diversity of NSC content in white clover.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The plant material used in this study was derived from the genetically diverse white clover variety Munida (Agroscope Reckenholz, Zurich, Switzerland). Munida seeds were obtained from the original variety source [31]. In total, 185 plants were grown from seed for 57 days and harvested at ED for carbohydrate content analysis. The plants were then allowed to regrow for eight days and were harvested for EN measurements. The 185 plants were divided equally into three glasshouse chambers. The first chamber faced north and received more morning sun, and the second chamber was positioned between the two chambers and received more afternoon sun, the third chamber faced south and received more evening sun. Of the 185 individual genotypes, twelve were selected and clonally propagated by dividing individual 70-day-old plants into 10 clonal replicates. Clones were allowed to recover and regrow to a similar growth stage as described above. To minimize environmental variation, all clones were grown in the glasshouse chamber, which was mentioned above as the one positioned in the middle and received afternoon sun in a completely randomized design. After 41 days, all 120 clones were harvested for ED measurements, allowed to regrow for eight days, and then harvested at EN. The plant growth medium was a mix of soil, peat and perlite at a ratio of 3:2:1. The soil consisted of 25% sterile topsoil, 25% tree-bark compost, 20% sand and 30% white peat (Ricoter Erdaufbereitung AG, Aarberg, Switzerland), the peat component was "Substrate 2" (Klasmann-Deilmann AG, Geeste, Germany) and the perlite added was at a size of 3–6 mm (GVZ-Rossat AG, Otelfingen, Switzerland).

Glasshouse growth conditions were the same as described previously for red clover [13]. Briefly, to simulate partially sunny growth conditions, direct intense sunlight was shaded to produce uniform light across the experimental growth area. The intensity of the uniform indirect sunlight was between 50 to 150 µmol m^{-2} s^{-1}, depending on time of day and position of the sun. Indirect sunlight was supplemented with 150–200 µmol m^{-2} s^{-1} light from Clean Arc© metal halide lamps (EYE Lighting International, Mentor, OH, USA). Total light intensity was between 250 to 350 µmol m^{-2} s^{-1}. Experiments were carried out under a 14/10 h light/dark photoperiod, with a 19–21 °C day, 14–15 °C night temperature regime. Experiments were carried out between 25 January and 29 April 2016, and the photoperiod was aligned with the maximum 14-hour day length observed in Eschikon, Switzerland at the end of April.

2.2. Quantitation of Glucose, Fructose, Sucrose, Starch and Biomass

Carbohydrate contents were quantified as described previously for red clover [13]. In short, leaves were harvested, flash frozen in liquid nitrogen, lyophilized, weighed, and homogenized into powder using a Mixer Mill MM 400 (Retsch, Haan, Germany). Biomass was determined as lyophilized leaf weight. WSC were extracted with two 80% (v/v) ethanol washes and two 50% (v/v) ethanol washes from 10 mg of the lyophilized powder. The remaining pellet was boiled in 0.2N KOH, and neutralized with 1 M acetic acid. The soluble starch was digested with α-amylase and amyloglucosidase (Sigma-Aldrich, St. Louis, MO, USA). Glucose, fructose and sucrose were quantified in triplicate,

based on the conversion of NADP to NADPH by hexokinase and glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, and invertase, which convert NADP to NADPH proportional to the glucose units (Sigma-Aldrich). NADPH was quantified by the absorption of light at 340 nm using an Enspire© plate reader (Perkin Elmer, Waltham, MA, USA).

2.3. Statistical Analysis of Phenotypic Data

All statistical analyses were carried out in R statistical software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria) [32]. The effect of the glasshouse environment (chamber 1–3) and the harvest time (ED, EN) on each of the carbohydrate contents in the 185 genotypes was determined using ANOVA with a Tukey HSD post-hoc test. Carbohydrate contents were log transformed to meet the assumptions of the ANOVA based on residual distribution. Harvest time and glasshouse environment were analysed independently due to a significant interaction. Correlations between NSC contents were determined using Spearman's rank correlation. To assess genetic diversity of NSC measurements, the mean-square of the variation between genotypes (σ^2_g) and the mean square of the variation within genotypes (mean-square of the error) were calculated by a one-way ANOVA per harvest time due to significant harvest time by genotype interaction. Repeatability was calculated by dividing the genotypic variance component (σ^2_g) with the sum of the σ^2_g and the effective mean square of the error [33]. Significant differences in NSC content between genotypes were determined using a Tukey HSD posthoc test.

2.4. Iodine Staining of Starch in Plants

To visualize starch in plant leaves, whole plants were removed from excess root material, washed and placed in 70% (*v*/*v*) boiling ethanol. After 2 h, the plants were removed and placed in Lugol's staining solution for 10 min. After 10 min stained plants were placed in water to reduce non-specific staining. Plants were then imaged on a light table.

3. Results

To evaluate the phenotypic variation of leaf NSC content in white clover, glucose, fructose, sucrose, and starch were analyzed in 185 genotypes of the variety *Munida*. *Munida* was used for this study, because the original *Munida* variety used enhanced genetic variation to increase the diversity of glucosinolate content [31]. To limit genotype-by-environment interactions, the experiments were carried out under uniform glasshouse conditions, and sampling was done at the end of the photoperiod at the end of the day (ED), and before the beginning of the photoperiod at the end of night (EN) to establish the maximum and minimum diel NSC content for individual genotypes.

3.1. Phenotypic Variation of Glucose, Fructose, Sucrose, and Starch Content in White Clover Leaves

At ED, glucose was the least abundant WSC in white clover leaves with a mean value of 2.18 mg g^{-1} DW and a phenotypic range from 0.0680 mg g^{-1} DW to 13.0 mg g^{-1} DW (Figure 1a). Fructose accumulated to a mean content of 4.81 mg g^{-1} DW in the population, with a broader range in comparison to glucose from 0.154 mg g^{-1} DW to 22.1 mg g^{-1} DW (Figure 1b). Sucrose was the most abundant WSC, with a mean accumulation of 11.3 mg g^{-1} DW and a range of 0.104 mg g^{-1} DW to 25.6 mg g^{-1} DW (Figure 1c). The mean WSC content derived from glucose, fructose, and sucrose was less than 2% of the DW (Figure 1a–c). At ED, the mean starch content was 154 mg g^{-1} DW with a range of 54.5 mg g^{-1} DW to 372 mg g^{-1} DW (Figure 1d). The summed-up mean of the measured NSC content observed in this population is 190 mg g^{-1} DW, with a maximum measured NSC content of 493 mg g^{-1} DW and a minimum measured NSC content of 55.2 mg g^{-1} DW. When comparing the mean total WSC content to the mean starch content at ED, over 89.4% of the total NSC content was derived from starch.

At EN, WSC and starch had lower mean values and ranges of variation than at ED. At EN, glucose and fructose were similar in their mean and range. Glucose accumulated to a mean value of

0.803 mg g^{-1} DW and a range of 0.130 mg g^{-1} DW to 4.62 mg g^{-1} DW. Fructose accumulated to a mean value of 0.862 mg g^{-1} DW with a range of 0.0247 mg g^{-1} DW to 3.97 mg g^{-1} DW (Figure 1a,b). Based on the distribution of the glucose and fructose contents at EN in the population, monosaccaride reserves were exhausted to below 0.1% of the total DW during nighttime mobilization in a majority of the genotypes (Figure 1a,b). Unlike glucose and fructose, sucrose changed less during the diel cycle, and over 50% of the population retained over 0.5% of its DW as sucrose at EN. At EN, sucrose was the primary WSC with a mean accumulation of 5.89 mg g^{-1} DW and a range of 0.223 mg g^{-1} DW to 11.1 mg g^{-1} DW (Figure 1c).

At EN, the mean starch content was 8.00 mg g^{-1} DW with a phenotypic range of 0.261 mg g^{-1} DW to 44.0 mg g^{-1} DW. At EN, starch contributed more to the mean measured NSC content and phenotypic variation than WSC, as WSC content had a mean value of 5.89 mg g^{-1} DW and phenotype range of 0.458 mg g^{-1} DW to 14.9 mg g^{-1} DW (Figure 1d). The difference between starch content and WSC content at EN was smaller than at ED, because 45% of the population contained less than 1.0 mg g^{-1} DW starch, which was below the observed mean WSC content. Therefore, unlike at ED, when starch content is the predominate NSC in all individuals, at EN, starch and WSC together contribute to NSC content and variation.

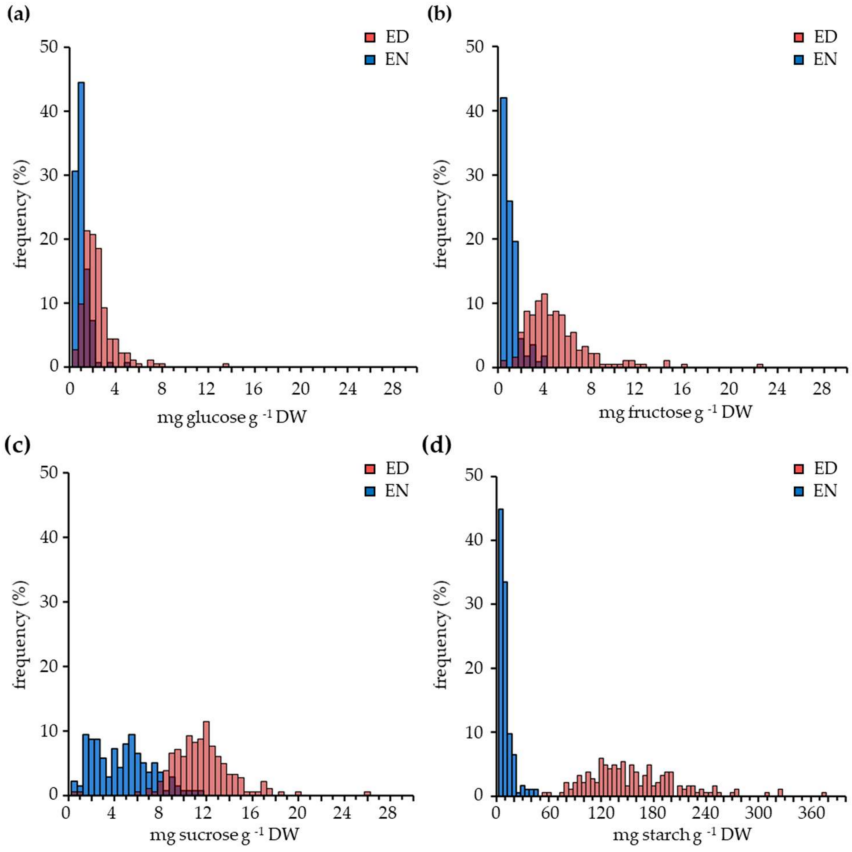

Figure 1. Frequency distribution of diel leaf glucose (**a**), fructose (**b**), sucrose (**c**), and starch (**d**) contents in 185 individual genotypes of the variety *Munida*, sampled at the end of the day (ED) and at the end of the night (EN). For measured water-soluble carbohydrate (WSC) contents, the scale was kept the same between the graphs for comparison. For (**a**–**c**), the purple sections of each histogram indicate regions where the frequency distribution of ED and EN overlap; DW: dry weight.

3.2. *Influence of Glasshouse Environment on Leaf Glucose, Fructose, Sucrose, and Starch Contents*

In order to test the effect that semi-controlled conditions have on NSC content, the 185 genotypes were equally divided into three glasshouse chambers. Due to a significant interaction between harvest

time and glasshouse chamber environment, significant effects on NSC content were determined separately. Harvest time and the glasshouse chamber environment significantly affected NSC content (Table S1). At ED, there was a significant difference ($p > 0.05$) in glucose, sucrose, and starch contents between some of the chambers, although the maximum differences between mean values observed were 24.5%, 16.3%, and 11.4%, respectively (Figure 2a,b). At EN, WSC and starch contents were less reproducible between glasshouse chambers, as significant differences ($p > 0.05$) between the chambers were observed for all WSC and starch, and maximum differences between mean values observed in the chambers were 17.6%, 51.7%, 42.4%, and 44.0% respectively (Figure 2a,b).

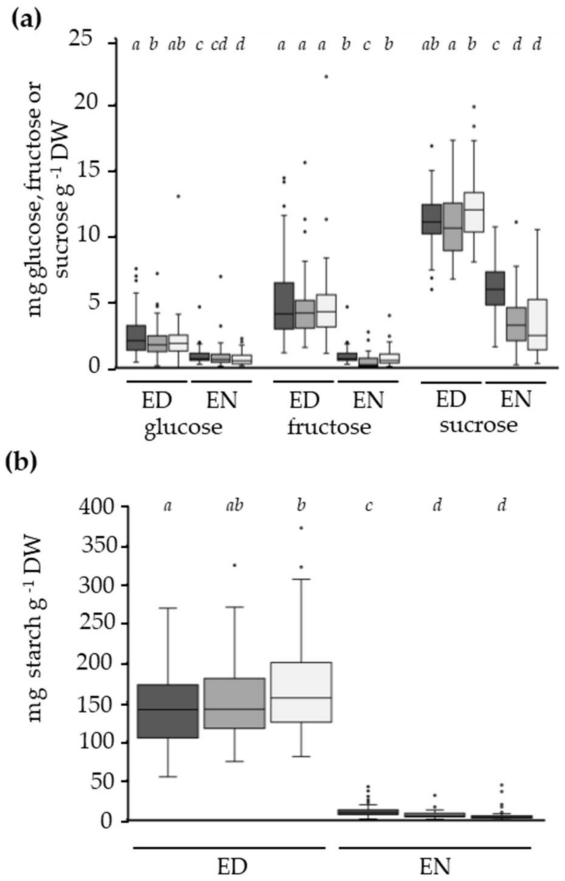

Figure 2. Glasshouse effects on variation in diel leaf glucose, fructose, and sucrose (**a**) contents and starch (**b**) content. One hundred and eighty-five individual genotypes were evenly distributed between three glasshouse chambers. The first chamber faced north and received more morning sun (dark grey), and the second chamber was positioned between the two chambers and received more afternoon sun (light-grey), and the third chamber faced south and received more evening sun (white). The letter pairs (*a*–*d*) indicate significant differences between glasshouse chambers ($p < 0.05$) determined by analysis of variance and Tukey's Honest Significant Difference (HSD) post-hoc comparison. Measurements for glucose, fructose, sucrose, and starch content were the same as described in Figure 1.

3.3. The Relationship between Glucose, Fructose, Sucrose, and Starch Contents in White Clover Leaves

The relationship between NSC contents was determined using Spearman's rank correlation. At ED, there was a strong correlation of $\varrho = 0.941$ ($p = 2.2 \times 10^{-16}$) between glucose and fructose (Figure 3a), while a low correlation was observed between sucrose and glucose ($\varrho = 0.298$, $p = 6.1 \times 10^{-5}$) and between sucrose and fructose ($\varrho = 0.291$, $p = 6.1 \times 10^{-5}$; Figure 3a). At EN, low correlations between WSCs were observed (glucose/fructose, $\varrho = 0.309$, $p = 8.7 \times 10^{-4}$; glucose/sucrose, $\varrho = 0.450$, $p = 3.1 \times 10^{-8}$; fructose/sucrose, $\varrho = 0.385$, $p = 2.5 \times 10^{-5}$; Figure 3b).

When total WSC content measured at ED and EN were compared, no correlation was observed ($\varrho = 0.0730$, $p = 0.400$; Figure 4a). There was also no correlation between ED and EN starch contents ($\varrho = -0.00457$, $p = 0.540$; Figure 4a). Therefore, higher ED NSC contents do not result in higher NSC contents at EN, or vice versa. At ED, no correlation was observed between the total measured WSC content and the starch content ($\varrho = -0.103$, $p = 0.16$; Figure 4b). Therefore, genotypes with more starch do not maintain larger WSC pools at ED. At EN, a low correlation of ($\varrho = 0.314$, $p = 2.5 \times 10^{-4}$) was observed between WSC content and starch content (Figure 4b). This correlation was mainly caused by a subset of genotypes with higher starch and higher WSC contents.

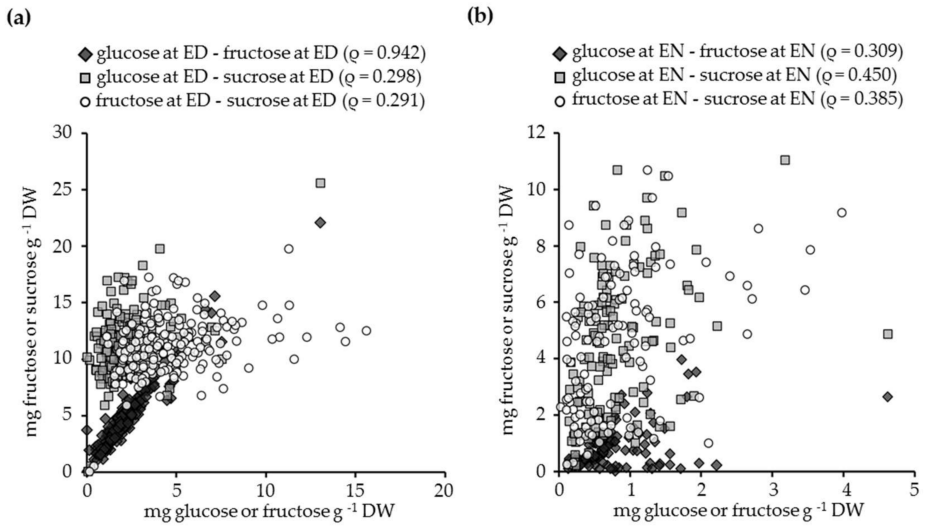

Figure 3. Relationship between individual diel leaf WSC contents in 185 individual genotypes sampled (**a**) at ED and (**b**) at EN. The corresponding *p*-values to the Spearman-rank correlation coefficients (ϱ) are indicated in the text. Measurements for glucose, fructose, sucrose, and starch contents were the same as described in Figure 1.

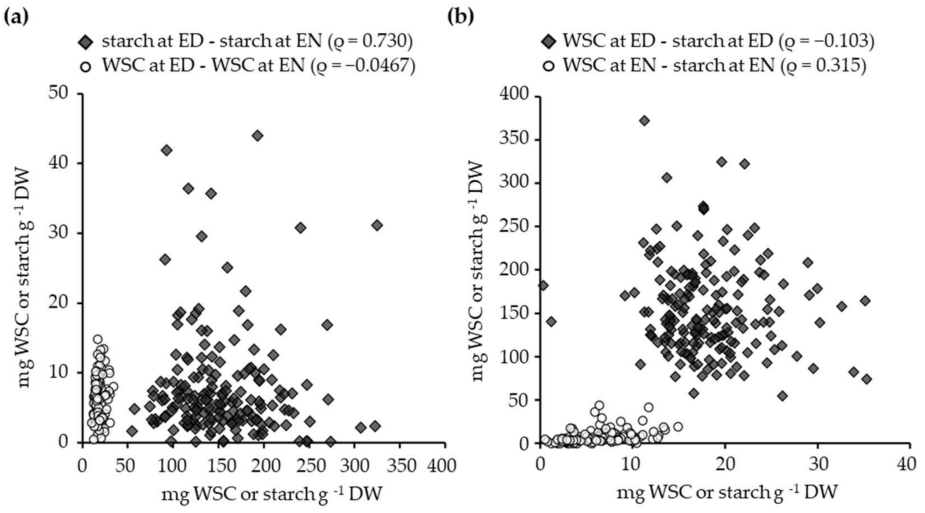

Figure 4. The relationship between combined WSC and starch contents at ED and at EN. (**a**) The relationship between WSC content at ED and EN, and the starch content at ED and EN; (**b**) the relationship between measured WSC and starch contents at EN at EN, and measured WSC and starch contents at ED. The corresponding *p*-values to the Spearman-rank correlation coefficients (ϱ) are indicated in the text. Measurements for glucose, fructose, sucrose, and starch content were the same as described in Figure 1.

3.4. The Relationship between Leaf Biomass, Water-Soluble Carbohydrates, and Starch Contents

Because starch and WSC support growth during the diel cycle, the relationship between starch and WSC contents were compared to the total fresh weight (FW) of the aerial tissue. At ED, WSC content did not correlate with biomass ($\varrho = -0.0200$, $p = 0.790$). At EN, there was no correlation between biomass and WSC content ($\varrho = -0.163$, $p = 0.0642$) (Figure 5a). This result is consistent with biomass and total WSC contents at the end of the diurnal and nocturnal cycles being independent traits. ED and EN starch contents did not correlate with leaf biomass (ED, $\varrho = 0.152$, $p = 0.0440$; EN, $\varrho = -0.0570$, $p = 0.467$; Figure 5b). Because plant growth conditions are very important in forage crops and can influence the ratio of fresh biomass to dry matter, the relationship between leaf DW per plant and leaf biomass per plant, and biomass of the aerial tissue per plant were analyzed (Figure S1). The correlation between leaf biomass DW and FW per plant was $\varrho = 0.964$ and the correlation between leaf biomass DW and aerial tissue biomass FW was $\varrho = 0.939$. These correlations support that, under the conditions used in this study, FW and DW biomass represent a similar reference for normalization.

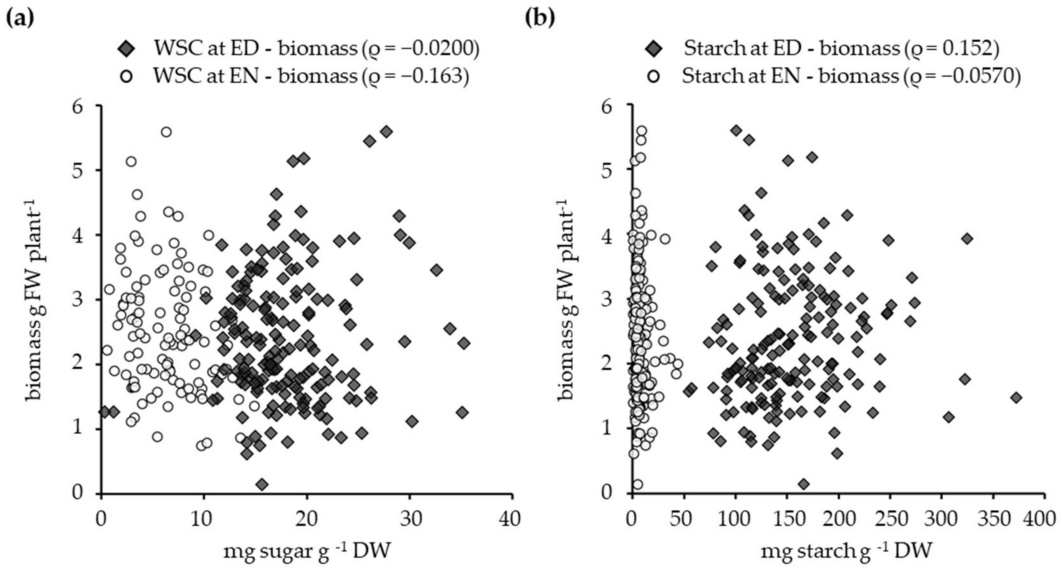

Figure 5. The relationship between WSC content, starch content, and biomass in 185 individual genotypes sampled at ED and at EN. (**a**) The relationship between combined WSC content at ED and EN and biomass; (**b**) the relationship between starch content at ED and EN and biomass. The corresponding *p*-values to the Spearman-rank correlation coefficients (ϱ) are indicated in the text. Measurements for glucose, fructose, sucrose, and starch content were the same as described in Figure 1.

3.5. Genetic Variation in Leaf Diel Leaf Glucose, Fructose, Sucrose, and Starch Contents

To estimate the genetic component of the phenotypic variation in WSC and starch contents, 12 individuals from the population were clonally propagated into 10 independent clones and assessed for NSC content at ED and EN. Although repeatability estimates are based on a rather low number of 12 genotypes, they indicate that the standard error for starch content is low for this experiment, which was carried out in a glasshouse environment (Table S2). In an agricultural setting, more genotypes would be needed to estimate repeatability. Here we use repeatability to illustrate the consistency of the growth environment, accuracy of the quantitation methodology, and genetic potential based on differences observed within a small set of genotypes. Moreover, due to the small genotype set a fixed linear model was used in the ANOVA. Due to the significant harvest time by genotype interaction, repeatability was calculated for each harvest time separately. Harvest time as well as genotype had a significant effect on all NSC measured (Table S2). At ED, sucrose had the lowest repeatability (0.753), followed by glucose (0.834) and fructose (0.867), while starch had the

highest repeatability (0.946; Table 1). Therefore, under glasshouse conditions, using this standard of quantification, the genetic variation between genotypes is the major factor contributing to phenotypic variation for glucose, fructose, sucrose, and especially starch. With the exception of EN glucose levels all NSC contents in the genotypes had a similar range to population experiments (Figure 1). This difference could be caused by more exogenous morning sun, which occurred during the longer days under which the genotype experiment was carried out. Because starch is the major NSC in white clover leaves at ED, and there was greater phenotypic variation between genotypes in starch than WSC, the genotypic variation in starch is responsible for 99% of the genotypic variation in measured NSC content. At EN, repeatability was lower for glucose (0.591), fructose (0.646), sucrose (0.709), and starch (0.799) than at ED (Table 1).

At ED, there was a significant difference between genotypes with higher glucose contents, such as *TrLB085* and *TrLB070*, and genotypes with lower glucose contents, such as *TrLB170*. At EN, there was no significant difference observed between any of the genotypes for glucose (Figure 6a). As with glucose at ED, genotypes *TrLB85* and *TrLB70* had significantly more fructose than genotype *TrLB170*. As with glucose, there were no significant differences between genotypes at EN in fructose content (Figure 6b). On average, there was a 3.2-fold increase in sucrose in the genotypes at ED when compared to EN. At ED, *TrLB082* had significantly more sucrose than 5 of the genotypes (Figure 6c). The average measured total WSC content was 15.1 mg g^{-1} DW at ED and 6.66 mg g^{-1} DW at EN.

At ED, starch accumulated to a mean content of 80.0 mg g^{-1} DW across all genotypes. *TrLB028* accumulated the most starch (144 mg g^{-1} DW), and *TrLB085* accumulated the least amount of starch (48.5 mg g^{-1} DW). Therefore, there was a threefold difference between the highest and lowest genotypes analyzed (Figure 6d). At EN, starch was retained to a mean content of 5.15 mg g^{-1} DW. *TrLB170* retained the most starch (9.11 mg g^{-1} DW), and *TrLB132* retained the least amount of starch (2.89 mg g^{-1} DW). At EN, the mean starch content across the clones was 40 times less than the ED mean content (Figure 6d). Since the mean content of starch was more than 5-fold the mean content of measured WSC at ED, starch is the major source of diel variation in NSC content. Phenotypic variation in the growth of clonal plants was also determined and biomass had a repeatability of 0.796 at ED and 0.805 at EN (Table 1). Between genotypes, no correlation between ED average starch content and average growth was observed (ϱ = 0.105, p = 0.740; Figure 7b).

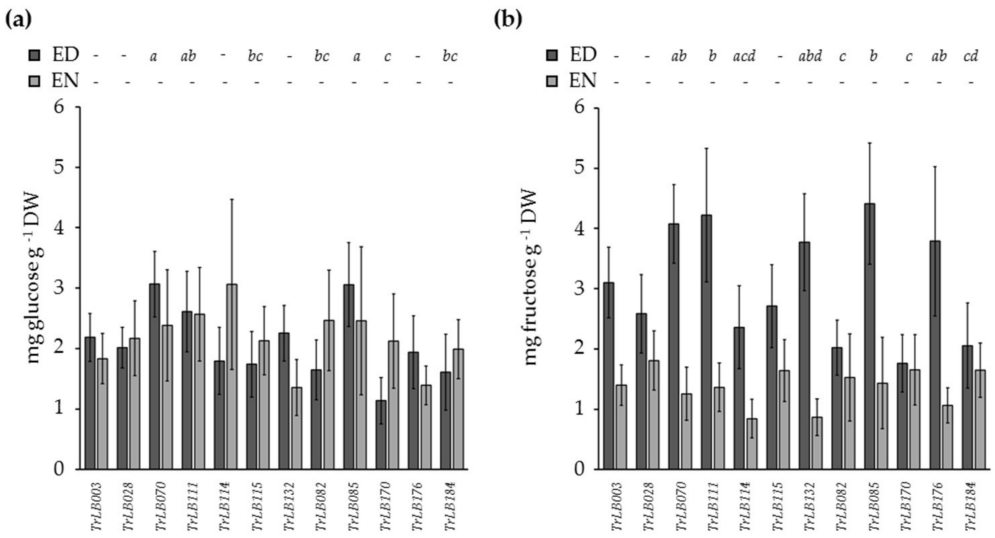

Figure 6. *Cont.*

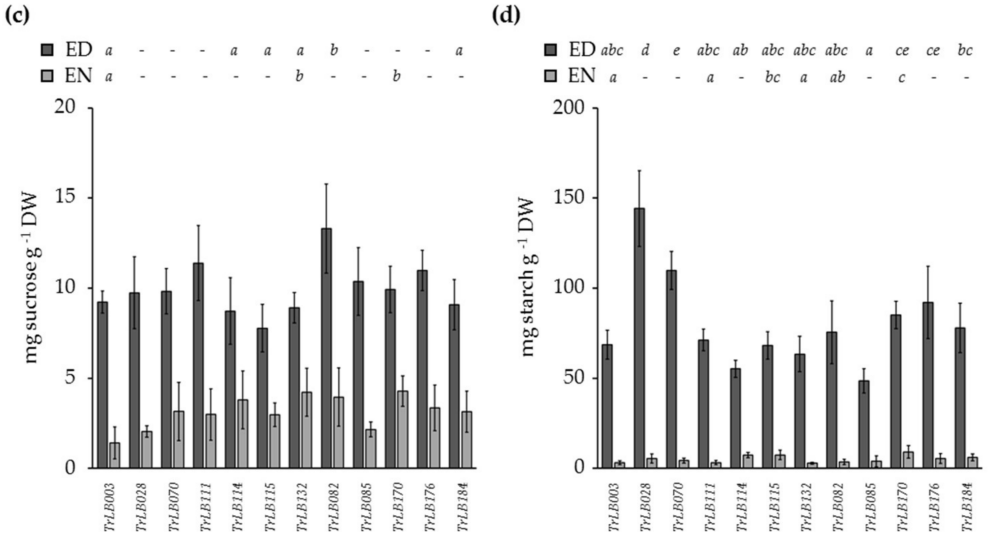

Figure 6. Diurnal leaf glucose (**a**), fructose (**b**), sucrose (**c**), and starch (**d**) contents in 12 selected genotypes. Ten clonal replicates of each of the 12 genotypes were harvested at ED and at EN. The letter pairs (*a*–*e*) indicate significant differences in NSC contents between genotypes (p = 0.05) derived from analysis of variance and Tukey's HSD post-hoc comparisons. For (**a**–**d**), a dash (–) was used to indicate no significant difference for example for ED glucose the dash (–) = *abc*. Error bars indicate 95% confidence intervals.

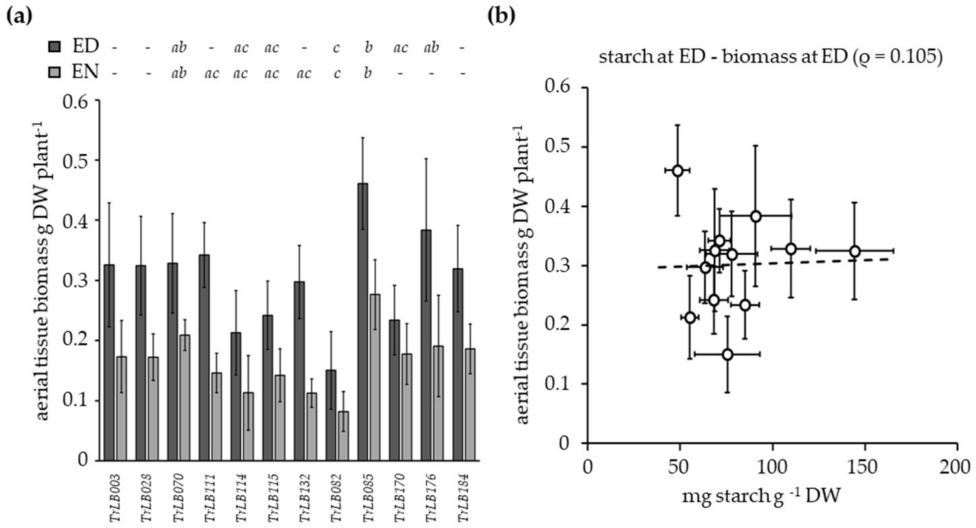

Figure 7. Relationship of starch and biomass in 12 selected genotypes, each represented with 10 clonal replicates. (**a**) Aerial biomass of the 12 genotypes described in Figure 6. Dry weights were determined at ED and at EN. The letter pairs (*a*–*c*) indicate significant differences in biomass between genotypes (p = 0.05) derived from analysis of variance and Tukey's HSD post-hoc comparisons. (**b**) The relationship between starch content and biomass at ED in clonal genotypes. The dash (–) indicates no significant difference as described for Figure 6. Error bars indicate 95% confidence intervals.

Table 1. Mean, estimate of genotypic variance, estimate of repeatability, and *F*-values as determined by ANOVA for WSC contents, starch content, total non-structural carbohydrate (NSC) content, and biomass DW: dry weight). * $p < 0.01$.

	End of Day						End of Night					
	Glucose	**Fructose**	**Sucrose**	**Starch**	**Total NSC**	**Biomass**	**Glucose**	**Fructose**	**Sucrose**	**Starch**	**Total NSC**	**Biomass**
Mean	2.01	3.09	9.86	79.9	94.8	0.298	2.16	1.38	3.12	5.28	15.1	0.165
Genotypic Variance	2.51	6.91	15.6	4830	4690	0.0429	1.65	0.792	6.03	37.2	33.8	0.0182
Repeatability	0.824	0.867	0.753	0.946	0.94	0.796	0.591	0.646	0.709	0.799	0.76	0.805
***F*-value**	4.70 *	6.51 *	3.04 *	17.4 *	15.6 *	3.91 *	1.44	1.70	2.44	3.97 *	3.24 *	4.13 *

3.6. The Pattern of Starch Content in White Clover Leaves

To further understand leaf starch content, one plant per genotype was stained with iodine at both ED and EN to qualitatively assess starch content and deposition. At ED, a higher starch content was observed than at EN, as visualized by the presence of the dark purple to black color in the leaves (Figure 8a–f). One of the general patterns of starch accumulation was that in young and juvenile tissue, starch content was generally lower than in adult tissue. The other observation was that at ED, regardless of overall starch content in the leaf, the plants fell into two types of accumulation patterns: In the first type, starch accumulates evenly throughout the leaves, such as is seen in *TrLB132*, *TrLB003*, *TrLB170*, *TrLB176*, and *TrLB184* (Figure 8a, Figure S2). The other observed accumulation pattern was less uniform with some leaf sectors showing high accumulation of starch, while other leaf sectors accumulated little to no starch, such as observed in plants *TrLB085*, *TrLB070, TrLB082*, and *TrLB114* (Figure 8b, Figure S2). Given that this uneven pattern was observed in multiple apical leaves of the same plant, the explanation that inconsistent starch deposition is caused by shading from more apical leaves does not explain the phenotype. A starch deposition pattern does not appear to affect the phenotypic variation in the total leaf starch content of the plant (Figure 6d). This observation is important for the accurate assessment of leaf starch content, because unlike model systems where single leaf measurements are representative of the whole plant starch content, in white clover, single leaf measurements may be misrepresentative of the whole plant starch content [29].

Iodine staining in the leaves was useful to distinguish between plants that accumulated significantly more starch at ED, such as *TrLB028*. Leaves in *TrLB028* were consistently dark purple to black. Plants with less starch, such as *TrLB085*, showed light purple or brown leaves (Figure 8a–c). Iodine staining was also useful to identify plants that retained starch at EN. In plants such as *TrLB28*, starch remains in some leaves at high regional concentrations at EN, whereas in other plants, the starch is uniformly degraded in all leaves (Figure 8d–f). Similar qualitative observation was seen in the other plants that were cloned (Figure S2).

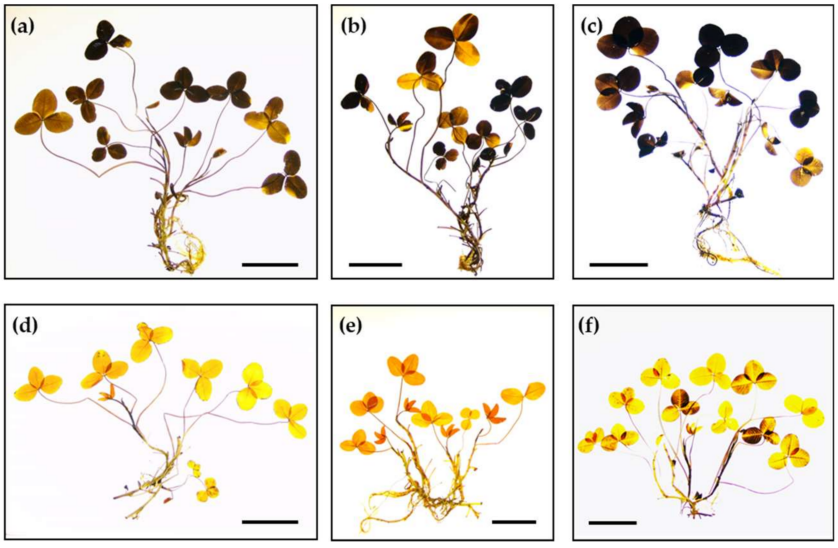

Figure 8. Staining of starch in white clover leaves at ED and at EN shows different patterns of localization across genotypes. Starch is indicated by the dark purple to black color in the leaves. Genotypes *TrLB132* (**a**); *TrLB085* (**b**); and *TrLB028* (**c**) stained at ED. Genotypes *TrLB132* (**d**), *TrLb085* (**e**), and *TrLB028* (**f**) stained at EN. Bars = 5 cm.

4. Discussion

The practical motivation for understanding NSC content in white clover and other forage legumes is to increase palatability and the overall energy content. Because WSC, starch, and biomass compete

for similar metabolite pools, the relationships between NSC content and growth need to be understood for high NSC white clover cultivars to be developed. Although NSC content is often considered when the quality of forage herbage is assessed, little work has focused on single genotypes [14]. A recently published review focused on how leaf starch content has the potential to supplement the feeding of high-energy grains, but it did not focus at the genetic relationships between WSC, starch, and growth [13]. Moreover, this review used red clover as a model, which is primarily used as a cut forage crop, so post-harvest losses must be considered [13]. One of the major problems with correlating diel NSC content and increased performance of grazing livestock is a lack of standardized methods for sampling, sample processing, and quantitation of leaf NSC. Reported NSC content in white clover and other forage legume herbage have ranged between 1 and 40% of the DW [13–15,22]. Here, we demonstrate the accuracy and precision of enzyme linked assays for quantitating NSC content and the benefit of adapting standardized sampling and processing strategies to identify white clover plants with characteristic NSC properties.

4.1. Defining NSC Content as a Trait in White Clover

Two lines of evidence support the accuracy and precision of the standardized sampling and NSC quantitation methods used in this study. The first line of evidence is the strong correlation ($\rho = 0.941$) observed between glucose and fructose, which are the major components of the hexose phosphate cytoplasmic and vacuolar glucose-6-phospate pools, which are kept in equilibrium by the activities of, phosphoglucomutase, and invertases [34]. Therefore, the close relationship between glucose and fructose is a property of these enzymatic reactions. Without accurate quantitation, this relationship, and the subsequent biochemical property of this equilibrium, would not have been observed. Although glucose, fructose, and sucrose pools are enzymatically linked by invertases within photosynthetically active cells, the concentration of sucrose in different subcellular pools combined with the role of sucrose in sugar transport may uncouple it from the glucose and fructose pools [35]. This uncoupling may explain the low to moderate correlation observed. The second line of evidence of the accuracy and precision of this method is the high repeatability of ED starch content (0.946) observed in cloned genotypes. This high repeatability is consistent with the combined variation caused by the environment and error from quantitation being a small proportion of the total variation observed in the experiment.

This data illustrates the importance of considering time of day for assessment of NSC potential in forage legumes. By comparing the differences between mean NSC contents in the genotypes, a 10-fold difference was observed between the EN and ED sampling, which is less than the threefold difference observed between the highest and lowest starch accumulating genotypes. This variation is predominantly due to the diel change in starch content. Moreover, all NSC contents had a higher repeatability at ED than at EN. The diel stability of sucrose observed in the 185 plants is consistent with its role in cellular carbohydrate transport, and glucose and fructose being used to maintain cellular respiration and function [35,36]), although in the cloned plants sucrose was the least stable WSC during the diel cycle. Starch contributing over 89.4% of the total NSC content is larger than the 30–60% previously reported for white clover [22]. The larger contribution of WSC to the NSC content observed at EN compared to ED would explain this difference. At EN, all measured NSC contents were lower, which is consistent with daytime accumulated NSC being consumed during the night to sustain growth and respiration [7,37]. Because this study only analyzed the theoretical diel minimum (EN) and maximum (ED), it did not address the contribution of WSC to the total NSC content during the photoperiod particularly at the beginning, where WSC pools typically replenish more rapidly when compared to starch, which accumulates steadily throughout the day to a maximum at ED [38,39].

Our study also demonstrates that ED glucose, fructose, and sucrose contents are not independent traits, but WSC content is independent from starch content (Figures 3 and 4). The low correlation supports the hypothesis that, although starch and WSC are biochemically coupled in the cell, they represent different carbohydrate pools with independent cellular functions [6]. A combination

of the best performing 5% of the population for ED glucose, fructose, and sucrose would result in WSC being less than 5% of the DW at ED. This WSC content is low in comparison to perennial ryegrass, where WSC content can reach over 20% of DW. The high WSC content in ryegrass is primarily constituted of fructans [17]. The best performing 5% of the white clover population contained, of their DW, more than 25% starch at ED. Because starch and WSC are independent traits, a combination of WSC and starch could result in a total NSC content of more than 30% of the biomass DW.

The other critical aspect that was assessed in this study was how NSC contents are most accurately assessed at the physiological level. Because of the high repeatability in starch content within genotypes and the relatively good reproducibility between glasshouse chambers at the population level, we conclude that inconsistent conditions that can create micro-environments in the greenhouse did not appear to be a major determinant of the mean NSC content at ED (Figure 2; Table 1) [40]. Therefore, in a single defined environment, NSC contents of white clover genotypes are reproducible and repeatable. We also demonstrate that as a trait NSC contents are maintained reproducibly at the whole plant level, even though at the level of single leaves or cellular production, starch contents can be highly heterogeneous. Based on iodine staining of starch at ED, there is not always a uniform accumulation of starch between leaves, as has been observed in model systems such as *Arabidopsis* [41]. In red clover, similar uneven starch distribution patterns were observed in plants measured at EN [13]. This observation is consistent with previous reports on the influence of leaf maturity on starch content in other species [42,43]. Because there is a high repeatability in NSC content at ED when total leaf biomass is analyzed, this uneven distribution of starch across the plant does not appear to influence the variation in whole-plant NSC content. We also observed starch retention at EN in some genotypes, which has been previously reported to occur to various degrees in red clover [13]. In *Arabidopsis*, uniform degradation of nighttime starch is tightly coupled to daytime synthesis, and almost complete degradation is typically observed under standard conditions [7,41].

4.2. Toward the Development of a Field Trait

Strategies that overcome high genotype-by-environment interactions, such as extensive genotype replication or measuring multiple times throughout the season to reduce the selection of false positives, are difficult in relation to NSC contents. The major challenge is the feasibility of sampling, processing, and quantitating NSC content for large sample numbers. Here, we show that, by focusing on ED starch content, the majority of the breeding potential for NSC content can be realized in a grazing system, where post-harvest losses are not a factor.

The primary advantage of starch over WSCs is its semi-crystalline structure, which is more energy dense, is osmotically neutral, and requires specialized amylases and glucosidases for degradation [44]. Because of its structure, starch is preferentially digested in the small intestine, where it synergistically promotes animal productivity with rumen-supplied protein. Better control of feed energy ratios that promote this synergistic action in the small intestine is one of the primary advantages of CFOs [3,27]. Consistently providing a 25–35% carbohydrate content that is primarily starch to grazing animal would arguably make pastures more competitive with CFOs, which are typically fed a carbohydrate content of 25–60% [5,27]. Although white clover leaves have the physiological potential to accumulate up to one-third of their DW as starch at ED, the development of a trait with this level of starch may not be realistic, because higher starch contents require an optimal environment for full realization.

As a direct product of photosynthesis, the environment strongly influences its metabolism and variation in its accumulation [6,13,28]. Under uniform glasshouse conditions, a high repeatability of starch content was observed. Therefore, starch content in the described genotypes can be associated with a single supplied environment. In field experiments with alfalfa, little heritability was observed between seasonal harvests in NSC contents, suggesting a high genotype-by-environment interaction [14]. To increase the reproducibility of field results, where multiple environments are observed, NSC contents need to be placed into the context of their associated environment, such as those produced reproducibly in a glasshouse setting. By applying recent advances in weather

monitoring, and eco-physiological modeling of NSC content in the field, NSC content can be placed into the proper diel and seasonal environmental context to address the low reproducibility observed for complex traits [45,46]. Therefore, the genotype-by-environment interactions, which influence the accumulation of leaf NSC and determine the heritability of NSC synthesis and degradation, become sub-traits based on environmental factors such as daytime light-intensity, day-length, nighttime temperature, and humidity. Because of its dependence on the environment, starch is arguably a poor target for conventional breeding; however, in combination with modern molecular breeding techniques, the development of leaf starch as a trait in white clover can be envisioned [13,47]. Moreover, automation of the enzyme-linked assays used here has been reported to provide high-throughput, accurate, and precise results [48]. Advances in field visualization and quantitation methods, such as hyperspectral scanning and imaging, could also provide rapid tracking of diel changes in starch in a non-destructive manner [49,50].

4.3. Relationship between Leaf NSC Content, Plant Growth, and Whole Plant Carbohydrate Homeostasis

In white clover, starch is stored beyond the diel cycle in stolons and roots [12]. This long-term storage starch is remobilized to support regrowth after cutting or grazing and is also critical for persistency [12]. Because biomass and persistency are two of the most important traits in white clover, the improvement of leaf starch content must have a minimal impact on long-term starch storage and biomass production. Although a negative correlation between ED leaf starch and biomass has been reported in Arabidopsis ecotype accessions, which are genetically homogeneous and inbred [51], little is known if a similar relationship functions in genetically heterogeneous outcrossing species. Here, we report no significant correlation between biomass and ED starch content at the single plant level, or across twelve clonally replicated genotypes. This observation is not unique to white clover as a similar observation was made in red clover [13]. Moreover, *starchless* mutants in the forage legume birdsfoot trefoil (*Lotus japonicus* L.) have been reported to have no growth penalty [52]. The high reproducibility of starch content, regardless of plant size, combined with the lack of starch staining in developing leaves, is consistent with the hypothesis that leaf starch homeostasis is highly stable and tightly coupled to the amount of source-to-sink biomass. Because starch content is stable within a genotype, and different genotypes show up to a threefold difference in starch content, white clover would be a good model to study the genetic relationship between relative growth rate and diurnal starch production. Such studies would go beyond model species, which do not contain stolon and root-based starch and would shed light on the control of the complex homeostasis between root, stolon, and diel leaf starch in one of the most important legume species worldwide.

5. Conclusions

White clover offers great opportunities to improve diurnal accumulation of leaf starch as a trait because, unlike forage grasses, forage legumes preferentially accumulate starch in their leaves over WSC. Moreover, white clover is primarily used in pastures, where breeding advances in leaf starch content would not need to endure substantial post-harvest losses. Post-harvest respiration can reduce starch content by 80–90% from dried or ensiled herbage [20]. Here, we show that there is a genetic basis for a potential ED NSC content of 30% by DW in individual genotypes. Such genotypes could be the basis for the development of varieties that have higher diurnal starch accumulation and content, which can be coupled with management practices of animal grazing to capture diel changes in the energy content of pasture herbage.

Supplementary Materials: The following materials are available online at http://www.mdpi.com/2073-4395/8/4/47/s1: Figure S1: The relationship between leaf fresh weight and dry weight. Figure S2 Iodine staining of genotypes at end of day and end of night: Table S1: Analysis of variance of the glasshouse effect on WSC and starch contents. Table S2: Analysis of variance of the genotype on WSC and starch contents.

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