ABSTRACT

Zeins are commercially important proteins found in corn endosperms. The objective of this study was to evaluate the effect of altering zeins levels in corn inbreds lines carrying endosperm mutations with differential allelic dosage and analyze its impact on the composition, nutritive value, and starch digestibility of whole plant corn silage (WPCS) at 5 storage length. Three inbred lines carrying 3 different endosperm modifiers \( \text{opaque-2 (o2)} \), \( \text{floury-2 (fl2)} \) and \( \text{soft endosperm-1 (h1)} \) were pollinated with 2 pollen sources to form pairs of near isogenic lines with either 2 or 3 doses of the mutant allele for each endosperm modifier. The experiment was designed as a split-plot design with 3 replications. Pollinated genotype was the main plot factor and storage length was the sub-plot level factor. Agronomic precautions were taken to mimic hybrid WPCS to the extent possible. Samples were collected at approximately 30% dry matter (DM) using a forage harvester and ensiled in heat-sealed plastic bags for 0, 30, 60, 120 and 240 d. Thus, the experiment consisted of 30 treatments (6 genotypes x storage lengths) and 90 ensiling units (3 replications per treatment). Measurements included nutrient analysis including crude protein, soluble crude protein, amylase-treated neutral detergent fiber, acid detergent fiber, lignin, starch, fermentation end products, zeins concentration, and in vitro starch digestibility (ivSD). The nutritional profile of the inbred-based silage samples was similar to hybrids values reported in literature. Significant differences were found in fresh (unfermented) sample kernels for endosperm vitreousness and zein profiles between and within isogenic pairs. The \( \text{o2} \) homozygous (3 doses of mutant allele) had the highest reduction in vitreousness level (74.5 to 38%) and zein concentration (6.2 to 4.7% of DM) compared with the heterozygous counterpart (2 doses of mutant allele). All genotypes showed significant reduction of total zeins and \( \alpha \)-zeins during progressive storage length. In vitro starch digestibility increased with storage length and had significant effect of genotype and storage length but not for genotype by storage length interaction, which suggest that the storage period did not attenuate the difference in ivSD between near isogenic pairs caused by zeins in WPCS. Both total zeins and \( \alpha \)-zeins showed a strong negative correlation with ivSD which agrees with the general hypothesis that the degradation of zeins increases ruminal starch degradability. Homozygous \( \text{o2} \) was the only mutant with significantly higher ivSD compared with the heterozygous version which suggest that, if all other conditions remain constant in a WPCS systems, substantial reductions in endosperm’s \( \alpha \)-zeins are required to significantly improve ivSD in the silo.

Key words: corn silage, ensiling, in vitro starch digestibility, prolamins

INTRODUCTION

Greater starch digestibility of the grain fraction of whole plant corn silage (WPCS) increases energy availability for dairy cows and thereby increases milk production, feed efficiency, or both (Firkins et al., 2001; Ferraretto et al., 2013). The starchy endosperm contained in the kernels is protected by the pericarp, which, if intact, is highly resistant to microbial attachment (McAllister et al., 1994). Mechanical processing of the kernels breaks up the pericarp which leaves the starchy endosperm exposed for ruminal bacterial attachment and fermentation. However, even the exposed endosperm is not fully digested as starch granules are tightly packed and encapsulated by prolamin proteins that form what is known as the starch-protein matrix. Prolamins, also referred to as zeins in corn, are the most abundant class of proteins in corn ker-
nels with 4 subclasses (α, β, γ, δ) defined according to their primary structure and different solubilities. As zein proteins develop and distend with advancing maturity, β- and γ-zeins cross-link and α- and δ-zeins penetrate their network, thereby encapsulating starch into a hydrophobic starch-protein matrix (Mu-Forster and Wasserman, 1998). Greater cross-linking of zein yields vitreous endosperm, which has been extensively studied due to its negative effect on starch digestibility. Kernels with higher vitreousness percentage have reduced starch degradability in vitro or in situ (Philippeau and Michaleot-Doreau, 1998; Ngogyamo-Majee et al., 2009; Lopes et al., 2009) and in vivo (Philippeau and Michaleot-Doreau, 1998; Taylor and Allen, 2005) compared with floury endosperm types.

In corn, the use of near isogenic lines with endosperm modifiers has been an empirical method to assess the effect of contrasting endosperm types over digestibility parameters focused mainly in the isolated grain portion for in situ and in vitro measurements (Ngogyamo-Majee et al., 2009) or as dry rolled kernels in situ and in vitro (Lopes., et al. 2009).

To evaluate the effect of the endosperm type within a whole plant feed ration, comparisons among commercial corn hybrids with different vitreousness levels have been reported (Der Bedrosian et al., 2012, Ferrareto et al., 2015; Coons et al., 2019). This type of studies provides valuable information about the overall effect of kernel types on feed digestibility and kinetics, but the direct effect of specific endosperm type is still susceptible to be confounded with the effect of the genotype as a whole (forage + grain). Different genotypes will likely have different in harvest indices which is defined as the mass of the grain portion divided by the total mass of the above ground biomass (grain + stover), also differences in pericarp thickness, intrinsic proteolytic machinery (plant enzymes or bacterial proteases), nutritional profile or other traits that could directly alter or at least interact with starch digestibility. In the case of long-term ensiling experiments, these factors could also affect the fermentation kinetics. This confounding is expected to hamper the ability to establish the effect of specific endosperm type on starch digestibility of WPCS.

In this study we propose the utilization of near isogenic inbred lines of corn to provide both, the forage and the grain material, to study the effect of endosperm attributes in the WPCS nutritive performance. Specifically, the first objective of this work is to study the effect of endosperm modifiers and the different allelic dosages can have on endosperm vitreousness, nitrogen fraction, fiber quality, prolamin content and in vitro starch digestibility (ivSD) using WPCS. Through controlled pollinations, we aim to alter specific endosperm traits such as vitreousness and zeins levels while keeping the same non-grain portion (stover) of the WPCS between isogenic lines.

During ensiling, the hydrophobic starch-protein matrices that surrounds starch granules are broken down, increasing starch accessibility for degradation by ruminal microorganisms (Kotarski et al., 1992). This response is intensified with prolonged storage (Kung et al., 2018). Several mechanisms are responsible for this proteolytic activity in the silo, including kernel proteases, microorganisms, and solubilization by fermentation acids (Simpson, 2001; Junges et al., 2017). Although it is well known that extended storage improves ruminal ivSD, the effect of ensiling time on ivSD and zeins concentration on WPCS produced from corn genotypes with contrasting endosperm vitreousness is not well understood. The second objective of this work is to assess fermentation profile, nitrogen fraction, ivSD, and zein degradation in WPCS of near-isogenic lines with dissimilar endosperm characteristics over multiple storage length times. Our hypothesis is that WPCS from genotypes with contrasting endosperm characteristics will show significant variability in ivSD at the beginning of the storage period, but these differences will diminish over time as ensiling progresses. By comparing WPCS from different genotypes, we aim to determine whether specific endosperm traits have a lasting impact on the digestibility of WPCS during ensiling. This information could be useful for optimizing the nutritional value of silage for livestock feed.

**MATERIALS AND METHODS**

**Silage production**

The WPCS material was generated using inbred lines carrying 3 endosperm modifiers. The inbred-modifier combinations selected were W64A o2o2 (opaque-2), Oh43 fl2fl2 (floury-2) and Oh43 h1h1 (soft endosperm-1).

Each of the inbred lines were treated with 2 different pollination regimens which resulted in 6 different silage sources to compare at harvest and during storage. The inbred lines were differentially pollinated to generate pairs of silage sources with contrasting endosperm characteristics maintaining the stover genetic material. To achieve that, half of the plots from each original inbred line were self-pollinated to attain maximum allelic dosage of the endosperm modifier (homozygosity), and the other half was cross pollinated with pollen from the isogenic wild-type version of each corresponding line (W64A and Oh43, respectively) which resulted in 2 copies of the maternal endosperm mutant allele and 1 paternal copy of the wild type allele. After pollination, each resulting silage source was considered a
unique silage genotype that is identified according to the final endosperm allelic dosage of the modifier (Self-pollinated: W64Ao2o2o2, Oh43f2f2f2, Oh43h1h1h1. Cross pollinated: W64Ao2o2wt, Oh43f2f2wt, and Oh43h1h1wt). Since all the measurements were performed after harvest, throughout the manuscript the 6 different pollinated inbred lines will be referred collectively as “genotypes” and the pairs of inbred lines carrying the same maternal endosperm modifiers, but different paternal (pollen) contribution will be referred as “near isogenic pairs” with the abbreviated mutant name (o2 for “opaque-2,” f2 for “floury-2” and h1 for “soft endosperm-1”).

Inbred lines were planted in 4 row plots, 4.8 m long and 0.75 m between rows to a planting density of 83,300 plants/ha at the University of Wisconsin-Madison West Madison Agricultural Research station in the spring of 2019. Each inbred line was planted twice within each of the blocks (reps) of the experiment thus allowing to get 1 “silage genotype” per block after pollination.

Isogenic wild-type versions of Oh43 and W64A were planted as borders with 2 delayed planted sections in the back of the experiment to provide pollen for the cross-pollinated plots. Once the plots started showing signs of transitioning to reproductive growth stage ear shoots were covered after emerging from the stalk and before silk appearance to avoid uncontrolled pollination. During this period all plants of the experiment were observed daily to look for exposed silks or shedding anthers on the tassel. If exposed silks were observed within the covered shoot and there was at least 5 cm of anthers shedding pollen, a brown paper bag was placed to cover the tassel to perform the cross the next day by taking down the brown paper bag and pouring the pollen over the silks. Each ear was pollinated on 3 consecutive days, providing excess of pollen during silk elongation to ensure optimal ear filling and minimizing the possibility of harvest index reduction due to lack of fertilization. Shoot bagging, tassel bagging and pollination were performed without removing any leaves from the plant nor breaking the tassel to mimic commercial growing conditions in silage production fields as closely as possible.

At harvest, each ear was visually inspected by pulling the husks, and if the ear was found not to be fully pollinated the whole plant was removed. When plants reached approximately 30% DM, the experiment was harvested using a research plot grade self-propelled forage harvester (JD 5830, John Deere, Moline, IL) equipped with a plot harvest sampler (Cibus TRM, Wintersteiger, Ried im Innkreis, Austria). Whole-plants were chopped at a theoretical length of cut of 1.9 cm. A full 100 L plastic container of WPCS of each chopped plot was immediately taken to the lab where samples of each plot were further mixed and subdivided to form the storage length treatments. Five-600g samples of chopped WPCS were allotted from each plot and immediately vacuum sealed in nylon-polyethylene standard barrier vacuum pouches (3.5-mm thickness, 25.4 x 35 cm, Doug Care Equipment Inc., Springville, CA) using an external clamp vacuum machine (Pro-3000, Weston, Southern Pines, NC). Each of the laboratory silos were identified with QR codes and stored at room temperature (approximately 20°C) in the dark for 0, 30, 60, 120 or 240 d. After the desired storage length was reached, the bags were immediately frozen and stored at −20°C in an horizontal freezer to stop fermentation until they were processed for laboratory analysis. All bags, including those ensiled for 240 d, were frozen for at least 21 d to ensure protocol consistency across samples. The experiment consisted of 6 genotypes x 3 blocks (reps) x 5 storage time points, which generated 90 laboratory silos. Laboratory silos are the experimental units.

**Kernel Sample Collection and Analysis**

On the day of harvest, 5 random plants were selected per plot of blocks 1 and 2 and excluded from harvest. Ears from these plants were air forced dried at 40°C for 72 h, then shelled, bulked and 10 representative kernels were used to measure vitreousness by manual dissection as described by Varela et al. (2022). Each kernel was soaked in distilled water for 3 min, then the pericarp and embryo were removed with a scalpel. The complete endosperm thus isolated was weighed on an electronic analytical balance. The floury endosperm component was manually removed using an electric rotary tool equipped with a 1/16 in round engraving accessory with the aid of magnifying glasses to enhance accuracy. After all the floury endosperm was carefully removed, the weight of the remaining vitreous endosperm was recorded to calculate vitreousness as a percentage of total endosperm weight.

**Fermentation Profile, Nutrients and Digestibility Analysis of Forage Samples.** After all storage length treatments were completed, including the 21 d treatment homogenization period in the freezer, each bag was opened and subdivided in 200g subsamples for the different wet lab analysis. Nitrogen fraction, fermentation end products and nutrients profile were performed by a commercial laboratory (Dairyland Laboratories Inc.; Arcadia, WI). Samples were analyzed for DM by oven-drying in forced air at less than 60°C until samples reach less than 6% moisture followed by National Forage Testing Association (NFTA, Method 2.1.4, Shere et al., 2006), CP (method 990.03; AOAC 2016a), borate-phosphate buffer soluble CP (Licitra et al., 1996), ADF (method 973.18 ; AOAC 2016a), floury-2 for “floury-2” and h1 for “soft endosperm-1”).
Varela et al.: Endosperm type affects corn digestibility.

When comparing traits at different storage length the samples at d 0 were excluded from the model to avoid confounding of ensiling time effects and interactions.

Because endosperm vitreousness can only be measured in intact unfermented kernels, this was measured only at storage length 0. Likewise, lignin was only measured in unfermented samples, therefore the experiment for these 2 traits was treated as a Randomized Complete Block Design as there was no subplot (storage length) factor used. Data were analyzed using the function “lm” from the package “stat” in R (R core Team, 2019).

The reduced linear model for vitreousness and lignin was:

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + e_{ij} + \tau_k + (\alpha \tau)_{ik} + \delta_{ijk} \]

where \( \mu \) = is the population grand mean, \( \alpha_i \) = is the fixed whole plot treatment effect of Genotype \( i \), \( \beta_j \) = is the fixed effect of Block \( j \), \( e_{ij} \) = error term for variation among whole plots (Genotypes), \( e_{ij} \sim N(0, \sigma^2_e) \), \( \tau_k \) = is the fixed subplot treatment effect for Storage Length \( k \), \( (\alpha \tau)_{ik} \) = is the fixed interaction effect between Genotype and Storage Length, \( \delta_{ijk} \) = error term for variation among Storage length (Subplot error), \( \delta_{ijk} \sim N(0, \sigma^2_\delta) \).

Factor means were determined using Least Square Means and the package “emmeans” (Lenth, 2022). Pairwise comparisons were computed using the “emmeans” function and the Dunn-Sidák correction was used to counteract the problem of multiple comparison.

RESULTS AND DISCUSSION

Chemical and Physical Characteristics of Fresh Samples

The average harvest DM concentration across all plots for the 6 genotypes evaluated was 28.1%, which is considered on the lower limit for optimum moisture for WPCS. The o2 isogenic pair was the only pair that showed significant differences within isogenic pairs where the homozygous version had less total DM (%) than the heterozygous version (Table 1). Opaque-2 mutants are reported to retain high content of water as compared with their normal and Quality Protein Maize (QPM) lines (Mehak et al., 2020). No differences were observed among genotypes for overall CP with an average of 9.86% of DM (Table 1).

Soluble CP showed significant differences within the o2 pair but not for the other genotypes (Table 1). Tsai et al. (1978) reported that o2 causes about 50% reduction in zein proteins compared with wild type. This type of proteins is innately insoluble in rumen environment (Larson and Hoffman, 2008), therefore, the difference in soluble CP observed for the 2 unfermented isogenic o2 samples is likely to be a consequence of reduced zeins of the homozygous version.
Concentration of aNDF and ADF (as % DM) were within the expected average range for WPCS hybrids commonly used and those previously reported in the literature including a meta-analysis of the effects of different WPCS hybrids on various fermentation and lactation performance indicators that found average NDF (% of DM) of 41.5 and ADF (% DM) of 24.2 across 126 and 107 experiments, respectively (Ferraretto and Shaver, 2015). Most of the fiber constituents including cellulose, hemicellulose and pectin are provided by the plant cell wall through the non-grain portion of the mix, with kernels having the lowest concentration of cell wall content compared with other tissues (Coors and Lauer, 2001). This relationship can help explain the high similarity of the fiber parameters within isogenic pairs observed in our study (Table 1).

Starch concentrations were low compared with hybrid-based WPCS but not outside the boundaries of possible values for corn hybrids. Low concentration of starch found in our inbred based WPCS is most likely attributed to a lower harvest index of inbred lines compared with heterotic hybrids. Despite significant efforts to exclusively harvest plants with fully pollinated ears, there is still an intrinsic disproportion of reproductive to vegetative tissue ratio between corn inbreds and hybrids probably due to the greater selection pressure that hybrids have been subjected to maintain high harvest index under modern production methods.

The protein fraction, fiber, lignin and starch levels of the inbred lines found in this study are similar to commonly reported commercial hybrids used for silage performance testing. With the data shown, we propose that under well-managed controlled conditions, inbred-based silage systems can be used as reliable models in research to investigate genetically controlled traits in a more effective way than using hybrids.

### Endosperm Vitreousness and Prolamins Levels in Unfermented Kernels

The homozygous o2 kernels had the highest reduction on endosperm vitreousness to almost half the level of its heterozygous counterpart (Table 2). This large effect was expected as this recessive mutation has been long and widely characterized to be a strong endosperm texture modifier. The O2 gene encodes a transcriptional activator that regulates the expression of several genes (Schmidt, 1993), including the 22-kDa α-zein protein (Schmidt et al., 1990) which concentration in the endosperm is positively associated with vitreousness levels. No significant difference in vitreousness level was observed among the fl2 isogenic pair (Table 2), with both dosage combinations showing extremely soft endosperm type. fl2 is a semi-dominant mutation that results from the expression of an abnormal 22-kDa α-zein which interferes with protein body assembly (Holding and Larkins, 2006). Unlike o2 which acts in a fully recessive manner, fl2 shows a partial dosage effect in which 2 doses of the mutant gene in the endosperm are needed to produce a mutant floury phenotype similar its corresponding homozygous state (Mertz et al., 1964, Jones, 1978).

Significant decrease on endosperm vitreousness was found for the homozygous h1 genotype compared with its heterozygous counterpart. This mutation is characterized as generating a “soft endosperm,” but to our knowledge this mutation has not been fine mapped nor cloned, therefore our phenotypic expectations of a heterozygous genotype were unknown, as the molecular mechanism of this mutation remains to be elucidated. Although not significant at the predefined α = 0.05, there was a 24% reduction on total zeins in the o2 homozygous compared with its heterozygous counterpart (P = 0.096, Table 2). This reduction became proportionally larger and significant for the α-zeins as expected due to the severe reduction of the 22-kDa α-zein family genes caused by the o2 mutation (P = 0.004,

### Table 1. Effect of genotype on dry matter, nitrogen fraction, fiber and lignin of unfermented WPCS

<table>
<thead>
<tr>
<th>Item</th>
<th>Genotype</th>
<th>SEM</th>
<th>Probability&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W64Ao2o2wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (% of as fed)</td>
<td>30.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CP (% of DM)</td>
<td>10.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble CP (% CP)</td>
<td>38.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>aNDF (% DM)</td>
<td>40.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADF (% DM)</td>
<td>22.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lignin (% DM)</td>
<td>2.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Starch (% DM)</td>
<td>24.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.56&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means values in the same row with different superscripts differ (P < 0.05) for Genotype effect.
<sup>1</sup>Probability of genotype effect (G).
Table 2). No difference was found for total zein content between the fl2 near isogenic pair, but significant differences were found in α-zeins levels when expressed as percentage of total zeins. Contrary to expectations, the fl2 homozygous line had higher values of α-zeins than the heterozygote counterpart. For the purpose of this research, this unexpected inversion does not affect the methods, as we are interested in capturing endosperm differences and its relations with digestibility performance regardless of which allelic dosage combination provides the highest or lowest phenotype. The h1 Isogenic lines showed no differences for any of the zein type comparisons (Table 2).

The 3 isogenic pairs analyzed in this experiment shows different associations levels between zeins and vitreousness (Table 2). For the o2 isogenic pair, the homozygous exhibited the well reported response of decreased α-zeins levels associated to a major reduction in vitreousness, which has been previously reported for several other endosperm mutants such as Defective endosperm B30 (De-B30) and Mucronate (Mc) (Holding and Larkins, 2006). Although the fl2 isogenic pair showed no changes in endosperm vitreousness, a slight increased level of α-zeins was observed for the homozygous line. Finally, the homozygous h1 exhibited significant decrease in endosperm vitreousness compared with the heterozygous with no significant change in any of the zein concentrations. Although there is a well described group of endosperm mutations that generate a vitreous endosperm through reduced zeins, the actual connection between vitreousness and endosperm types is just recently being elucidated in a limited number of mutants and much remains to be learned from most endosperm mutants. For example, there is a group of endosperm mutations that generate a soft phenotype including opaque1 (o1) and floury1 (1) that do not show reduced zein accumulations (Holding, 2014).

### Fermentation Profile and Nitrogen Fraction During Ensiling

Storage length was significant for pH ($P < 0.001$, Table 3). The pH rapidly decreased from 4.64 at d 0 to an average of 3.67 at 30 d of storage and then increased only slightly from there to 3.71 at 240 d of storage (Table 3). A pH of 4.64 is particularly low for what we aimed to be a fresh “unfermented” sample. We believe that these unusually low values might be a consequence of an accelerated start of the fermentation process pushed by the instant depletion of oxygen generated with the vacuum machine and maybe also affected by the relatively high moisture of our samples. The narrow range of pH values among isogenic lines is perhaps an indicator of a correct acid balance during ensiling and the similarity among genotypes in terms of composition and harvest index.

Lactic acid (% DM) was found in the normal range for ensiled WPCS with no significant effect of storage length ($P = 0.09$, Table 3), nor storage length by genotype interaction ($P = 0.59$, Table 3) in agreement with the values found by Der Bedrosian et al. (2012).

Storage length and genotype had a significant effect on acetoc acid concentration ($P < 0.001$ and $P = 0.003$, respectively, Table 3 and 4), but no interaction was detected. The concentration of acetoc acid increased steadily with length of storage from 1.61 at 30 d to 1.95 at 240 d. No significant difference within isogenic pairs were found for acidity, organic acids or ethanol (Table 4) which supports the notion that the allelic dosage treatment applied here did not interfere with the fermentation performance of the genotypes. These results provide a baseline to establish appropriate comparisons of prolamin degradations and digestibility.

Genotype by storage length interaction was observed for ethanol ($P = 0.016$), soluble CP ($P = 0.03$) and

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**Table 2. Effect of genotype on endosperm vitreousness, crude protein and zein profile of unfermented kernels**

<table>
<thead>
<tr>
<th>Item</th>
<th>W64Ao2o2wt</th>
<th>W64Ao2o2wt</th>
<th>Oh43fl2fl2wt</th>
<th>Oh43fl2fl2wt</th>
<th>Oh43h1h1wt</th>
<th>Oh43h1h1h1</th>
<th>SEM</th>
<th>Probability(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreousness (%)</td>
<td>74.5(^a)</td>
<td>38(^a)</td>
<td>31.5(^a)</td>
<td>33(^a)</td>
<td>56.5(^b)</td>
<td>35(^a)</td>
<td>1.39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CP (% of DM)</td>
<td>13.23</td>
<td>12.76</td>
<td>11.93</td>
<td>12.12</td>
<td>12.09</td>
<td>12.26</td>
<td>0.31</td>
<td>0.10</td>
</tr>
<tr>
<td>Zein Protein Profile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total zein (% of DM)</td>
<td>46.93</td>
<td>46.93</td>
<td>40.3</td>
<td>42.62</td>
<td>44.02</td>
<td>38.09</td>
<td>2.97</td>
<td>0.24</td>
</tr>
<tr>
<td>Total zein (% of CP)</td>
<td>30.57(^b)</td>
<td>30.57(^b)</td>
<td>19.7</td>
<td>26.25(^b)</td>
<td>22.89(^b)</td>
<td>21.23(^ab)</td>
<td>1.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>α-zeins (% of DM)</td>
<td>65.26(^c)</td>
<td>65.26(^c)</td>
<td>49.03(^c)</td>
<td>61.53(^c)</td>
<td>51.97(^c)</td>
<td>55.59(^abc)</td>
<td>2.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>α-zeins (% of CP)</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>β,γ,δ zeins (% DM)</td>
<td>34.73(^c)</td>
<td>34.73(^c)</td>
<td>48.17(^bc)</td>
<td>38.5(^ab)</td>
<td>48.03(^bc)</td>
<td>44.43(^abc)</td>
<td>2.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>β,γ,δ zeins (% CP)</td>
<td>13.23</td>
<td>13.23</td>
<td>13.23</td>
<td>13.23</td>
<td>13.23</td>
<td>13.23</td>
<td>1.39</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^a\)Means values in the same row with different superscripts differ ($P < 0.05$) for Genotype effect.

\(^b\)Probability of genotype effect (G).

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ammonia ($P = 0.018$). Ferraretto et al. (2015) also found significant levels of genotype by storage length interactions for ethanol when comparing 3 hybrids with contrasting grain endosperm properties. In this work, we found no differences within genotypes at 30 or 60 d after storage, but significant genotype mean separation were observed within storage length for 120 and 240 d (Figure 1A).

Ammonia-N and soluble CP steadily increased during storage (Figure 1B and 1C). Increased concentration of soluble CP and ammonia-N with prolonged storage time have been previously reported in the literature (Der Bedrosian et al., 2012; Young et al., 2012) and suggest the occurrence of proteolysis or solubilization of proteins (Hoffman et al., 2011). The observed increase in soluble CP of homozygous $o2$ compared with its heterozygous at all storage lengths (Figure 1C) might be an indirect consequence of the effect of the mutation on endosperm protein balance. Soluble protein was measured in the WPCS ration that includes forage and kernels, and assuming that the forage part is almost identical between near isogenic pairs, which was demonstrated previously, we could report a significance influence of endosperm type over total soluble CP of the silage mixture. The extensively reported reduction synthesis of non-soluble α-zeins proteins in $o2$ mutants comes accompanied by a series of changes including an increased synthesis of soluble non-zeins proteins. It has been suggested that the increase of non-zein proteins in $o2$ endosperm is a consequence of the diversion of N from the zein to the non-zein fraction (Habben et al., 1993).

Misra and Oaks (1981) reported increased level of ammonia in homozygous $o2$ corn compared with a wild type near isogenic line. Although not statistically significant, it is interesting to point out that a similar trend was observed for ammonia-N in our experiment at 0–30–60 and 120 d of storage (Figure 1B) when comparing the $o2$ near isogenic pairs.

Propionic and butyric acid were below the limit of detection (<0.01% DM basis) for all samples at all storage times (Data not shown) which is a good indicator of well-fermented silage, probably free of clostridial fermentation (Kung et al., 2018). This was particularly important for this experiment as it is known that forages ensiled with high-moisture have a greater risk for clostridial growth. Methanol, 1-propanol, 1,2-propanediol and 2-butanol were also below the limit of detection (<0.01% DM basis) for all samples at all storage times (Data not shown). Overall, the fermentation values obtained in this study show that under well-managed controlled conditions, inbred-based WPCS behaves similarly to hybrid systems.

### Prolamin Degradation and Starch Digestibility

Significant differences were found among genotypes, storage length and genotype by storage length interaction for total zeins (Table 3 and 4). All genotypes decreased total zeins in kernels with increased storage.

#### Table 3. Effect of storage length on nutrient composition, fermentation profile and starch digestibility in whole-plant corn silage and prolamin fraction in kernels.

<table>
<thead>
<tr>
<th>Item</th>
<th>Storage Length</th>
<th>Probability$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0d</td>
<td>30d</td>
</tr>
<tr>
<td>DM (% of as fed)</td>
<td>28.07</td>
<td>28.22$^a$</td>
</tr>
<tr>
<td>CP (% of DM)</td>
<td>9.86</td>
<td>10.13</td>
</tr>
<tr>
<td>Soluble CP (% CP)</td>
<td>43.92</td>
<td>57.03$^a$</td>
</tr>
<tr>
<td>Ammonia-N (% of CP)</td>
<td>2.05</td>
<td>4.16$^a$</td>
</tr>
<tr>
<td>Starch (% of DM)</td>
<td>21.51</td>
<td>21.67</td>
</tr>
<tr>
<td>ivSD (% of Starch)</td>
<td>53.57</td>
<td>62.62$^a$</td>
</tr>
<tr>
<td>pH</td>
<td>4.64</td>
<td>4.67$^a$</td>
</tr>
<tr>
<td>Lactic Acid (% DM)</td>
<td>1.26</td>
<td>6.46</td>
</tr>
<tr>
<td>Acetic Acid (% DM)</td>
<td>0.37</td>
<td>1.61$^a$</td>
</tr>
<tr>
<td>Ethanol (% DM)</td>
<td>0.2</td>
<td>0.55$^a$</td>
</tr>
<tr>
<td>Total Acids (% DM)</td>
<td>1.62</td>
<td>8.07$^a$</td>
</tr>
<tr>
<td>ADF (% DM)</td>
<td>23.13</td>
<td>23.64$^a$</td>
</tr>
<tr>
<td>NDF (% DM)</td>
<td>41.28</td>
<td>41.3$^a$</td>
</tr>
<tr>
<td>CP of grain (% DM)</td>
<td>12.4</td>
<td>10.41$^a$</td>
</tr>
<tr>
<td>Total zein (% of DM)</td>
<td>5.14</td>
<td>4.92$^a$</td>
</tr>
<tr>
<td>$\alpha$ - zeins (% of DM)</td>
<td>2.89</td>
<td>2.27$^a$</td>
</tr>
<tr>
<td>$\beta,\gamma$ - zeins (% of DM)</td>
<td>2.25</td>
<td>2.27$^a$</td>
</tr>
</tbody>
</table>

$^1$Probability of treatment effects: SL = Effects of storage length; G x SL = interaction between genotype and storage length.

$^{ab}$Means values in the same row with different superscripts differ ($P < 0.05$). Treatment comparison between 30, 60, 120, and 240 d.
DM (% of as fed) 29.94 c 29.46bc 26.01a 27.24ab 29.83c 28.48bc 0.49 <0.01 0.88


c - zeins (Table 3 and 4). Its concentration decreases at genotype by storage length interaction was observed for isogenic lines. Even between the isogenic lines.

No difference in total zeins at any storage length was compared with the other 2 pairs across storage length. isogenic lines had intermediate values between the isogenic pairs. The average difference in total zeins across storage length, with almost parallel lines between isogenic pairs seem relatively constant. 2B). The o2 and f2 isogenic pairs showed significant α - zeins difference at all storage stages with remarkably parallel trends within pairs. Although significant, genotype by storage length interaction was slightly below the significance threshold (P = 0.04, Table 4) and most likely that probability value was slightly influenced by the crossing over between the W64ao2o2o2 and the h1 isogenic pairs. The parallel response within isogenic lines with significantly different total zeins and α-zeins is important as it suggests that the differential efficiency of starch degradation inside the rumen will not be compensated by ensiling time, assuming that the negative effect in starch degradation is imposed by the prolamin matrix.

It is worth noting that while the results reported in this study demonstrate consistent degradation trends among all genotypes and significant differences among them, it is important to acknowledge that there might be potential inaccuracies in the estimation of prolamins as these were determined in kernels and not in WPCS mixtures. These inaccuracies could arise from the challenge of fully isolating the grain from the non-grain portion, despite the meticulous care taken during kernel separation.

Significant genotype and storage length effect were found for ivSD (Table 3 and 4). The o2 isogenic pair had significant difference in ivSD, whereas f2 and h1 displayed no difference in ivSD value (Table 4). Even with significant differences in α-zeins across all storage length (Figure 2B), the f2 isogenic lines were not different in terms of ivSD which is suggesting, that under advanced storage times similarly to total zeins (Figure 2B). The o2 and f2 isogenic pairs showed significant α - zeins difference at all storage stages with remarkably parallel trends within pairs. Although significant, genotype by storage length interaction was slightly below the significance threshold (P = 0.04, Table 4) and most likely that probability value was slightly influenced by the crossing over between the W64ao2o2o2 and the h1 isogenic pairs. The parallel response within isogenic lines with significantly different total zeins and α-zeins is important as it suggests that the differential efficiency of starch degradation inside the rumen will not be compensated by ensiling time, assuming that the negative effect in starch degradation is imposed by the prolamin matrix.

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equal conditions of comparisons, strong differences in zeins concentrations are required to generate a significant impact in starch digestibility in a whole-plant ration. Our results indicate that, for the mutations and dosages used in this experiment, the levels of α-zeins plays a more preponderant role in starch ivSD than the kernel vitreousness endosperm level. This can be seen by comparing the α-zeins and vitreousness level of the

Figure 1. Interaction between storage length and genotype for Ethanol (A; \( P = 0.02 \)), Ammonia-N (B; \( P = 0.02 \), SEM = 0.5), Soluble CP (C; \( P = 0.03 \), SEM = 1.26). Means within the same day with different letters (a-c) differs \(( P < 0.05 \), SEM = 0.22\), n.s = not significant.

Figure 2. Interaction between storage length and genotype for Total Zeins (A; \( P < 0.01 \), SEM = 0.44), α - zeins (B; \( P = 0.04 \), SEM = 0.22). Means within the same day with different letters (a-c) differs \(( P < 0.05 \))
3 isogenic pairs and their respective ivSD values. The h1 isogenic pair had significant difference in endosperm vitreousness (Table 2) and no difference in α-zein levels, which resulted in no difference in ivSD performance (Table 4) at any storage length. Contrasting, the fl2 isogenic pair, which had no vitreousness differences but intermediate level of α-zeins difference showed an intermediate ivSD separation on average compared with the other 2 pairs, although not statistically significant (Table 4). Finally, o2 showed the maximum separation having both, high vitreousness differences and the highest α-zein differences within the pairs. These inferences related to the effect of endosperm vitreousness and ivSD should be bounded to this specific situation as the preparation for this technique implied grounding at 4mm before exposure to the ruminal fluid, which might have attenuated the differences in ivSD of a vitreous endosperm.

Genotype by storage length interaction was not significant (P = 0.47, Table 3) for ivSD which indicates that fermentation in the silo did not eliminate the difference caused by floury and vitreous endosperms or high or low level of zeins. These results support research evaluating storage length of corn hybrids varying in endosperm type (Der Bedrosian et al., 2012; Ferraretto et al., 2015; Coons et al., 2019) and refute the long-existing industry observations that fermentation in the silo eliminates any differences between floury and vitreousness endosperm of unfermented kernels. Der Bedrosian et al. (2012) reported that BMR corn silage reached similar in vitro starch digestibility to conventional corn silage only after 270 d of fermentation when both were harvested at 32% of DM, but this phenomenon was not observed even after 360 d of fermentation when both hybrids were harvested at 41% of DM. Likewise, prolonged storage (240 d) did not attenuate the negative effects of vitreousness on starch digestibility of WPCS (Ferraretto et al., 2015). Coons et al. (2019) reported that a BMR floury hybrid had 5.6%-units greater in vitro starch digestibility than a non-floury BMR and a conventional corn hybrid after 120 d of fermentation despite having lower digestibility at 0 d. These authors speculated that perhaps instead of reducing differences between hybrids, storage length may increase this effect. But regardless of endosperm properties, prolonged storage improved starch digestibility of all hybrids across all these different studies and is a viable practice to enhance WPCS starch digestibility.

Both total zeins content and α-zeins showed strong negative correlations with ivSD (Figure 3A and 3B, r = −0.69 and r = −0.72, respectively) which agrees with the general hypothesis that the degradation of hydrophobic zeins proteins increases ruminal starch degradability by increasing accessibility of starch granules to rumen microorganisms (Philippeau and Michalet-Doreau, 1998).

In vitro starch digestibility linearly increased (3C and 3D) with soluble CP (r = 0.76; P < 0.001) and ammonia-N (r = 0.67; P < 0.001) which agrees with Ferraretto et al. (2015). The published study compared 8 hybrids using the same digestibility assessment used in this work (4-mm ivSD). Similar results were observed for high-moisture corn and thought to be related to the lower concentration of zein-proteins associated with greater ammonia-N and soluble-CP concentration after 240 d of ensiling (Hoffman et al., 2011). These data suggest that ammonia-N and soluble CP are good indicators of starch digestibility and may be used in future models as predictors of starch digestibility as proposed by Ferraretto et al. (2015).

CONCLUSIONS

The present study shows the first continued assessment of zeins during storage of WPCS. Soluble protein and ammonia-N concentrations increased through storage for all genotypes supporting the idea that extended fermentation time is beneficial for dairy farmers independently of the hybrid type. The o2 near isogenic pair showed the highest difference in zeins profile and was the only pair able to significantly show differences in starch digestibility. All genotypes decreased total zeins and α-zeins with storage length. Interestingly the 2 pairs with significant difference at all storage lengths for α-zeins (o2 and fl2) showed a parallel response, which would suggest that extended ensiling times will not alter the negative effects of α-zeins on ivSD. The present study shows that for 4mm ground ivSD, α-zein levels plays a more preponderant role than vitreousness in terms of starch digestibility. Finally, total zeins, α-zeins, ammonia-N and soluble CP were all significantly correlated to ivSD, which implies that the different measurements of protein content can be used in future models instead of ivSD, reducing labor and cost for commercial laboratories and dairy farmers. Overall, these results emphasize the importance of endosperm type and ensiling time to achieve maximum ivSD of WPCS.

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