# WORKING WITH WHITE WHEAT: IMPACTS ON AGRONOMIC AND QUALITY TRAITS FROM BULK BREEDING AND COLOR SORTING

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**MASTER OF SCIENCE**

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## ABSTRACT

Though the NDSU spring wheat breeding program developed some adapted HWSW germplasm in the last 30 years, significant improvement in baking quality is needed to meet the milling and baking industry's needs. To make improvements, elite HRSW cultivars are commonly crossed to HWSW germplasm. This study used an optical color sorter to create four color-sorted subpopulations from a cross between Glenn (HRSW) and NDSW0932 (HWSW) in addition to unsorted and single seed descent subpopulations. The NaOH test determined pericarp color for 1927 $F_{5:6}$ plants and found differences with generations and intensity of sorting. From these subpopulations, 265 $F_{5:7}$ inbred lines were compared in a yield trial with two replicates across three ND locations in 2020. Measured traits included heading, height, lodging, yield, test weight, protein, SRC, GlutoPeak, BLS, and PPO. Significant subpopulation means and variance of traits were analyzed and interpreted. Breeding applications and future work was considered.

## ACKNOWLEDGMENTS

I would like to acknowledge the teamwork, help, and guidance of the NDSU HRSW Team led by my advisor, Dr. Andrew Green, and my coworkers Andre Luis Miranda, Thor Selland, and Jesse Underdahl. The creation and growth of the NDSU Big Data Pipeline Unit has been important during the time this research has unfolded, and particular thanks go to Dr. Ana Maria Heilman Morales and Didier Murillo. The research done by Kelly McMonagle and DeLane Olsen at the NDSU Wheat Quality Lab was crucial to this study and was overseen by Dr. Senay Simsek at the time of this work. I have been happy to have my graduate committee of Tom DeSutter, Richard Horsley, Gideon Francois Marais, as well as Dr. Green, to learn from through the years. John Rickertsen of the NDSU Hettinger Research Extension Center has shown interest and drive to assist in developing HWSW germplasm and has been an important partner in getting new populations and cultivars grown in their target environments. Edil Vidal Torres was a fantastic colleague who helped organize and formalize some procedures used in this research. Numerous student employees through the years have been the helping hands needed at the Spring Wheat Breeding lab, including Lucas Huss, Kory Johnson, Katarina Jones, Cade Kapphahn, Emily Lind, Jacob Mehlhoff, Vanessa McNutt, Kayla Provencher, Siddhant Rana, Hannah Swenson, and Michelle Witt. And finally, thanks and pay raises should go out to the NDSU Department of Plant Sciences faculty and staff, who step up regularly. I appreciate you all.

## DEDICATION

To my late mother, Mary Lou Walz, for inspiration.

To my dad, Gary Walz, for dedication.

To my daughter, Liliana Walz Seviour, for curiosity.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| AU | Absorbance Units (from spectrophotometer) |
| B | Block, or replication |
| BLS | Bacterial Leaf Streak, *Xanthomonascampestris pv. Translucens* |
| BLUE | Best Linear Unbiased Estimate (for fixed effects) |
| BLUP | Best Linear Unbiased Predictor (for random effects) |
| BU | Brabender Units |
| CAR | Carrington, ND plots located at ND Research Extension Center |
| F | Filial generation |
| G | Genotype |
| GP | GlutoPeak test (Brabender Instruments) |
| HET | Hettinger, ND plots located at ND Research Extension Center |
| HRSW | Hard Red Spring Wheat |
| HRWW | Hard Red Winter Wheat |
| HWSW | Hard White Spring Wheat |
| HW | Hard White |
| L | Location |
| NaOH | Sodium Hydroxide |
| ND | North Dakota, USA |
| NDAES | North Dakota Agriculture Experiment Station |
| NDSU | North Dakota State University |
| NZ | New Zealand |

PPO ..... Polyphenol Oxidase

PRO ..... Prosper, ND plots located at ND Research Extension Center

S ..... Subpopulation

SpATS ..... Spatial Analysis of field Trials with Splines

SRC ..... Solvent Retention Capacity test

SSD ..... Single Seed Descent

## LIST OF SYMBOLS

Ɛ.....error

μ .....mean

y.....variable, or trait value

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# INTRODUCTION

As a staple of our food system and a major agricultural product in our state, NDSU has been breeding hard red spring wheat (HRSW; *Triticum aestivum* L.) for over 100 years. The program has aimed to release improved cultivars for North Dakota's farmers with suitable baking quality and agronomic characteristics. In addition, there has been a small hard white spring wheat (HWSW) breeding program since the 1990s. Although some HWSW germplasm adapted for the Northern Plains has been developed, significant improvement needed in whole-grain baking quality is needed to meet the milling and baking industry's needs for whole-grain flour. The importance of this work affects three aspects of food production. First, it provides a new option for growers to secure contracts that may give a premium price. Second, HWSW whole grain flour would expand markets for our region's milling and baking industry. Third, society would benefit from additional whole wheat flour options that create more widely desirable products and a healthier population (Prasadi and Joye, 2020).

## Wheat Breeding History and Techniques

Wheat cultivation in the Great Plains began in the 1800s (Paulsen and Shroyer, 2008). Early cultivars adapted to this region were HRSW. They were increased through selection and eventually became accepted as the highest-quality bread-baking wheat varieties. This tradition continued with the establishment of public land grant university breeding programs, which steadily improved and released HRSW and HRWW cultivars through the twentieth century (Paulsen and Shroyer, 2008; Taylor et al., 2005). These efforts have steadily improved agronomic and disease-resistance traits while maintaining high quality for millers and bakers (Underdahl et al., 2008). While hard white (HW) wheat cultivars were grown in Australia, it was not until Elmer G. Heyne – leader of the wheat improvement program at Kansas State University

from 1946 to 1982 – that HW wheat began to play a role in American breeding programs (Paulsen and Shroyer, 2008). In 1990, the USDA officially recognized HW wheat as a distinct class. The US milling and baking industries have indicated an interest in using HW because of higher flour extraction rates, sweeter tasting bread, and suitability for Asian noodles and steamed bread (Taylor et al., 2005). These advantages encouraged incentives from the US government and contracting programs from industry.

Many breeding programs for wheat employ a modified pedigree method, as does the HRSW program at NDSU. This method allows for early generation selection ($F_2 – F_3$) of highly heritable qualitative traits. It leads to fewer inbred lines that are unacceptable for simply inherited traits, such as plant height or rust (incited by multiple Puccinia spp.) resistance. However, it is thought to restrict variation for complex quantitative traits. Bulk breeding is another strategy employed to efficiently advance populations to near-inbred lines ($F_5 – F_6$) before selection is imposed. Its application maintains a high genetic variability for quantitative traits, such as yield or baking quality.

## Genetic and Environmental Factors Affecting HWSW Breeding

Seed coat (pericarp) color in wheat is conferred by three major homoeologous genes on chromosomes 3A, 3B, and 3D (Metzger and Silbaugh, 1970). While there are other colors, only red and white seed coats will be discussed. The seed coat forms from maternally-derived tissue, so the identification of seed color indicates the phenotype of the maternal plant. This trait is additive, where only a single dominant red allele at one of the three loci will confer red color, making it unacceptable for use as HW flour. Thus, a white seed will be homozygous recessive at all three loci. Table 1 gives the expected genetic frequencies of white seed for crosses between a white parent and a red parent with one, two, and three red alleles (Brabec et al., 2017). With

populations segregating for all three red alleles, and because of the very low frequencies of HWSW found in the early stages of inbreeding (through $F_5$ plants), the population size of HWSW plants is more restricted than a typical breeding population (e.g. compared to a HRSW population or a HWSW population with all HWSW parents). Suppose the minimum number of plants from which to select in a population is 1000. In that case, the number of HWSW plants expected within that population is small when considering three segregating red alleles: for an $F_3$, 16, an $F_4$, 53, and an $F_5$, 84. This leads to a smaller pool of plants to find genotypes with desirable traits.

**Table 1.** Expected genetic frequencies of white seed for segregating populations of wheat.

| Segregating Loci | Expected Frequency of White Seed | | | | | |
|---|---|---|---|---|---|---|
| | **$F_1$** | **$F_2$** | **$F_3$** | **$F_4$** | **$F_5$** | **$F_6$** |
| 1 Red allele | 0.000 | 0.000 | 0.250 | 0.375 | 0.438 | 0.469 |
| 2 Red alleles | 0.000 | 0.000 | 0.0625 | 0.141 | 0.191 | 0.220 |
| 3 Red alleles | 0.000 | 0.000 | 0.016 | 0.053 | 0.084 | 0.103 |

*Note:* The table is adapted from Brabec et al., 2017.

In addition to low frequencies of white seeds when a HRSW parent is crossed with a HWSW parent, environmental factors complicate the determination of seed color because the effects of preharvest rain and the length of time the crop stands post-maturity (weathering). These weathering effects cause increased lightness in the seed coat and decreased vitreousness, meaning that pre-harvest environmental conditions can degrade pericarp color (McCaig, et al., 2006). Though genetic factors control pericarp color, this environmental effect causes some samples from HRSW and HWSW genotypes to overlap in color within locations, so that visual identification of color might lead to misidentification (Wu et al., 1999). When choosing locations and dedicating harvest resources for breeding programs which have both red and white germplasm, these factors should be considered.

## Color Screening Methodologies

Because of the low proportions of HWSW in HRSW-by-HWSW populations, and the confounding effects of phenotypic expression in variable environments, different techniques are used to select for HWSW in these populations both during the inbreeding and selection generations ($F_2$ to $F_5$), and to screen inbred plants before entry to a yield trial ($F_6$ or $F_7$).

### Optical Color Sorting Systems

Optical color sorting systems separate differently colored seeds from bulk samples by accepting or rejecting individual seeds during sample sorting. The threshold of acceptable seed is determined by calibrating the color sorter with reference seed lots, one for the acceptable seed color, and one with seeds that should be rejected. The optical color sorter used in this study was constructed by a collaboration between USDA/ARS and the National Manufacturing Company. It uses compressed air to divert seeds that meet the rejection threshold into a separate container from the acceptable seed lot (Pearson et al., 2008).

Populations resulting from HRSW-by-HWSW crosses can be sorted into red and white seeds. Because of phenotype variability due to environment (Brabec et al., 2017; Knott et al., 2008; Talbert et al., 2013)., the calibrations should use known red and white genotypes from the same location and for the population of interest. Processing errors occur during sorting, but when using a moderate sorting rate, Pearson et al. (2008) could sort seeds with accuracy above 90% consistently. This holds even when the color contrast is low. To achieve higher accuracy, a slower rate of sorting or multiple sorts can be used (Pearson, et al., 2008). After repeated testing and use, it was determined that much of the error occurred in the container for the retained seed. To compensate for this, the first sort can be completed for the dominant color (red), with the

rejected seed (white) kept from the rejection container. This can be repeated, as was done in this study, with the final sort keeping the desired color (white).

### Sodium Hydroxide (NaOH) Color Test

The potassium hydroxide test (USDA-FGIS, 2006) has been used to measure of red and white seed phenotypes due to its reaction with tannins in the pericarp (Brabec et al., 2017). It helps alleviate the differentiation of color in environments where the phenotype is unclear. Ram, et al. (2002) compared methods of performing a color test using variations in time, soak temperature, chemicals, and chemical concentration. Their study helped inform, adapt, and optimize this research's NaOH color test procedure. It can be used at any generation, but because the seed is inviable after testing, it is unsuitable for segregating populations in early generations. It should be used to affirm the color of inbred lines with ample seed supply.

### Marker Assisted Selection (MAS)

Closely linked microsatellite markers have been discovered for each locus controlling pericarp color, and were validated in a population containing germplasm from Montana State University (Sherman et al., 2008). Due to the segregation of heterozygotes, this technique cannot be used in early generations to confirm that a line is red, but can confirm that a line is white. Because of the resources needed for MAS, screening inbred lines upon entry to a preliminary yield trial or to use when backcrossing is reasonable, but not for screening each plant in early generations (Talbert et al., 2013).

## HWSW Breeding Objectives

NDSU seeks to develop HWSW cultivars with yield, baking qualities, and disease resistance similar to the HRSW cultivars it releases for production in ND. One difference in HWSW end-use quality is a focus on whole wheat milling and baking. Small-scale tests, such as

Solvent Retention Capacity (SRC) and GlutoPeak, are critical for breeding programs to predict industrial-scale baking performance of experimental inbred lines (AACC 56-11.02, Wang, et al., 2018).

## Research Objectives

This work will answer three questions critical to improving HWSW for crosses which involve a HRSW parent and utilize bulk breeding combined with an optical color sorter for selection of HWSW:

1) Do subpopulations of HWSW developed through SSD or different generations of first sorting or number of color sorts per generation affect the proportion of HWSW $F_5$ plants?
2) Do those subpopulations differ in agronomic and quality characteristics?
3) Do any of those subpopulations have higher overall agronomic and quality performance so that higher proportions of genotypes would be advanced in a typical selection scheme?

For questions one and two above, this study will test the null hypothesis that no color-sorted subpopulation of $F_{5:7}$ inbred lines derived from different generations of sorting or sorts-per-generation differs from the unsorted subpopulation or SSD subpopulation with respect to a higher proportion of $F_{5:6}$ HWSW lines, and subpopulation means for agronomic or end-use quality performance.

Question three above places this work in a breeding program context, and is worth considering due to potential implications of the above questions for a breeding program, but is considered in a more subjective way.

## METHODOLOGY

The population from which all subpopulations and experimental lines were developed for this experiment ('W16-0006'), is the result of a cross between 'Glenn' (Reg. no. CV-974, PI 639273) and 'NDSW0932', an unreleased HWSW line with the pedigree NDSW0345/ND2831 (Figure 1). The cross was made in February 2016 in a glasshouse room at the Jack Dalrymple Agricultural Research Complex greenhouse. Glenn was chosen as a HRSW parent for consistently dark red color expression and high end-use quality traits. In contrast, NDSW0932 was chosen as the HWSW parent due to consistent white seed color expression across environments and lower-than-average protein. Based on white and red seed segregation ratios in previous HRSW by HWSW crosses, Glenn is a HRSW, assumed to have three dominant red alleles, and NDSW0932, as all HWSW cultivars, has three recessive white alleles. Based on Table 1, the expected genetic frequency of white seed in the population without selection will be: 0.000 for $F_2$, 0.016 for $F_3$, 0.053 for $F_4$, 0.084 for $F_5$, and 0.103 for $F_6$.

a)

b)

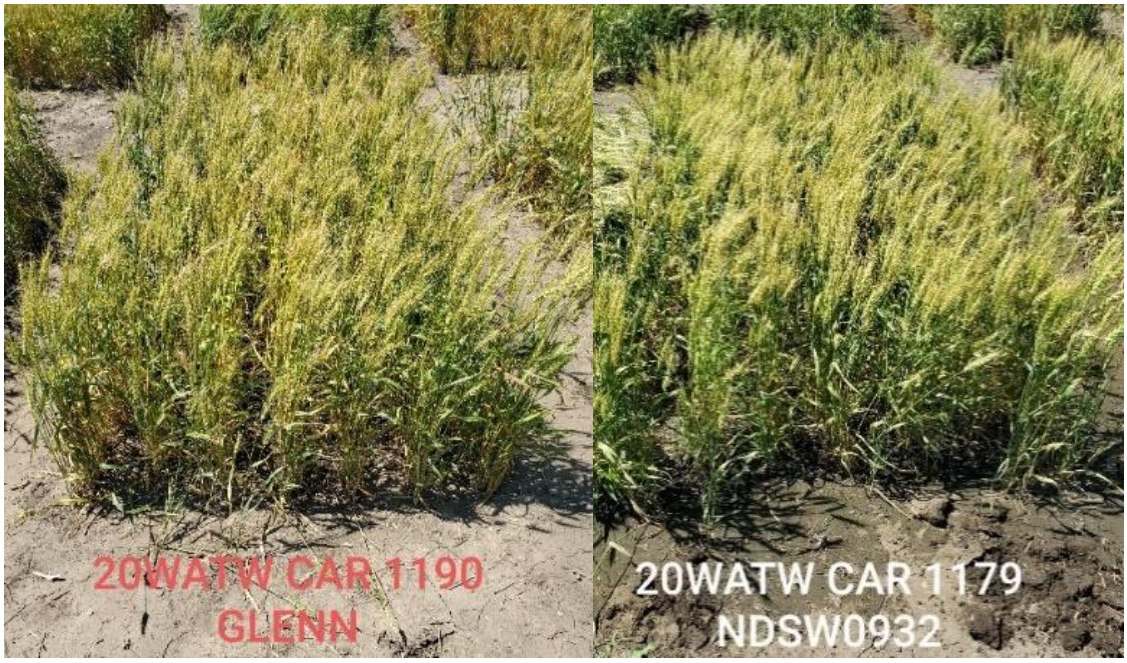



**Figure 1**. Parents of cross W16-0006, Glenn (left) and NDSW0932 (right) from Carrington, ND: a) seed, b) plants on 23 July 2020 during grain fill, Zadoks 85.

**Subpopulation Development**

In this paper, "Subpopulations" refers to the below-described fixed subpopulations derived from this particular cross W16-0006 with the associated color sorting details. Additionally, Subpopulation 1 is abbreviated as "S1," and similarly for the remaining Subpopulations.

From the initial cross, 15 $F_1$ seeds were sown for increase in June 2016 at the Jack Dalrymple Agricultural Research Complex greenhouse. This increase provided all the seeds for developing six Subpopulations (Table 2), that represent typical combinations of sorting generation and number of sorts per generation that are common to bulk breeding and selection for a HRSW by HWSW population in the NDSU Spring Wheat Breeding program. For each Subpopulation, the generations of color-sorting and number of sorts per generation are shown. The purpose of S6 was to achieve inbreeding of a control population of plants with minimal natural selection pressure compared to the other Subpopulations, which had natural selection due to field conditions but no plant selection. S1 is included as a field-grown control with no color-sorting.

**Table 2.** Color sorting details for the six subpopulations descended from the cross between Glenn and NDSW0932†.

| Subpopulation | Description | Generation(s) of Color Sorting |
|---|---|---|
| 1 | Unsorted | none |
| 2 | $F_3$ Double-sort‡ | $F_3$, $F_4$, $F_5$ |
| 3 | $F_3$ Triple-sort‡ | $F_3$, $F_4$, $F_5$ |
| 4 | $F_4$ Triple-sort‡ | $F_4$, $F_5$ |
| 5 | $F_5$ Triple-sort‡ | $F_5$ |
| 6 | Single Seed Descent (SSD) | none |

†All subpopulations descended from a bulk of the same $F_2$ population, W16-0006. ‡Double-sort went through the color sorter twice each generation of sorting, while Triple-sort went through the color sorter three times per generation of sorting.

No additional selection was performed at each generation and across all Subpopulations, except color-sorting at the stated generations. Additionally, any subsampling for the purpose of sowing a next generation was done as randomly as possible. Typical greenhouse, field, and crop management practices were used to produce plants at all locations consistent with local breeding or seed increase recommendations with regard to plots and rows (Wiersma and Ransom, 2005).

Color sorting for S2 was doubled, having gone through the color sorter twice each generation of sorting, while S3, S4, and S5 had triple sorting, having gone through the color sorter three times per sorting generation. The ranking of color sorting intensity is assumed to be S3 > S2 > S4 > S5 >> S1 = S6. That is, color sorting intensity was highest for S3, with triple sorting at the $F_3$, $F_4$, and $F_5$ generations, followed by S2 with double sorting at the $F_3$, $F_4$, and $F_5$ generations, followed by S4 with triple-sorts at the $F_4$, and $F_5$ generations, followed by S5 having a triple-sort at the $F_5$ generation. No color sorting was done with S1 and S6; thus, the proportions of HWSW in them are due to the parent's genetics and any sampling or natural selection effects.

**Field-developed Subpopulations (S1 – S5)**

An offseason nursery near Leeston, New Zealand (-43° 43' 59.0514", 172° 21' 37.0152") was used for the $F_2$ to $F_4$ seed increases of S1 through S5 to achieve quicker inbreeding within the Subpopulations by use of an off-season growing cycle. Additionally, pericarp phenotype expression had high penetrance, and we sought to remove as much sorting error due to environmental factors as possible in the earliest generations. At the $F_2$ stage (November 2016 – February 2017), one 2 m row was grown with 6 g of seed from the original $F_1$ greenhouse increase. That $F_2$ row was bulk harvested, and the $F_3$ seed was randomly split into the five field-developed Subpopulations according to Table 3. The $F_3$ Subpopulations were grown (November 2017 – February 2018) in three 2 m rows per Subpopulation, with 492 seeds per Subpopulation.

**Table 3.** Population sizes, from $F_3$ through $F_5$, of all subpopulations descended from a bulk of the same F2 population, W16-0006.

| Subpopulation | Advanced $F_2$ to $F_6$ | Color sorting description | $F_3$ seeds planted | $F_4$ seeds planted | $F_5$ seeds planted | $F_{5:6}$ spikes harvested |
|---|---|---|---|---|---|---|
| 1 | field | Unsorted | 492 | 1476 | 1200 | 316 |
| 2 | field | F3 Double-sort | 492† | 2117† | 1200† | 255 |
| 3 | field | F3 Triple-sort | 492† | 1563† | 1200† | 284 |
| 4 | field | F4 Triple-sort | 492 | 959 † | 1200† | 271 |
| 5 | field | F5 Triple-sort | 492 | 1476 | 537 † | 337 |
| 6 | greenhouse | SSD‡ | 476 | 475 | 471 | 464 |

† Source seed was color sorted before planting. ‡SSD = Single Seed Descent.

The $F_4$ and $F_5$ plants of the field-developed Subpopulations were grown at the NDSU Research Extension Center in Hettinger, ND (46°00'46.3"N 102°38'40.5"W, soil: clay loam - E0617B—Belfield-Wyola-Daglum complex) with six row plots of area 6.9 $m^2$ (74 square-feet) and having 25 cm (about 9.84 in) row spacing. Three $F_4$ plots per Subpopulation were sown in April 2018 and bulk harvested in August 2018. Three $F_5$ plots per Subpopulation were planted in April 2019 and one spike from each plant in each Subpopulation was harvested in August 2019. Those spikes (with one $F_6$ seed representing each plant) were run through the NaOH color test, and a proportion of them were increased at the New Zealand winter nursery from November 2019 to February 2020 for entry to the 2020 yield trial as $F_{5:7}$ inbred lines.

**Greenhouse-developed Subpopulation 6**

From the remnant $F_2$ seed from the initial greenhouse increase in the summer of 2016, 492 seeds were chosen randomly to establish the Single Seed Descent (SSD) subpopulation, S6. Those initial 492 $F_2$ seeds were grown in 492 individual cone-tainers (Ray Leach Cone-tainer SC10- 3.8 cm X 21 cm, Stuewe and Sons, Inc. Tangent, Oregon). Upon maturity, one random seed per spike was reserved and resown to each subsequent generation as such for four generations, from $F_2$ to $F_6$, in the Jack Dalrymple Agricultural Research Complex greenhouse

from January 2018 through August 2019. Of the initial 492 lineages, a small number were lost each generation (Table 3), and 464 $F_6$ plants were harvested in August 2019, which was the most of any $F_6$ Subpopulation.

**NaOH Color Tests**

The NaOH color test, used to determine pericarp color for all the $F_{5:6}$ spikes from all Subpopulations, is detailed in Appendix A. As shown in Fig. 2, one seed was taken from each spike to represent each $F_{5:6}$ plant, as the $F_{5:6}$ lines were considered sufficiently inbred. From the NaOH Color test, genotypes were classified as HRSW or HWSW and counted within each Subpopulation. The Pearson Chi-Square test was used to compare the proportions of HWSW genotypes within each Subpopulation against each other. The null hypothesis was tested at the 95% level of confidence.

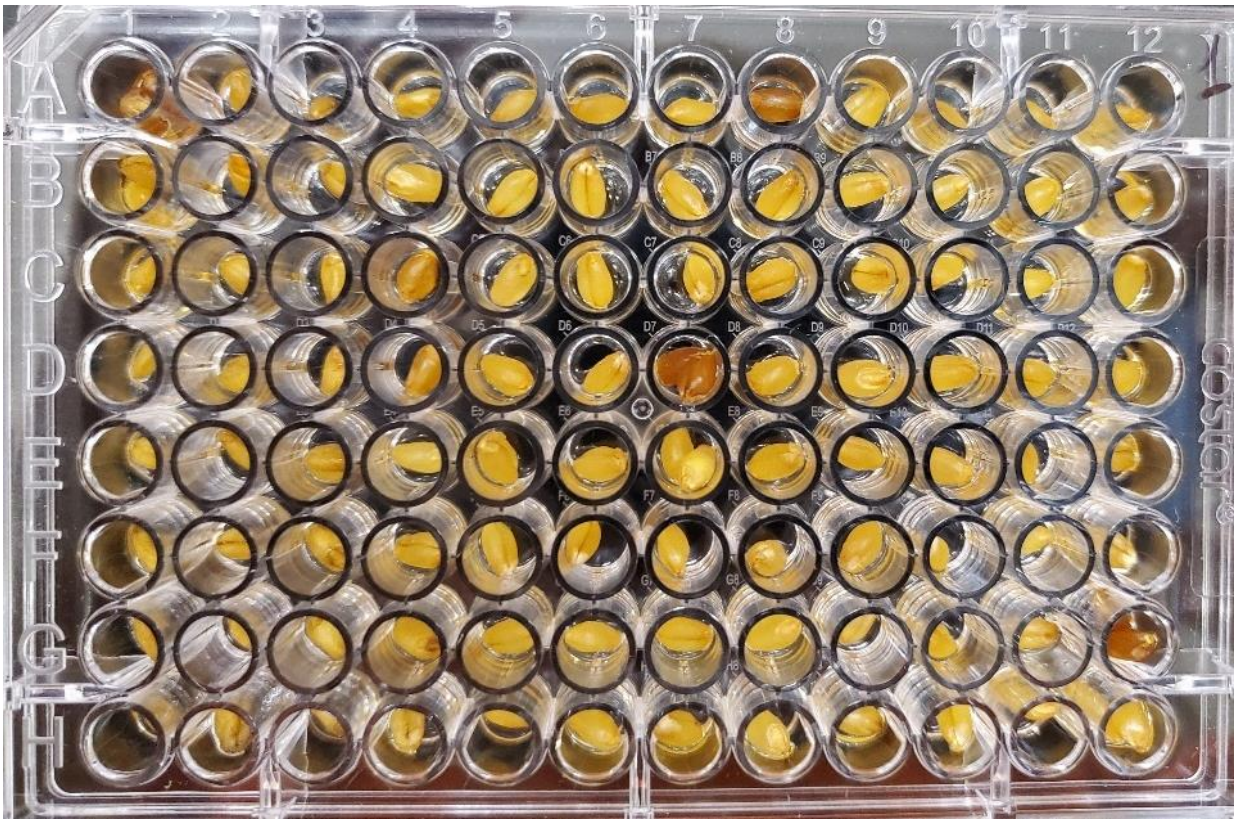



**Figure 2**. Microplate example of NaOH Color Test with a HRSW x HWSW population. Light-yellow seeds indicate a white pericarp, while darker seeds (e.g. D7, G12) indicate a red pericarp.

### Inbred Line Selection of Yield Trial Entries

Only $F_{5:6}$ spikes determined by the NaOH color test to be white were considered for entry into the yield trial. From the available HWSW spikes for S2, S3, S4, and S5, 70 white $F_{5:6}$ spikes per Subpopulation were chosen randomly to be increased in single two m rows near Leeston, New Zealand from November 2019 through February 2020. S1 and S6 had fewer than 70 HWSW lines, so all available white $F_{5:6}$ spikes were sown in New Zealand for those Subpopulations. Once individual rows were harvested, lines with sufficient $F_{5:7}$ seed were considered for use as entries to the yield trial. For S1, 40 lines produced sufficient seed for inclusion in the yield trial. S2 through S6 resulted in more than 45 inbred lines per Subpopulation, so entries were randomly selected amongst those with sufficient seed quantity.

## Yield Trial

### Experimental Design

Because the seed amount of many genotypes was limited, a partially-replicated design was used to split entries into groups across the three locations with two replicates (blocks) per location. The field randomizations were performed using FielDHub (Murillo et al., 2021). This allowed the maximum number of genotypes to compare Subpopulations in the experiment. Entries were equally split across Subpopulations within each location, and repeating spatial checks were used for added spatial analysis due to the size of the trials (560 plots per location). Each genotype was grown in at least two locations, and 21 genotypes per Subpopulation were grown in all three locations. Across Subpopulations, 265 total genotypes were grown (S1 had 40, with S2 through S6 having 45 genotypes each), plus six checks and the two parents.

**Locations and Agronomy**

The $F_{5:7}$ HWSW Subpopulation lines, parents, and checks were grown in yield trials in three distinct ND environments in 2020, with two replicates per location. Hettinger Research Extension Center (REC) represented the western ND environment, Carrington REC (47.508340, -99.136726) represented the central ND environment, and Prosper (47.001769, -97.118908) was the eastern ND location. All locations were seeded at approximately 3.7 million live seeds per hectare. Carrington and Prosper had seven-row plots with 19 cm row-spacing, 2.7 m in length (area 3.509 $m^2$). Hettinger had seven -row plots with 17.78 cm row-spacing, 3.658 m length (area 5.909 $m^2$). Standard research management practices were followed for fertilizer and herbicides, and no fungicides were applied.

The Carrington yield trial field was managed cooperatively by the Carrington REC and the NDSU Spring Wheat Breeding program. The trial was seeded 30 April 2020 and harvested on 12 August (rep 1) & 13 August (rep 2) 2020.

The field for the Hettinger yield trial was managed cooperatively by the Hettinger REC and the NDSU Spring Wheat Breeding program. This is notably the only no-till land where this experiment was grown and the most similar location to the HWSW target environment. The trial was seeded on 29 April 2020 and harvested 17 August 2020.

The field for the Prosper yield trial was managed cooperatively by the Agronomy Seed Farm and the NDSU Spring Wheat Breeding program. The trial was seeded 20 May 2020 and harvested 27 August 2020.

**Data Collection**

Data were collected as described below and stored in the NDSU Hard Spring Wheat Breeding program database, with details of traits listed in Table 4.

### *Field Traits*

Field traits were measured as follows. Bacterial leaf streak (BLS) was rated on a scale of severity from 0 to 9, with a rating of 0 lacking any sign of the disease, to 9 being completely susceptible, with extensive leaf coverage by chlorotic and necrotic lesions (Saari and Prescott, 1975). For days to heading (DH), days were recorded between sowing and heading, when 50% of the plants in the plot had spikes emerged from the leaf sheath (Feekes stage 10.3). Height was measured at maturity from the soil to the tip of the glumes, in inches and converted to centimeters. The degree of lodging was rated immediately before harvest on a 0 to 9 scale, with a rating of 0 being completely upright plants across the entire plot and nine having the entire plot lying down. Combine traits were measured during harvest on the H2 HarvestMaster system by Juniper Systems (Logan, UT), which was calibrated August 2020. Combine yield was measured in pounds and converted to kilograms. Combine test weight was recorded in pounds per bushel and converted to kilograms per hectoliter. Combine moisture was recorded as a percentage.

In locations with the absence of variability, some traits were not measured. In Carrington, notes for days to heading, height, lodging and combine yield were recorded. In Prosper, all traits were recorded. In Hettinger, only days to heading and height were noted. Because of the variation in tiller maturity in Hettinger, the H2 combine weighing system became repeatedly obstructed with unripe kernels and green vegetative matter, so field data collection for combine traits was impossible. Thus, weights for yield in Hettinger were measured after cleaning the seed in the lab and combine test weight and moisture were not taken. For the moisture-adjusted yield, combine or scale-measured weights were adjusted to 13.5% moisture and reported in tons per hectare.

**Table 4.** Trait measurements.

| Trait | Abbreviation | Data type | Description | Trait Locations† recorded |
|---|---|---|---|---|
| Bacterial Leaf Streak | BLS | Ordinal | 0 to 9 scale, 0 = no sign, 9 = fully susceptible | PRO |
| Days to Heading | DH | Continuous | Number of days after planting, when 50% of plants were headed | CAR, HET, PRO |
| Height | HT | Continuous | cm, converted from inches, measured at maturity from the soil to tip of glumes | CAR, HET, PRO |
| Lodging | LODG | Ordinal | 0 to 9 scale, 0 = completely upright plants, 9 = entire plot laying down | CAR, PRO |
| Combine yield | CombYLD | Continuous | kg, converted from lbs (H2 HarvestMaster system) | CAR, HET, PRO |
| Combine moisture | CombMoist | Continuous | %, (H2 HarvestMaster system) | CAR, PRO |
| Combine test weight | CombTWT | Continuous | kg/hectoliter, converted from lbs/bushel, (H2 HarvestMaster system) | CAR, PRO |
| Moisture-adjusted Yield | AdjYLD | Continuous | tons/hectare, adjusted for 13.5% moisture | CAR, HET, PRO |
| Test Weight | TWT | Continuous | kg/hectoliter, converted from lbs/bushel, measured on Foss Infratec NOVA | CAR, HET, PRO |
| Grain Protein | Protein | Continuous | Whole grain wheat protein at 12% moisture basis | CAR, HET, PRO |
| Flour Extraction | EXT | Continuous | percentage of refined flour to weight of grain milled | PRO (full), HET & CAR (partial) |
| GlutoPeak: Aggregation Energy | GP-AggE | Continuous | Area under curve (sq. cm) 15 seconds before and after BEM | PRO & CAR (full), HET (partial) |
| GlutoPeak: PreMaximum Torque | GP-PreMax | Continuous | Pre-maximum torque 15 seconds before BEM in Brabender Units (BU) | PRO & CAR (full), HET (partial) |
| GlutoPeak: Torque maximum | GP-Max | Continuous | Maximum recorded torque in Brabender Units (BU) | PRO & CAR (full), HET (partial) |
| GlutoPeak: Plateau energy | GP-PlateauE | Continuous | Area under the curve (sq. cm) for the GP Plateau | PRO & CAR (full), HET (partial) |
| GlutoPeak: Post-maximum Torque | GP-PostMax | Continuous | Post-maximum torque 15 seconds after BEM in Brabender Units (BU) | PRO & CAR (full), HET (partial) |

**Table 4**. Trait measurements (continued).

| Trait | Abbreviation | Data type | Description | Trait Locations† recorded |
|---|---|---|---|---|
| GlutoPeak: Peak maximum time | GP-PeakTime | Continuous | Seconds until maximum torque (BEM) is reached | PRO & CAR (full), HET (partial) |
| GlutoPeak: Start-up Energy | GP-StartUpE | Continuous | Area under the curve (sq. cm) for the GP Start Up | PRO & CAR (full), HET (partial) |
| SRC: Lactic Acid‡ | SRC-Lactic | Continuous | Weight (g) of solvent retained in flour | PRO & CAR (full), HET (partial) |
| SRC: Sodium Carbonate‡ | SRC-SoCa | Continuous | Weight (g) of solvent retained in flour | PRO & CAR (full), HET (partial) |
| SRC: Sucrose‡ | SRC-Sucrose | Continuous | Weight (g) of solvent retained in flour | PRO & CAR (full), HET (partial) |
| SRC: Water‡ | SRC-Water | Continuous | Weight (g) of solvent retained in flour | PRO & CAR (full), HET (partial) |
| Polyphenyl Oxidase | PPO | Continuous | absorbance units, measured at 475 nm wavelength | PRO |

†CAR = Carrington, HET = Hettinger, and PRO = Prosper. ‡SRC = Solvent Retention Capacity testing.

***Post-harvest Processing and Data Collection***

After harvest, the yield trial plot seed was cleaned using a Clipper Office Tester (Seedburo, Des Plaines, IL) to remove shrunken and broken kernels, inorganic and plant matter, insects, and other foreign material. Test weight and grain protein were measured with the Foss Infratec NOVA using standard settings for whole wheat kernels, which has been approved by the USDA Grain Inspection, Packer and Stockyards Administration (GIPSA). The PPO methodology, shown in Appendix B, was adapted from Dr. Senay Simsek's wheat quality group based on AACC International Method 22-85.01, Fuerst et. al, 2006, and Anderson, et. al, 2001. Milling of 50 g subsamples was done on the Brabender Quadrumat Jr. (Ashland, VA) laboratory mill. Flour extraction was calculated as a percentage of refined flour to weight of grain milled. The Solvent Retention Capacity tests used all four solvents, lactic acid, sodium carbonate,

sucrose, and water, as shown in AACC International Method 56-11.02. The GlutoPeak (Brabender) test was run according to Chandi and Seetharaman, 2012, and measured Aggregation Energy, Pre-maximum torque, Torque maximum, Plateau energy, Post-maximum torque, Peak maximum time, and Start-up energy.

**Data Analysis of Yield Trial traits for Subpopulation Comparisons**

The primary point of inquiry was to determine differences between Subpopulations, thus a randomized nested block analysis was used to compare them. Even though the Subpopulations started from the same bulked $F_2$ seed source, the individual genotypes derived within each Subpopulation were considered fixed effects because of the color-sorting and generation of sorting that occurred during development from the $F_3$ Subpopulations to $F_{5:7}$ lines. Using this design allows us to compare Subpopulation groups to one another and genotypes to other genotypes within the same Subpopulation, but not genotypes between different Subpopulations.

***Assessing Assumptions and Locations' Validity for Across Location Analysis***

Because normality affects homogeneity of variances tests, and is assumed for the analysis of variance (ANOVA), trait distributions were assessed using QQ Plots. Additionally, Bartlett's test for homogeneity of variances across locations were used for each trait. In some cases Levene's test was used due to the sensitivity of Bartlett's test to non-normality. Comparisons across locations were considered significant at $P<0.01$. For traits with non-homogenous variances, data transformations were used to attempt to find a transformation that would adjust for heterogeneity, including square root, natural log, and arcsine transformations, according to Mowers et. al, 2023. In conjunction with biological factors within each environment and the above aspects of distribution, variance, and mean within location, valid locations for across-

location analysis were determined. These same considerations were used to determine which individual trait locations should be considered for within-location Subpopulation comparisons.

***Comparison of Subpopulations across Locations***

Terms for across-location analysis are: $y$ = trait value, μ = mean, $L$ = location, $B/L$ = bloc within location, $S$ = Subpopulation, $L * S$ = location by Subpopulation, $G/S$ = genotype within Subpopulation, $L * G/S$ = location by genotype within Subpopulation, and Ɛ = error, as shown in Formula 1, where genotype within Subpopulation and location by genotype within Subpopulation are random effects, while all others are fixed effects.

$$y = \mu + L + B/L + S + L * S + G/S + L * G/S + \varepsilon \quad (1)$$

***Comparison of Subpopulations within Location***

Terms for within-location analysis are: $y$ = trait value, μ = mean, $B$ = bloc or rep, $S$ = Subpopulation, $G/S$ = genotype within Subpopulation, and Ɛ = error, as shown in Formula 2 with all terms as fixed effects.

$$y = \mu + B + S + G/S + \varepsilon \quad (2)$$

**Analysis for Genotype Acceptability and Advancement**

In order to determine if Subpopulation differences were associated with the proportions of acceptably performing HWSW lines in a typical selection scheme, spatial analysis (SpATS) in MrBean was used to determine genotype means across environments irrespective of Subpopulation (Rodriguez-Alvarez, et al., 2018; Aparicio, et al., 2024). SpATS uses smoothing splines for row, column, and interaction effects, that help estimate field-wide trends but can retain smaller local effects within the field while adding additional fixed or random model effects such as genotype and block. Because of the complexity of the model, there is no simplified model statement to give here. Reference Rodriguez-Alvarez, et. al, 2018 for details. Each

location was analyzed to obtain the fixed genotypic BLUEs and weights. Using this information from each environment, a combined analysis was done to calculate genotypic BLUPs for each trait (Möhring and Piepho, 2009).

An independent truncation method was used for advancement decisions, similar to the typical selection scheme used by the NDSU HSW breeding program for a first-year yield trial, which normally advances around 8 to 12% of genotypes. Because repeating spatial checks with known agronomic and quality characteristics were used, BLUP comparisons to checks were used to make informed advancement decisions for each economically important trait. Genotypes determined to be acceptable generally have acceptable end-use quality and agronomic performance.

**Trait Correlations**

In order to determine potential relationships between traits, correlations were calculated within individual locations of the yield trial alongside the SpATS analysis in MrBean. Correlations were calculated using Pearson's second moment correlation coefficients from the BLUEs for the following independent traits of economic importance: moisture-adjusted yield, days to heading, height, lodging, test weight, grain protein, flour extraction, lactic acid SRC, water SRC, polyphenyl oxidase, and bacterial leaf streak.

## RESULTS

### NaOH Color Test

The numbers of $F_{5:6}$ lines rated HRSW or HWSW using the NaOH color test are listed in Table 6. Except for S1 (Unsorted) and S6 (SSD), each Subpopulation's proportion of HWSW differed significantly from each other Subpopulation ($P \leq 0.05$). Notably, the color sorting process used for S3 ($F_3$ triple-sort) removed all HRSW, giving 100% HWSW. Only one $F_{5:6}$ line showed an inconclusive NaOH result in S4.

**Table 5.** Sodium Hydroxide (NaOH) test results to determine pericarp color for $F_{5:6}$ lines across all six subpopulations of W16-0006.

| Subpopulation | Description | Total $F_{5:6}$ Spikes harvested | Red (HRSW) $F_{5:6}$ lines (NaOH Test) | White (HWSW) F5:6 lines (NaOH Test) | % White lines† | |
|---|---|---|---|---|---|---|
| 1 | Unsorted | 316 | 272 | 44 | 13.9% | e |
| 2 | $F_3$ Double-sort | 255 | 8 | 247 | 96.9% | c |
| 3 | $F_3$ Triple-sort | 284 | 0 | 284 | 100.0% | a |
| 4 | $F_4$ Triple-sort | 271 | 4 | 266 | 98.5% | b |
| 5 | $F_5$ Triple-sort | 337 | 22 | 315 | 93.5% | d |
| 6 | SSD‡ | 464 | 407 | 57 | 12.3% | e |

†Values followed by the same letter are not significantly different at $P \leq 0.05$ as determined using Pearson's Chi-square test. ‡SSD = Single Seed Descent.

### Yield Trials

North Dakota was generally warmer and drier during the growing season, from April through September. The departure from average precipitation for the growing season in Foster County (Carrington, ND) was -13.3 cm, while Adams County (Hettinger, ND) was approximately -12.6 cm, and Cass County (Prosper, ND) was approximately -4 cm. (USDA, National Agricultural Statistics Service, North Dakota Field Office, 2021) Trait data at some locations were not recorded or removed from analysis due to location effects known from abiotic

and biotic field conditions. Though precipitation in Prosper was not as low as in the other two locations, the later planting date and relatively dry conditions limited yield in what typically would be the highest-yielding location of the three by a considerable amount. Hettinger had a dry spring that delayed emergence and had minimal rainfall in May. Late season rain caused high variability in maturity on plot edges and between plot ranges so when the majority of spikes were ready to be harvested, simultaneous green tillers and milk-to-dough-stage seed were present. Thus, yield traits (moisture-adjusted and combine-measured) were removed in Hettinger.

To address the assumptions of analysis of variance, QQ plot assessment of normality and analysis of means for variance (ANOMV) are shown in Table 6, as are determinations for across-location analysis. All location data for combine moisture and combine test weight have non-normal distributions, so those traits were removed from across location analysis. For other traits in general, Carrington and Prosper were reasonable for combined analysis of agronomic traits, and either all locations or Carrington and Hettinger were acceptable for combined analysis for quality traits.

The Bartlett Chi-squared test, and the Levene's test, were used to analyze variance assumptions regarding the homogeneity of variances across locations. Table 7 displays results and interpretations for homogeneity of variances across locations. According to these results, most trait error variances were homogenous across locations. However, days to heading, grain protein, and flour extraction had heterogenous variances between locations, indicating that each location should be analyzed separately. Trait data collected at only one location, i.e. bacterial leaf streak and polyphenyl oxidase, were analyzed using a single within-location analysis.

**Table 6.** Determinations for across-location analysis for multi-location traits for 2020 North Dakota yield trials in Carrington, Hettinger, and Prosper.

| Trait† | QQ Plot: locations with homogenous variances‡ | QQ Plot: locations with heterogenous variances‡ | ANOMV‡ | Across-Location Analysis‡ | Within-Location Analysis‡ |
|---|---|---|---|---|---|
| Moisture-Adjusted Yield | CAR, PRO | HET | unequal (HET) | CAR, PRO | |
| Combine Moisture | | CAR, PRO | | | |
| Combine Test Weight | | CAR, PRO | | | |
| Combine Yield | CAR, PRO | | | CAR, PRO | |
| Days to Heading | CAR, HET | PRO | unequal | CAR, HET | |
| Height | CAR, HET, PRO | | unequal | all | |
| Lodging | CAR, PRO | | | CAR, PRO | PRO |
| Test Weight | CAR | HET, PRO | unequal (PRO) | CAR, HET | |
| Protein | CAR, HET, PRO | | unequal (PRO) | CAR, HET | |
| Flour Extraction | CAR, HET, PRO | | unequal (PRO) | CAR, HET | |
| ln(GP-Aggregation Energy) | CAR, HET, PRO | | | CAR, HET | |
| GP Pre-Maximum Torque | CAR, HET, PRO | | unequal | CAR, HET | |
| GP Torque Maximum | CAR, HET | PRO | unequal | CAR, HET | |
| ln(GP Plateau Energy) | CAR, HET | PRO | equal | all | |
| ln(GP Post-Maximum Torque) | CAR, HET, PRO | | equal | all | |
| sqrt(GP Peak Maximum Time) | CAR, HET, PRO | | equal | all | |
| GP Start-up Energy | HET | CAR, PRO | equal | all | |
| SRC Lactic Acid | CAR, HET, PRO | | equal | all | |
| SRC Sodium Carbonate | CAR, HET, PRO | | equal | all | |
| SRC Sucrose | CAR, HET, PRO | | equal | all | |
| SRC Water | CAR, HET, PRO | | equal | all | |
| Bacterial Leaf Streak | PRO | | | | PRO |
| Polyphenyl Oxidase | PRO | | | | PRO |

†GP=GlutoPeak, SRC=Solvent Retention Capacity. ‡CAR = Carrington, HET = Hettinger, PRO = Prosper.

**Table 7.** Bartlett and Levene's tests for homogeneity of variance across locations for all trait data and select transformed trait data.

| Trait | Across-Location Analysis‡ | Bartlett Probability > F | Bartlett (Mowers Interpretation) >0.01 | Levene's Probability > F | Levene's (Mowers Interpretation) >0.01 |
|---|---|---|---|---|---|
| Moisture-Adjusted Yield | CAR, PRO | 0.1108 | COMPARE ACROSS LOCS | | |
| Combine Yield | CAR, PRO | 0.0862 | COMPARE ACROSS LOCS | | |
| Days to Heading | CAR, HET | <0.0001*** | LOCATION VARIANCE HETEROGENOUS | <0.0001*** | LOCATION VARIANCE HETEROGENOUS |
| Height | all | 0.0150 | COMPARE ACROSS LOCS | | |
| Lodging | CAR, PRO | 0.9107 | COMPARE ACROSS LOCS | | |
| Test Weight | CAR, HET | 0.4459 | COMPARE ACROSS LOCS | | |
| Protein | CAR, HET | <0.0001*** | LOCATION VARIANCE HETEROGENOUS | <0.0001*** | LOCATION VARIANCE HETEROGENOUS |
| Flour Extraction | CAR, HET | 0.0002*** | LOCATION VARIANCE HETEROGENOUS | 0.0019** | LOCATION VARIANCE HETEROGENOUS |
| ln(GP-Aggregation Energy)† | CAR, HET | 0.5702 | COMPARE ACROSS LOCS | | |
| GP Pre-Maximum Torque† | CAR, HET | 0.0013*** | LOCATION VARIANCE HETEROGENOUS | 0.1123 | COMPARE ACROSS LOCS |
| GP Torque Maximum† | CAR, HET | 0.5176 | COMPARE ACROSS LOCS | | |
| ln(GP Plateau Energy) † | all | 0.5625 | COMPARE ACROSS LOCS | | |
| ln(GP Post-Maximum Torque) † | all | 0.0851 | COMPARE ACROSS LOCS | | |
| sqrt(GP Peak Maximum Time) † | all | 0.0624 | COMPARE ACROSS LOCS | | |
| GP Start-up Energy† | all | 0.9852 | COMPARE ACROSS LOCS | | |
| SRC Lactic Acid† | all | 0.7095 | COMPARE ACROSS LOCS | | |
| SRC Sodium Carbonate† | all | 0.1613 | COMPARE ACROSS LOCS | | |
| SRC Sucrose† | all | 0.1105 | COMPARE ACROSS LOCS | | |
| SRC Water† | all | 0.1096 | COMPARE ACROSS LOCS | | |

**, *** Significant at P≤0.05, and P≤0.01, respectively. †GP=GlutoPeak, SRC=Solvent Retention Capacity ‡CAR = Carrington, HET = Hettinger, and PRO = Prosper.

**Multi-location Comparisons of Subpopulations**

The full analysis of variance (ANOVA) is in Appendix C. Fixed effects (for location, block within location, Subpopulation, and location by Subpopulation interaction) are in Table C1, and random effects are shown in Table C2. From Table C1, the subsets of Subpopulation effects for each multi-location trait are shown in Table 8, which gives the probability of a greater *F* to test the null Hypothesis that color sorting has a significant effect on Subpopulation. Significant Subpopulation effects were found for height, lodging, and test weight.

**Table 8.** Subpopulation effects for analysis of variance (ANOVA) across selected trait locations for multi-location traits for 2020 North Dakota yield trials in Carrington, Hettinger, and Prosper.

| Trait | Locations‡ | DF Numerator | DF Denominator | F Ratio | Prob > F |
|---|---|---|---|---|---|
| Moisture-Adjusted Yield | CAR, PRO | 5 | 240.0791 | 1.5903 | 0.1635 |
| Combine Yield | CAR, PRO | 5 | 235.9472 | 0.9921 | 0.4232 |
| Height | all | 5 | 259.0048 | 5.1432 | 0.0002*** |
| Lodging | CAR, PRO | 5 | 251.5259 | 2.9181 | 0.0140** |
| Test Weight | CAR, HET | 5 | 259.8565 | 6.0638 | 0.0000*** |
| ln(GP-Aggregation Energy)† | CAR, HET | 5 | 228.0245 | 1.0498 | 0.3892 |
| GP Pre-Maximum Torque† | CAR, HET | 5 | 196.6991 | 0.5154 | 0.7645 |
| GP Torque Maximum | CAR, HET | 5 | 204.3694 | 0.9010 | 0.4814 |
| ln(GP Plateau Energy) † | all | 5 | 248.7007 | 0.5246 | 0.7576 |
| ln(GP Post-Maximum Torque) † | all | 5 | 395.6139 | 0.7547 | 0.5830 |
| sqrt(GP Peak Maximum Time) † | all | 5 | 363.8934 | 1.5500 | 0.1736 |
| GP Start-up Energy† | all | 5 | 355.5013 | 0.7777 | 0.5663 |
| SRC Lactic Acid† | all | 5 | 360.9370 | 1.2198 | 0.2992 |
| SRC Sodium Carbonate† | all | 5 | 366.0539 | 2.0451 | 0.0717 |
| SRC Sucrose† | all | 5 | 403.2073 | 0.8746 | 0.4980 |
| SRC Water† | all | 5 | 398.3421 | 1.4891 | 0.1922 |

**, *** Significant at P≤0.05, and P≤0.01, respectively. †GP=GlutoPeak, SRC=Solvent Retention Capacity. ‡CAR = Carrington, HET = Hettinger, and PRO = Prosper.

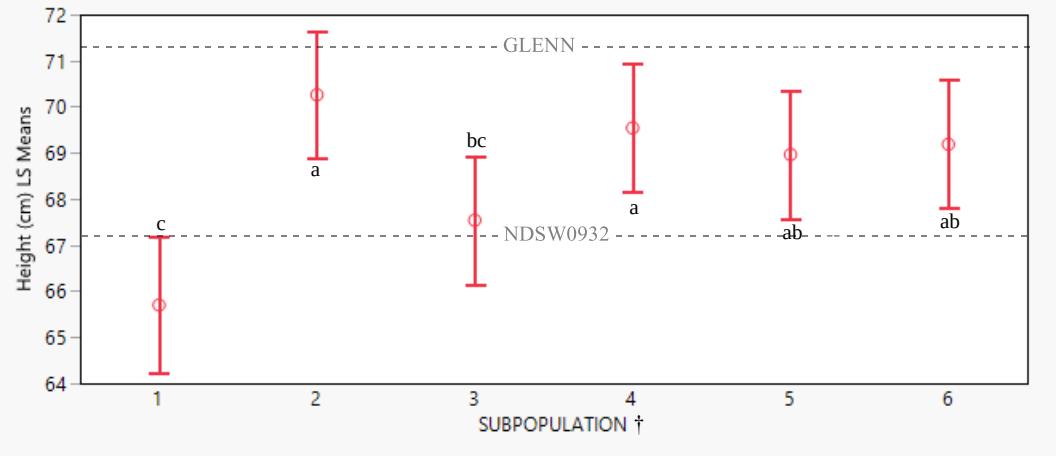
Table 9 gives the least square means for each Subpopulation. Means separation was performed using the Student's t test on traits with significant Subpopulation effects and graphed in Fig. 3, where different letters indicate significantly different Subpopulation means ($p<0.05$). For plant height, Subpopulation means ranged from 65.8 cm for S1 to 70.3 cm in S2. The tallest were S2 and S4, and differed from S1 and S3, which were the shortest, while S3, S5, and S6 were closer to the experiment mean and did not differ from each other. Notably with Lodging, the only Subpopulation that differed was S1, with a mean score of 4.5, which was lower than all other Subpopulations, while the highest mean was 5.1 for S6. The highest means for test weight were in S2 and S3, with 80.1 kg/hl, while the lowest mean was 79.2 kg/hl for S4. Mean test weights for S1, S5, and S6 were intermediate.

**Table 9.** Least Squares Means estimates for each Subpopulation and trait, and the means of all Subpopulations from analysis of variance (ANOVA) across selected trait locations for 2020 ND yield trials in Carrington, Hettinger, and Prosper.

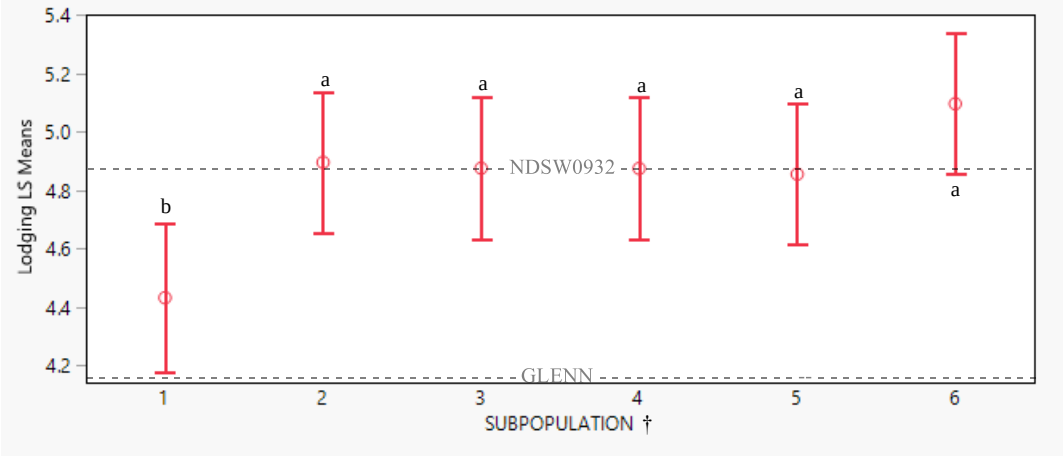
| Trait | Locations‡ | Subpopulation Least Square Means⁋ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | Mean of Subpopulations |
| Moisture-Adjusted Yield (t/ha) | CAR, PRO | 4.0 | 3.8 | 3.9 | 4.0 | 4.0 | 3.9 | 3.9 |
| Combine Moisture (%) | CAR, PRO | 13.3 | 13.7 | 13.8 | 13.4 | 13.6 | 13.8 | 13.6 |
| Combine Test Weight (kg/hl) | CAR, PRO | 70.9 | 70.6 | 71.1 | 71.3 | 71.6 | 71.1 | 71.1 |
| Combine Yield (kg) | CAR, PRO | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 |
| Days to Heading (days after planting) | CAR, HET | 54.8 | 55.6 | 55.2 | 55.3 | 55.2 | 55.5 | 55.3 |
| Height (cm) | all | 65.8 c | 70.3 a | 67.6 bc | 69.6 a | 69.0 ab | 69.2 ab | 68.6 |
| Lodging (0 – 9 scale) | CAR, PRO | 4.5 b | 4.9 a | 4.9 a | 4.9 a | 4.9 a | 5.1 a | 4.9 |
| Test Weight (kg/hl) | CAR, HET | 79.6 b | 80.1 a | 80.1 a | 79.2 c | 79.8 ab | 79.8 ab | 79.8 |
| Protein (%) | CAR, HET | 16.1 | 16.2 | 16.0 | 16.5 | 16.3 | 16.3 | 16.2 |
| Flour Extraction (%) | CAR, HET | 69.3 | 70.1 | 69.9 | 69.4 | 69.3 | 69.6 | 69.6 |
| ln(GP-Aggregation Energy)† (sq.cm) | CAR, HET | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| GP Pre-Maximum Torque† (BU) | CAR, HET | 56.0 | 55.0 | 54.9 | 56.1 | 55.9 | 55.8 | 55.6 |
| GP Torque Maximum† (BU) | CAR, HET | 63.3 | 61.9 | 62.3 | 63.4 | 63.2 | 62.3 | 62.7 |
| ln(GP Plateau Energy) † (sq.cm) | all | 5.7 | 5.6 | 5.7 | 5.8 | 5.6 | 5.6 | 5.7 |
| ln(GP Post-Maximum Torque) † (BU) | all | 4.0 | 4.0 | 3.9 | 4.0 | 3.9 | 4.0 | 4.0 |
| sqrt(GP Peak Maximum Time) † (sec.) | all | 10.4 | 11.1 | 11.0 | 10.7 | 10.5 | 10.6 | 10.7 |
| GP Start-up Energy† (sq.cm) | all | 922.2 | 913.2 | 834.5 | 981.6 | 970.8 | 1014.7 | 939.5 |
| SRC Lactic Acid† (g) | all | 143.1 | 143.8 | 141.2 | 140.6 | 140.5 | 142.9 | 142.0 |
| SRC Sodium Carbonate† (g) | all | 89.6 | 88.4 | 88.0 | 87.7 | 89.6 | 88.9 | 88.7 |
| SRC Sucrose† (g) | all | 114.3 | 112.8 | 114.1 | 113.2 | 114.0 | 113.9 | 113.7 |
| SRC Water† (g) | all | 74.2 | 73.3 | 73.0 | 73.1 | 73.8 | 73.7 | 73.5 |

†GP=GlutoPeak, SRC=Solvent Retention Capacity. ‡CAR = Carrington, HET = Hettinger, and PRO = Prosper. ⁋Values followed by the same letter are not significantly different at P≤0.05 as determined using Students-*t* test. Subpopulations are descended from cross W16-0006 using fixed color-sorting methods as follows: 1 is unsorted, 2 is $F_3$ double-sorted, 3 is $F_3$ triple-sorted, 4 is $F_4$ triple-sorted, 5 is $F_5$ triple-sorted, and 6 is single-seed descent.

a)




b)




c)

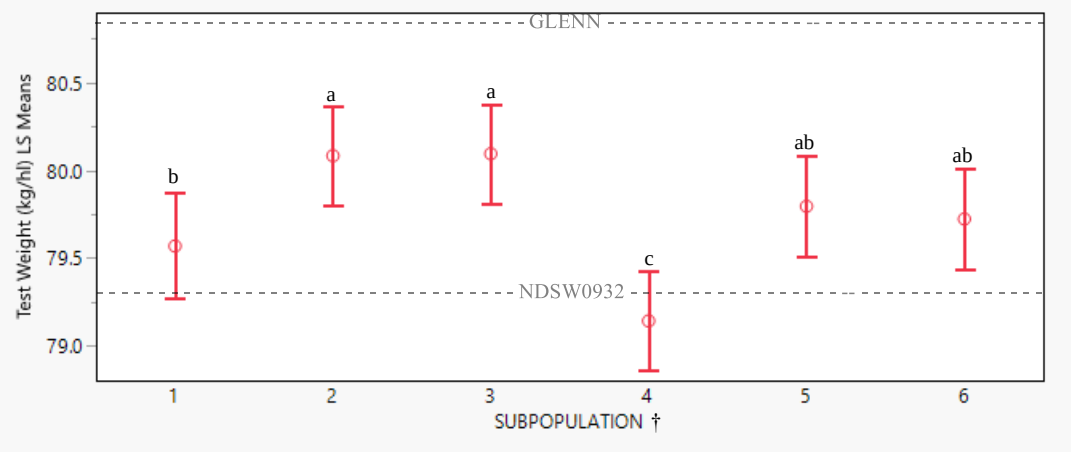



**Figure 3**. Least square means with confidence limits for multi-location traits for 2020 ND yield trials at Carrington (CAR), Hettinger (HET), and Prosper (PRO). a) Height in cm (CAR, HET, PRO), b) Lodging rated on a 0 to 9 scale (CAR, PRO), c) Test Weight in kg/hl (CAR, HET). †Means with the same letter are not significantly different at P≤0.05 as determined using Students-*t* test. Subpopulations are descended from cross W16-0006 using fixed color-sorting methods as follows: 1 is unsorted, 2 is $F_3$ double-sorted, 3 is $F_3$ triple-sorted, 4 is $F_4$ triple-sorted, 5 is $F_5$ triple-sorted, and 6 is single-seed descent.

## Within-location Comparisons of Subpopulations

### *Prosper-specific Trait Analysis: BLS, PPO, and Lodging*

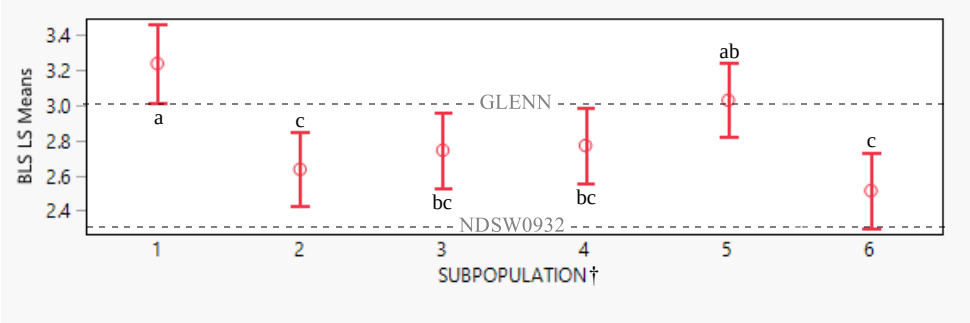
Individual location analysis of variance (ANOVA) was performed in Prosper for bacterial leaf streak ratings, polyphenyl oxidase, and lodging, and results are displayed in Table 10. Subpopulation was significant for each, and means are graphed in Fig. 4, with mean separation performed using the Student's-*t* test and significantly different Subpopulation means indicated with differing letters. The range of BLS ratings spanned from 3.24 as the maximum of S1, to 2.51 as the minimum for S6. Differences were significant with Subpopulations 1 and 5 more susceptible, while Subpopulations 2 and 6 were more resistant. Polyphenyl Oxidase ranged from 0.78 in S1, to 0.87 in S4. Differences were found between S1 and every other Subpopulation, as well as between Subpopulations 2 and 4, which were different from each other. Lodging showed some similarity because S1 had the lowest mean score of 6.68 and was different from every other Subpopulation. Additionally, S6 had the highest mean lodging score, with a mean of 7.57. Subpopulations 2, 5, and 6 also differed from Subpopulations 3 and 4.

**Table 10.** Fixed effects for analysis of variance (ANOVA) for Prosper, ND yield trial 20WATW in 2020.

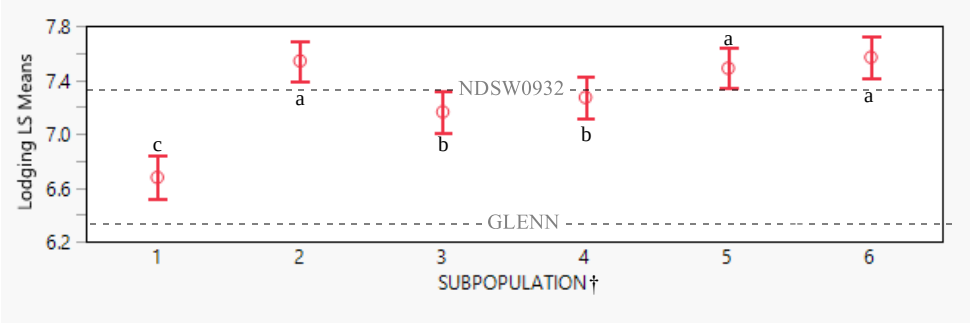
| Trait | Source of Variation | Prob. > F | |
|---|---|---|---|
| bacterial leaf streak | block | 0.0572 | * |
| bacterial leaf streak | SUBPOPULATION | <0.0001 | *** |
| bacterial leaf streak | Genotype within Subpopulation | <0.0001 | *** |
| lodging | block | 0.1126 | |
| lodging | SUBPOPULATION | <0.0001 | *** |
| lodging | Genotype within Subpopulation | <0.0001 | *** |
| polyphenyl oxidase | block | <0.0001 | *** |
| polyphenyl oxidase | SUBPOPULATION | 0.0001 | *** |
| polyphenyl oxidase | Genotype within Subpopulation | <0.0001 | *** |

*, *** Significant at P≤0.10, and P≤0.01, respectively.

a)




b)




c)

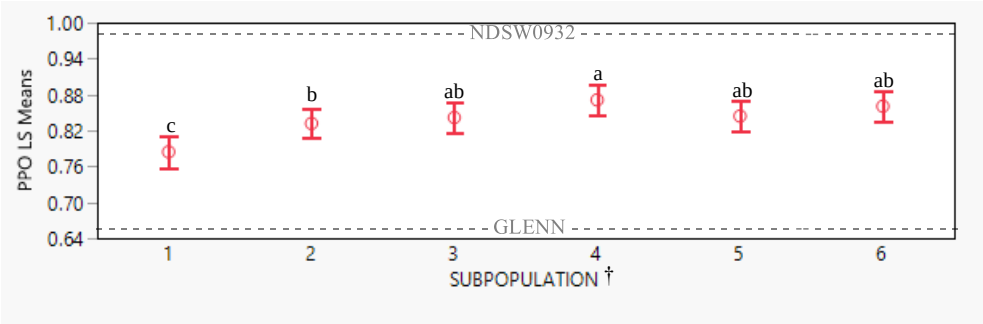



**Figure 4.** Least Square means with confidence limits for single-location traits for a 2020 yield trial at Prosper, ND: a) BLS (Bacterial Leaf Streak) score (0 to 9), b) Lodging score (0 to 9), and c) PPO (Polyphenyl Oxidase, absorbance units).
†Subpopulations are descended from cross W16-0006 using fixed color-sorting methods as follows: 1 is unsorted, 2 is $F_3$ double-sorted, 3 is $F_3$ triple-sorted, 4 is $F_4$ triple-sorted, 5 is $F_5$ triple-sorted, and 6 is single-seed descent.

***Within Location Analysis due to Heterogenous Variances***

Because of heterogeneous variances across locations for days to heading, grain protein, and flour extraction, within location analyses were done for each location. Table 11 shows the analysis of variance tables for those traits within location and indicates that Subpopulation is significant for each trait and location.

**Table 11**. Within location Analysis of variance (ANOVA) for days to heading, grain protein, and flour extraction within a 2020 yield trial at Carrington, Hettinger, and Prosper, ND.

| Location | Trait | Source of Variation | Prob > F | |
|---|---|---|---|---|
| Carrington | Days to Heading | bloc | 0.0001 | *** |
| | | SUBPOPULATION | <0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Carrington | Grain Protein | bloc | 0.0002 | *** |
| | | SUBPOPULATION | <0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Carrington | Flour Extraction | bloc | 0.0001 | *** |
| | | SUBPOPULATION | 0.0010 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Hettinger | Days to Heading | bloc | 0.9572 | |
| | | SUBPOPULATION | 0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Hettinger | Grain Protein | bloc | 0.3086 | |
| | | SUBPOPULATION | <0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Hettinger | Flour Extraction | bloc | 0.0656 | * |
| | | SUBPOPULATION | 0.0419 | ** |
| | | Genotype within Subpopulation | 0.0014 | *** |
| Prosper | Days to Heading | bloc | 0.0467 | ** |
| | | SUBPOPULATION | <0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Prosper | Grain Protein | bloc | <0.0001 | *** |
| | | SUBPOPULATION | <0.0001 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |
| Prosper | Flour Extraction | bloc | 0.0734 | * |
| | | SUBPOPULATION | 0.0006 | *** |
| | | Genotype within Subpopulation | <0.0001 | *** |

*, **, *** Significant at P≤0.10, P≤0.05, and P≤0.01, respectively.

Figure 5 displays results of least square means and mean separation using Student's t for days to heading, flour extraction, and grain protein at each location. For days to heading, Prosper had a much lower range of heading date means, from 44.00 DAP in S1 to 44.97 DAP in S2, while Carrington ranged from 52.77 DAP in S1 to 53.84 DAP in S2, and Hettinger had the

highest means, from 56.75 DAP in S1 to 57.32 DAP in S6. The Subpopulation means for grain protein were the highest and least variable in Carrington, ranging from 16.6% in S6 to 16.8% in S4, while Hettinger ranged from 15.5% in S3 to 15.9% in S4, and Prosper had the lowest means from 15.2% in S3 to 15.6% in S4. Flour extraction means were lowest in Prosper, ranging from 65.97% in S1 to 67.37% in S6, while Subpopulation means at Hettinger and Carrington were both in a similar range, 69.3% in S1 to 70.4% in S3 at Carrington, 69.3% in S5 to 70.4% in S2 at Hettinger.

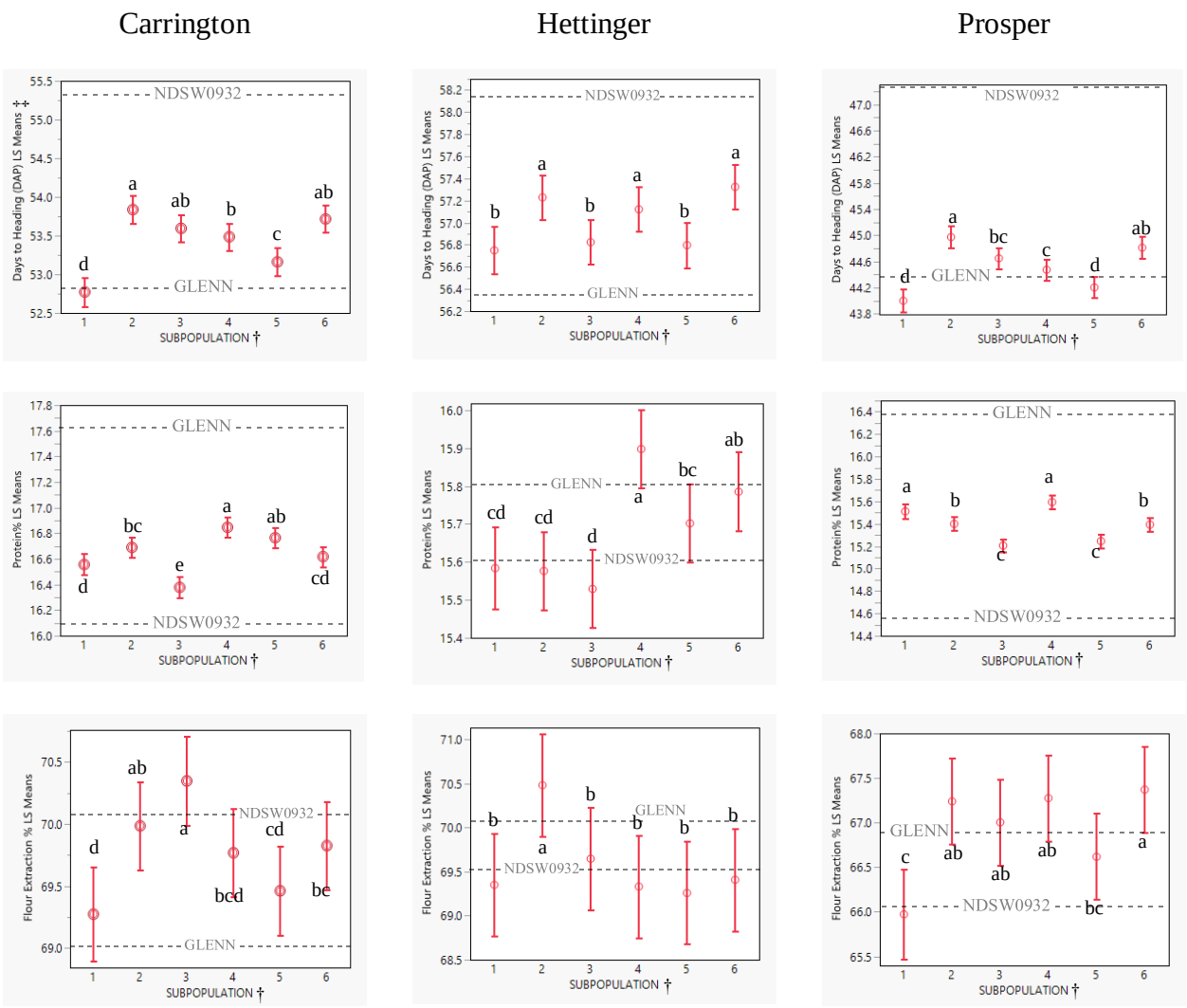



**Figure 5.** Least square means with confidence limits for days to heading, protein, and flour extraction for a 2020 ND yield trial at Carrington, Hettinger, and Prosper.
†Least square means for Subpopulations with different letters within a location are significantly different at P≤0.05 as determined using Student's-*t*. Subpopulations are descended from cross W16-0006 using fixed color-sorting methods as follows: 1 is unsorted, 2 is $F_3$ double-sorted, 3 is $F_3$ triple-sorted, 4 is $F_4$ triple-sorted, 5 is $F_5$ triple-sorted, and 6 is single-seed descent. ‡ DAP = Days After Planting.

**Genotypic Analysis of Yield Trial Data**

Some traits had variability, but were acceptably high across genotypes, as was the case for test weight and protein, so no limit was imposed for those traits. The thresholds for trait acceptability based on experiment means, checks, and breeding objectives were as follows:

- moisture-adjusted yield > 3.6 tons/hectare,

- height < 72.3 cm,
- lodging < 4.8,
- days to heading < 53.5 DAP,
- bacterial leaf streak < 3.5,
- polyphenyl oxidase < 0.85,
- lactic acid SRC > 136.9, and
- water SRC > 72

Table 12 displays BLUPs for genotypes judged to be acceptable based on the overall performance and breeding objectives. The parents, Glenn and NDSU0932, are at the top, while the experimental mean, threshold of acceptability, and mean of acceptable genotypes are listed for each trait at the bottom. Overall performance of genotypes was factored into decisions. So, if one or two agronomic or quality traits failed to meet the threshold of acceptability, but the overall performance of a genotype was strong, it may meet the overall objectives and be ruled acceptable. One example is genotype W16-06-6262, which exceeded the maximum thresholds for lodging and polyphenyl oxidase, but had excellent moisture-adjusted yield and bacterial leaf streak means, while it otherwise met the trait thresholds (Table 12).

**Table 12.** BLUPs for acceptably-performing genotypes of 20WATW from Carrington, Hettinger, and Prosper, ND in 2020.

| Genotype | AdjYLD† (t/ha) | DH† (DAP) | HT† (cm) | Lodging (0 – 9) | BLS† (0 – 9) | SRC-LA† (g) | SRC-W† (g) | PPO† (AU) |
|---|---|---|---|---|---|---|---|---|
| Glenn | 3.49 | 51.3 | 71.2 | 4.07 | 3.22 | 151.4 | 73.5 | 0.659 |
| NDSW0932 | 3.60 | 53.6 | 67.0 | 4.82 | 2.18 | 140.0 | 73.4 | 0.984 |
| W16-06-1088 | 4.02 | 51.5 | 63.6 | 3.92 | 1.94 | 141.1 | 73.9 | 0.843 |
| W16-06-1089 | 3.84 | 53.1 | 69.5 | 3.98 | 2.05 | 146.1 | 73.8 | 0.563 |
| W16-06-1138 | 3.90 | 51.4 | 65.5 | 4.08 | 2.07 | 138.7 | 72.6 | 0.803 |
| W16-06-1147 | 3.75 | 51.0 | 61.1 | 4.07 | 3.05 | 140.8 | 75.0 | 0.703 |
| W16-06-1150 | 3.91 | 51.7 | 64.8 | 4.55 |  | 145.2 | 75.2 |  |
| W16-06-1155 | 3.91 | 51.1 | 60.8 | 4.21 | 1.81 | 139.6 | 75.2 | 0.79 |
| W16-06-1187 | 3.83 | 52.0 | 70.4 | 4.43 | 4.34 | 139.2 | 72.2 | 0.717 |
| W16-06-1202 | 3.61 | 50.2 | 59.9 | 3.36 | 3.23 | 140.3 | 76.1 | 0.746 |
| W16-06-1253 | 3.90 | 52.0 | 65.3 | 4.80 | 3.22 | 138.5 | 75.7 | 0.786 |
| W16-06-2027 | 3.77 | 52.7 | 67.4 | 4.07 | 1.79 | 145.0 | 73.5 | 0.851 |
| W16-06-2034 | 3.79 | 52.0 | 65.7 | 4.32 | 2.67 | 137.6 | 75.3 | 0.845 |
| W16-06-2035 | 3.86 | 51.2 | 64.7 | 4.14 | 1.74 | 148.7 | 72.6 | 0.701 |
| W16-06-2042 | 3.83 | 51.8 | 67.0 | 3.94 | 2.43 | 142.6 | 74.0 | 0.729 |
| W16-06-2221 | 3.81 | 52.9 | 63.3 | 4.19 | 2.57 | 144.6 | 71.8 | 0.75 |
| W16-06-2250 | 3.80 | 53.9 | 64.4 | 4.18 | 2.57 | 143.4 | 73.5 | 0.663 |
| W16-06-3003 | 3.73 | 51.4 | 69.2 | 3.98 | 2.47 | 145.0 | 76.3 | 0.799 |
| W16-06-3243 | 3.80 | 51.9 | 62.2 | 4.49 | 2.69 | 144.2 | 73.1 | 0.938 |
| W16-06-4236 | 3.62 | 52.1 | 70.7 | 4.15 |  | 153.1 | 71.8 |  |
| W16-06-5025 | 3.87 | 49.8 | 65.3 | 4.68 | 2.22 | 137.0 | 73.6 | 0.746 |
| W16-06-6262 | 4.08 | 53.1 | 66.6 | 4.85 | 1.78 | 149.2 | 76.5 | 0.879 |
| W16-06-6300 | 3.94 | 52.5 | 68.8 | 4.89 | 1.91 | 140.1 | 71.2 | 0.939 |
| W16-06-6422 | 3.68 | 50.4 | 66.2 | 4.60 |  | 150.9 | 73.2 |  |
| Mean of all lines | 3.69 | 51.7 | 68.6 | 4.85 | 2.82 | 141.3 | 73.4 | 0.841 |
| Threshold | >3.6 | <53.5 | <72.3 | <4.80 | <3.50 | >136.9 | >72.0 | <0.85 |
| Mean of Selected | 3.83 | 51.8 | 65.56 | 4.27 | 2.45 | 143.22 | 73.93 | 0.78 |

†AdjYLD = Moisture-adjusted yield, DH = days to heading, DAP = Days After Planting, HT = height, BLS = bacterial leaf streak, SRC-LA = Solvent Retention Capacity lactic acid, SRC-W = SRC water, PPO = polyphenyl oxidase.

A summary of the number advanced and percent of total lines within Subpopulation is given in Table 13. The total number of lines advanced from all Subpopulations is 22 (8.3% of 265 tested lines). S1 had the most with nine, and S4 and S5 had the fewest with only one each.

**Table 13**. Count and percentage of genotypes within each Subpopulation determined to meet acceptable agronomic and end use quality parameters for advancement to subsequent yield trials.

| Subpopulation† | Total # 2020 genotypes | Selected genotypes | % Selected from 2020 |
|---|---|---|---|
| 1 | 40 | 9 | 22.5% |
| 2 | 45 | 6 | 13.3% |
| 3 | 45 | 2 | 4.4% |
| 4 | 45 | 1 | 2.2% |
| 5 | 45 | 1 | 2.2% |
| 6 | 45 | 3 | 6.7% |
| **TOTAL:** | **265** | **22** | **8.3%** |

†Subpopulations are descended from cross W16-0006 using fixed color-sorting methods as follows: 1 is unsorted, 2 is $F_3$ double-sorted, 3 is $F_3$ triple-sorted, 4 is $F_4$ triple-sorted, 5 is $F_5$ triple-sorted, and 6 is single-seed descent.

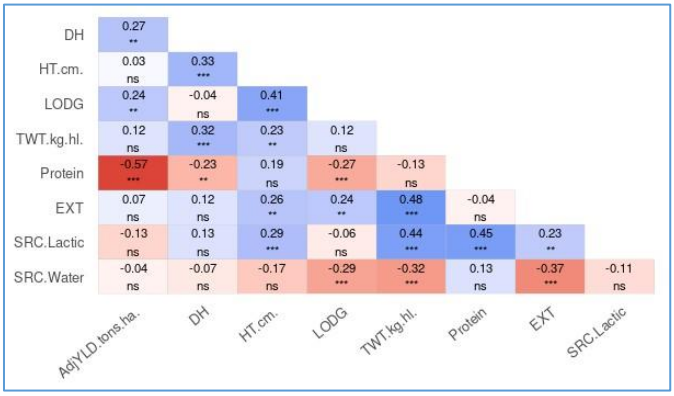
**Trait Correlations**

Trait correlations for selected traits of importance within each location are shown in Figure 6. In Carrington, positive correlations above 0.4 were found between lodging and height, test weight and flour extraction, test weight and SRC-Lactic Acid, protein and SRC-Lactic Acid, while negative correlation below -0.4 was found between adjusted yield and protein. In Hettinger, positive correlations above 0.4 were found between test weight and flour extraction, while negative correlation below -0.4 was found between adjusted yield and protein. In Prosper, positive correlations above 0.4 were found between lodging and height, flour extraction and height, flour extraction and test weight, protein and SRC-Lactic Acid, while a negative correlation below -0.4 was found between adjusted yield and protein.

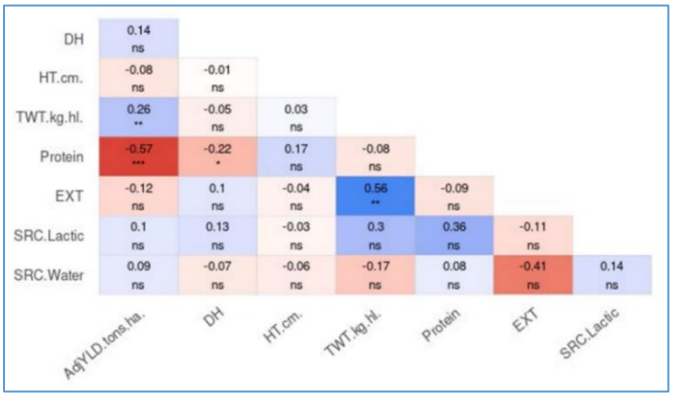
Other correlations only found in Carrington and Prosper were between height and test weight, while negative correlations below -0.3 are found with test weight and SRC-Water, as

well as flour extraction and SRC-Water. Additionally, though bacterial leaf streak was only measured in Prosper, it had correlations of 0.34 with protein, -0.39 with moisture-adjusted yield, and -0.37 with test weight.

a) Carrington




b) Hettinger




c) Prosper

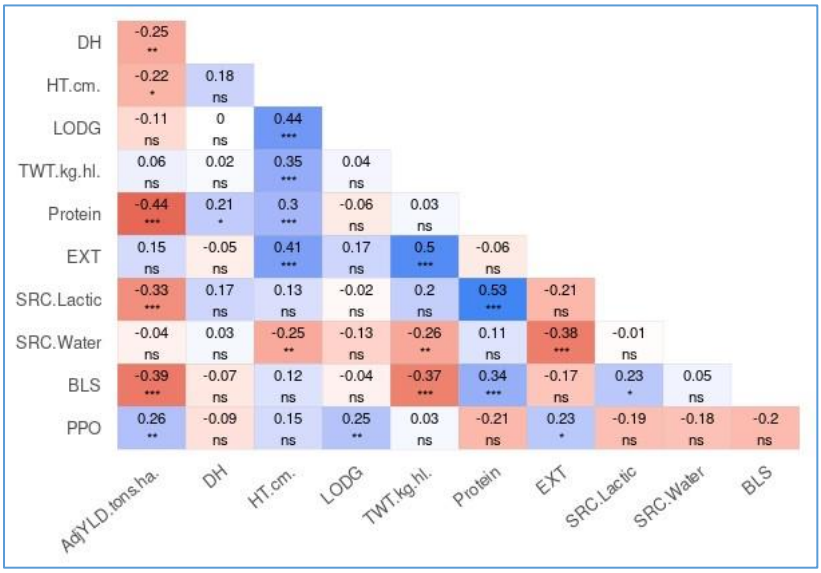



**Figure 6**. Correlations between traits† within locations for 2020 yield trial in ND.
†AdjYLD = Moisture-adjusted yield, DH = days to heading, HT = height, LODG = lodging, TWT = test weight, Protein = grain protein, EXT = flour extraction, BLS = bacterial leaf streak, SRC-Lactic = Solvent Retention Capacity lactic acid, SRC-Water = SRC water, PPO = polyphenyl oxidase.

# DISCUSSION

## Subpopulation Development

### Genetic Bottlenecks

Because of the inherently low frequencies of white seed in a HRSW by HWSW population, there is an inevitable population bottleneck at some point during breeding efforts. There were up to three bottlenecks throughout the development of the Subpopulations. The first was caused by the attempt to control for differential effects caused by low population number, which was to start all field $F_3$ Subpopulations with the same number of seeds. That limitation, 492 seeds, was due to the number of white seeds available for S2 and S3 after color-sorting the $F_3$ seeds. This was done in order to treat each Subpopulation the same at the start ($F_{3)}$. The second bottleneck occurred for S4 and S5 only, at the generation for which they were color-sorted. The third bottleneck applied to all Subpopulations at the $F_{5:6}$ stage, when lines were eliminated using the NaOH Color test. For S1 and S6, their population size was most restricted at that point.

### Proportions of HWSW

Subpopulations developed with different color sorting intensities significantly affected the proportion of HWSW $F_5$ plants, as was shown in Table 5, thus we reject the null hypothesis. Because of the different proportions of HWSW between Subpopulations due to color sorting intensity, we can surmise that for pericarp color both Leeston, NZ and Hettinger, ND are consistent locations for phenotypic selection of HWSW.

When comparing the experimental and expected proportions from Table 1, the proportions of HWSW are closest to the expected frequency for a population with one parent having three red alleles for the red color (Glenn), and one having three white alleles

(NDSW0932 for the white color). Even though genotyping results for the number of color alleles from the parents have been inconclusive, the assumption that Glenn has three red alleles and NDSW0932 has three white alleles can be presumed.

***Color Sorting Implications for Bulk Breeding HWSW***

With a goal of developing populations for HWSW inbred line development, all of the color sorted Subpopulations (S2, S3, S4, and S5) had proportions of HWSW above 90% so as to make plant selection in $F_5$ bulk plots successful for finding HWSW genotypes. The Unsorted and SSD Subpopulations (S1 and S6) resulted in a very low proportion of HWSW in the $F_5$ plants, such that agronomic selection and line derivation in $F_5$ plots, as is the norm for the NDSU Hard Spring Wheat Breeding program, might result in only about 10-13% of the selected plants for entry to a first-year HWSW yield trial being white lines. For populations with a HWSW parent crossed with a HRSW parent with one or two red alleles the proportion should be significantly higher, but still with an expected frequency less than 0.5. For populations with one HWSW and one HRSW parent, to select plants in $F_5$ breeding plots that are reliably white, color sorting at the $F_3$, $F_4$, or $F_5$ generations will give acceptable proportions of HWSW plants.

The ranking of experimental proportions of $F_5$ HWSW plants within Subpopulations was S3 > S4 > S2 > S5 >> S1 = S6. Regarding double- versus triple-sorting, the results contrast an expectation that double-sorting one additional generation would remove more HRSW from the population than a triple-sort starting one generation later. This would suggest that if the ability to color sort is limited to one or two generations, triple-sorting will significantly improve the proportion of HWSW compared to double-sorting.

## Yield Trial

Upon reflection of the location analysis and homogeneity of variances, location differences help draw conclusions about the effects of biotic and abiotic stresses on the yield trial results. Late sowing in Prosper likely affected grain fill and reduced days to heading, test weight, flour extraction, and some quality traits. Yield and combine traits at Hettinger could not be used due to the aforementioned drought and late tillering. Bacterial Leaf Streak was only present in Prosper and was not recorded at other locations. Lodging was not observed at Hettinger, as all plots were upright. While these stresses, or lack thereof, reduced the overall availability of data for these yield trials in 2020, significant differences were able to be found. While a more typical range of moisture across ND may have resulted in greater differences between lines at a location, specifically for yield, conclusions for the color sorting effects across this suite of traits can be drawn. Additionally, because the target environment for commercial HWSW production in ND is Western ND, which is typically drier than Eastern ND, the yield trials for 2020 may be a fairly typical representation of the target environment.

### Trait Expression

When using a nested block analysis, genotypes from the same Subpopulation can be compared, but genotypes from different Subpopulations cannot be compared. Subpopulation effects from the ANOVA presented in Table C2 indicate that significant effects for genotype are present for every trait except combine moisture, combine test weight, and combine yield. For the most important agronomic and quality traits, the quality of data and number of genotypes used in this study was sufficient to identify genotypic differences and find Subpopulation means, which allowed us to make comparisons due to the ranges of expressed trait values.

## Trait Correlations

Correlations among interrelated agronomic and quality traits were the only ones to show very strong associations above 0.7, such as adjusted yield and combine yield, or within GlutoPeak and SRC traits. The typical negative correlation between yield and grain protein content in wheat was found to be consistent across locations, as was the positive correlation between test weight and flour extraction. The positive correlations seen with height and lodging are typical of those observed for first-year yield trial genotypes in NDSU's breeding program. Correlation values between grain protein and Lactic Acid SRC ranged from 0.36 to 0.53, which indicates higher overall grain protein lends itself toward a more functional protein for baking bread but follows that protein quantity is not the only factor for baking quality.

In general, when comparing Subpopulation means between correlated traits, the ranking in the correlated trait were as expected, e.g. Prosper height and lodging have a 0.44 correlation, and S1 is grouped with lowest mean for both traits. That doesn't hold for all Subpopulations across each set of correlated traits, but this is reasonable since the correlations between traits were relatively weak (0.2 to 0.6 and -0.2 to -0.6). Despite this, it's worth noting that S4 and S5 were repeated outliers when it came to expected rankings between correlated traits, particularly between test weight and flour extraction for S5, TWT and BLS for S4 and S5, and TWT and height for S4, as well as BLS and protein for S4 and S5. Logical relationships aligned more closely for S1, S2, S3, and S6, which suggests that there are not tight linkages between any of these traits and there are not severe restrictions imposed by color sorting.

## Acceptable Genotypes

When comparing subpopulations, differences were seen between populations for height and lodging, two traits with agronomic importance. When applying selection thresholds for

genotypic BLUPs to find acceptably-performing genotypes, S1 had many more lines with favorable height and lodging, and made up just over 40% of all acceptably-performing lines found across all six Subpopulations. The unintended changes within the unsorted S1 led to a much higher selection proportion than all other Subpopulations.

**Subpopulation Effects from Color Sorting**

From the multi-location analysis, we concluded that differences were found for Subpopulation means within height, lodging, and test weight. Likewise, traits that required within location analysis also showed differences for days to heading, protein, flour extraction, BLS, and PPO. Thus, we need to reject the null hypothesis for those traits. Notably, we fail to reject the null hypothesis when it comes to moisture-adjusted yield, SRC and GlutoPeak traits, which did not show significant Subpopulation effects.

The Subpopulation with the most consistent differences between lines for multiple traits is S1 (Unsorted), which had no optical color sorting at any generation. Notably, for lodging, S1 was different from all other Subpopulations including from the S6 (SSD), which was greenhouse-grown and not color-sorted. The differences seen between S1 and S6 regarding both lodging and height may reveal some effects of natural selection from field conditions for those Subpopulations, though this does not explain differences between S1 and the other field-grown Subpopulations (S2, S3, S4, and S5) with respect to height and lodging. In the within-location analysis, S1 and S6 also differed in Carrington for DH and EXT, Hettinger for DH and protein, and Prosper for DH, protein, EXT, BLS, and PPO. This may indicate that, since no color sorting was used for S1 and S6, the differences and effects seen in days to heading, height, lodging, test weight, protein, flour extraction, PPO, and BLS are not associated with color sorting intensity or

environmental selection, rather may merely reveal the effects from genetic bottlenecks by shifting the genetic variation for traits at random within each Subpopulation.

Regarding the potential negative impact on important complex quantitative traits, such as yield and end-use quality, this study suggests that any color sorting generation or intensity typically used by the NDSU Spring Wheat Breeding program should not significantly impact those traits for the typical Western ND environment. Because yield expression was lower due to drought, that may not hold true when a more favorable environment allows expanded expression of these traits.

### *Considerations of Within Location Analysis Applied to Breeding*

With respect to Subpopulation effects within locations, significant differences were observed for days to heading, protein, and flour extraction. Considering the effects seen across Subpopulations with many traits, differential phenotypic expressions in early generation environments are a potentially key aspect that could shift population means during the breeding process, whether inadvertently due to genetic drift and natural selection, or purposefully by using selection techniques amongst and between populations. As seen with careful use of location and phenotype for pericarp color with color sorting, the selection environments for other traits of importance should be carefully considered.

## Suspected Mechanisms in Response to Color Sorting

While working with HWSW germplasm and color sorting methodologies, it was suspected that the optical nature of the imager and rejection calibrations, in conjunction with the tendency for slight but meaningful rejection errors, may be associated with negative test weight and grain protein responses. This could be because lighter color red kernels that remained in the population could have had lower test weight or grain protein content, and remained in the

population to potentially segregate to HWSW in subsequent generations, thereby reducing the overall test weight or protein content, while the deeper-colored, more vitreous kernels with higher test weight or protein may be more easily screened out due to consistently dark red kernel color. The Subpopulation means for test weight and protein did not show consistent reductions with higher intensity color sorting, so this research does not confirm this suspicion.

## Breeding Strategies

While differences were found in Subpopulation means for some traits, the impacts seem to be in line with changes from typical field pressure and population bottlenecks due to the small proportion of white genotypes within early generations during population development. Two important aspects to consider when using an optical color sorter are:

1) When selecting environments for color sorting, good phenotypic expression of pericarp color is necessary. However, determining how those environments impact the range of economically important traits should be included in decision-making to maintain desirable genetic variability.
2) Maintaining as large a population size as possible during early generations for HWSW in populations that have some level of HRSW parentage.

Integrating color sorting into early-generation population development achieves high proportions of HWSW in a population, but the fewer generations that color sorting is used, the more important it will be to screen for HRSW in the derived lines. If feasible, HWSW breeding programs should use the adapted NaOH color test procedure on all plant selections before entry to a first-year yield trial.

## Conclusions

In summary, the questions posed by this study's objectives are concluded as such:

1) The null hypothesis is rejected because the number of color sorts per generation and generations of first sorting do affect the proportion of HWSW within the fixed Subpopulations of $F_{5:6}$ plants in comparison to the unsorted and SSD controls. S2, S3, S4, and S5 differ in proportion of HWSW from S1 and S6, as well as from each other.
2) The null hypothesis is rejected for height, lodging, and test weight across location, because the fixed Subpopulations do express differences. It is also rejected for within-location analysis because of differences for days to heading, protein, flour extraction, BLS, and PPO. The null hypothesis fails to be rejected for moisture-adjusted yield, SRC, and GlutoPeak traits because they did not show significant Subpopulation effects.
3) The fixed Subpopulations did show subjective differences when selecting for advancement into subsequent year's yield trials, particularly because the high proportion of lines advanced in S1.

## Reflection and Future Work

This work successfully tested hypotheses for effects from using an optical color sorter, and will help inform strategies for developing new HWSW populations. The yield trials in 2020 were large, and the experimental design addressed the research's primary concerns, though use of bulked Subpopulation entries may have saved space. This would have allowed Subpopulation means to be found but would lack understanding of the genotypic variance of derived $F_{5:7}$ lines. In order to validate these results in multiple populations with different parents, Subpopulation bulks could be tested against one another, saving the space that individual genotypes used in this trial. It may be found that similar differences are shown across many populations, or conversely, that particular parent sets reveal differences for traits specific to that combination.

Though not practical, very large populations with thousands of individual plants would be ideal for crosses between white and red parents to allow typical selection for important traits in white wheat. Much more practical will be combining what we have learned here with increasing use of genomic prediction and marker-assisted selection to allow more rapid population development across the many traits important for white wheat. Moving forward with these approaches will be more sustainable for developing hard white spring wheat to fill the demand for better tasting whole grain products.

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## APPENDIX A. NaOH COLORTEST PROCEDURE

***Setup:***

- Place one seed per well into a 96-well microplate with a cover.
- 70 ºC oven
- 20% NaOH solution mixed from pellets, then brought to a temperature (of 70 ºC) in a water bath

***Procedure:***

a) Add 0.2 ml of NaOH solution to each well with 12-channel pipette.

b) Place the microplate into a 70 ºC oven for 8 minutes (3 microplates can be run each period in the oven).

c) After the 8-minute soak, the solution was removed from each well with a multi-pipette.

d) Rate seed in each well HRSW (brownish) or HWSW (yellowish).

e) Take a picture of plates on a solid-color background after soaking.

## APPENDIX B. POLYPHENOL OXIDASE (PPO) PROCEDURE

- Procedure assistance from NDSU Wheat Quality (Dr. Senay Simsek et al.).

***Reagents***

1. MOPS buffer (50mM, pH 6.5) preparation:

   a. Dissolve 11.56 g of MOPS in 850 ml of deionized water.

   b. Adjust pH to 6.5 using 1 M HCl.

   c. Adjust volume to 1 L.

   d. Use within two weeks.

2. Reaction buffer (10mM L-DOPA, 50mM MOPS & 0.02% Tween-20, pH 6.5) preparation:

   a. L-DOPA may take 1 hour to dissolve.

   b. Dissolve 0.0985 g L-DOPA in 40 ml MOPS buffer.

   c. Add 10 µL Tween-20 and stir to dissolve.

   d. Bring volume to 50 ml.

   e. Prep and use *daily*.

3. Blank buffer (50mM MOPS w/ 0.02% Tween-20, pH 6.5) preparation

   a. Add 10 µL Tween-20 to 40 ml MOPS buffer.

   b. Bring volume to 50 ml.

   c. Prep and use *daily*.

***Procedure***

1. Add 4 kernels to two wells of a 96-well deep-well microplate for each entry.

   a. One well is for the reaction with L-DOPA.

   b. The second well is for the reaction blank (Blank buffer).

2. Add 1.2 ml (1200 μL) Reaction buffer to each Reaction well (use a 12-channel pipette across the plate row).

3. Add 1.2 ml Blank buffer to each Blank well (use a 12-channel pipette across the plate row).

4. Seal & check deep-well plate(s) for leaks.

5. Shake deep-well plate(s) on their side at room temperature for 1 hour.

6. Pipette 0.4 ml (400 μL) from each well into their respective well in a NUNC Edge flat-bottomed microplate.

   a. Note: The electronic pipette speed was changed from default to 4 for dispensing, and 5 for uptake. While pipetting to NUNC plate, do it slowly. If bubbles are noted, wait a few seconds to let them settle, and then pipette the next row. Take the plate without the cover and place it on the spectrophotometer.

7. Measure absorbance at 475 nm.

8. Discarded solution must be dumped in HAZARDOUS WASTE container, labeled as Toxic hazard class and MOPS BUFFER (pH 6.5) with L-Dopa, then kept inside the fume hood.

***References***

Fuerst, E. Patrick, James V. Anderson, and Craig F. Morris. 2006. Delineating the role of polyphenol oxidase in the darkening of alkaline wheat noodles. J. of Agricultural and Food Chemistry 54.6: 2378-2384. DOI: 10.1021/jf0526386.

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## APPENDIX C. ANALYSIS OF VARIANCE (ANOVA) FOR NESTED MULTI-LOCATION ANALYSIS

**Table C1.** Fixed effects for analysis of variance (ANOVA) for multi-location analysis.

| Trait | Locations‡ | Source of Variation | Prob. > F | |
|---|---|---|---|---|
| Moisture-Adjusted Yield | CAR, PRO | location | 0.8160 | |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.1635 | |
| | | location*SUBPOPULATION | 0.0565 | * |
| Combine Moisture | CAR, PRO | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.0007 | *** |
| | | location*SUBPOPULATION | 0.0374 | ** |
| Combine Test Weight | CAR, PRO | location | 0.0000 | *** |
| | | Block within Location | 0.0052 | *** |
| | | SUBPOPULATION | 0.2458 | |
| | | location*SUBPOPULATION | 0.0116 | ** |
| Combine Yield | CAR, PRO | location | 0.0153 | ** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.4232 | |
| | | location*SUBPOPULATION | 0.1080 | |
| Days to Heading | CAR, HET | location | 0.0000 | *** |
| | | Block within Location | 0.0012 | *** |
| | | SUBPOPULATION | 0.0403 | ** |
| | | location*SUBPOPULATION | 0.1624 | |
| Height | all | location | 0.0000 | *** |
| | | Block within Location | 0.3300 | |
| | | SUBPOPULATION | 0.0002 | *** |
| | | location*SUBPOPULATION | 0.0051 | *** |
| ln(GP-Aggregation Energy)† | CAR, HET | location | 0.0019 | *** |
| | | Block within Location | 0.0193 | ** |
| | | SUBPOPULATION | 0.3890 | |
| | | location*SUBPOPULATION | 0.1731 | |

**Table C1**. Fixed effects for analysis of variance (ANOVA) for multi-location analysis (continued).

| Trait | Locations‡ | Source | Prob > F | |
|---|---|---|---|---|
| ln(GP Plateau Energy)† | all | location | 0.0160 | ** |
| | | Block within Location | 0.3201 | |
| | | SUBPOPULATION | 0.7576 | |
| | | location*SUBPOPULATION | 0.3007 | |
| ln(GP Post-Maximum Torque)† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.5832 | |
| | | location*SUBPOPULATION | 0.2673 | |
| Lodging | CAR, PRO | location | 0.0000 | *** |
| | | Block within Location | 0.0363 | ** |
| | | SUBPOPULATION | 0.0140 | ** |
| | | location*SUBPOPULATION | 0.0016 | *** |
| Flour Extraction | CAR, HET | location | 0.0765 | * |
| | | Block within Location | 0.0001 | *** |
| | | SUBPOPULATION | 0.5423 | |
| | | location*SUBPOPULATION | 0.8026 | |
| GP Pre-Maximum Torque† | CAR, HET | location | 0.5303 | |
| | | Block within Location | 0.7584 | |
| | | SUBPOPULATION | 0.7645 | |
| | | location*SUBPOPULATION | 0.6775 | |
| GP Torque Maximum† | CAR, HET | location | 0.0009 | *** |
| | | Block within Location | 0.0041 | *** |
| | | SUBPOPULATION | 0.4814 | |
| | | location*SUBPOPULATION | 0.5006 | |
| GP Start-up Energy† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0093 | *** |
| | | SUBPOPULATION | 0.3599 | |
| | | location*SUBPOPULATION | 0.7242 | |
| SRC Lactic Acid† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.2992 | |
| | | location*SUBPOPULATION | 0.2306 | |

**Table C1**. Fixed effects for analysis of variance (ANOVA) for multi-location analysis (continued).

| Trait | Locations‡ | Source | Prob > F | |
|---|---|---|---|---|
| SRC Sodium Carbonate† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.0717 | * |
| | | location*SUBPOPULATION | 0.9979 | |
| SRC Sucrose† | all | location | 0.0000 | *** |
| | | Block within Location | 0.1832 | |
| | | SUBPOPULATION | 0.4980 | |
| | | location*SUBPOPULATION | 0.0641 | * |
| SRC Water† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.1922 | |
| | | location*SUBPOPULATION | 0.9781 | |
| sqrt(GP Peak Maximum Time)† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0002 | *** |
| | | SUBPOPULATION | 0.1736 | |
| | | location*SUBPOPULATION | 0.6246 | |
| sqrt(GP Peak Maximum Time)† | all | location | 0.0000 | *** |
| | | Block within Location | 0.0002 | *** |
| | | SUBPOPULATION | 0.1736 | |
| | | location*SUBPOPULATION | 0.6246 | |
| Test Weight | CAR, HET | location | 0.0000 | *** |
| | | Block within Location | 0.0000 | *** |
| | | SUBPOPULATION | 0.0000 | *** |
| | | location*SUBPOPULATION | 0.0848 | * |
| Protein | CAR, HET | location | 0.0000 | *** |
| | | Block within Location | 0.0027 | *** |
| | | SUBPOPULATION | 0.0485 | ** |
| | | location*SUBPOPULATION | 0.0353 | ** |

*, **, *** Significant at P≤0.10, P≤0.05, and P≤0.01, respectively. †GP=GlutoPeak, SRC=Solvent Retention Capacity. ‡CAR = Carrington, HET = Hettinger, and PRO = Prosper.

**Table C2.** Random effects for analysis of variance (ANOVA) for multi-location analysis.

| Trait | Locations‡ | Variance Component | Wald p-Value | |
|---|---|---|---|---|
| Moisture-Adjusted Yield | CAR, PRO | Genotype within Subpopulation | 0.0688 | * |
| | | location*Genotype within Subpopulation | 0.1681 | ** |
| | | Residual | | |
| | | Total | | |
| Combine Moisture | CAR, PRO | Genotype within Subpopulation | 0.0016 | *** |
| | | location*Genotype within Subpopulation | 0.6658 | |
| | | Residual | | |
| | | Total | | |
| Combine Test Weight | CAR, PRO | Genotype within Subpopulation | 0.9950 | |
| | | location*Genotype within Subpopulation | 0.0000 | *** |
| | | Residual | | |
| | | Total | | |
| Combine Yield | CAR, PRO | Genotype within Subpopulation | 0.8445 | |
| | | location*Genotype within Subpopulation | 0.0000 | *** |
| | | Residual | | |
| | | Total | | |
| Days to Heading | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0052 | *** |
| | | Residual | | |
| | | Total | | |
| Height | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0010 | *** |
| | | Residual | | |
| | | Total | | |
| Lodging | CAR, PRO | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0042 | *** |
| | | Residual | | |
| | | Total | | |

**Table C2**. Random effects for analysis of variance (ANOVA) for multi-location analysis (continued).

| Trait | Locations‡ | Variance Component | Wald p-Value | |
|---|---|---|---|---|
| Flour Extraction | CAR, HET | Genotype within Subpopulation | 0.0023 | *** |
| | | location*Genotype within Subpopulation | 0.5740 | |
| | | Residual | | |
| | | Total | | |
| ln(GP-Aggregation Energy)† | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.5084 | |
| | | Residual | | |
| | | Total | | |
| GP Pre-Maximum Torque† | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0900 | * |
| | | Residual | | |
| | | Total | | |
| GP Torque Maximum† | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.2985 | |
| | | Residual | | |
| | | Total | | |
| ln(GP Plateau Energy)† | all | Genotype within Subpopulation | 0.1521 | |
| | | location*Genotype within Subpopulation | 0.0000 | *** |
| | | Residual | | |
| | | Total | | |
| ln(GP Post-Maximum Torque)† | all | Genotype within Subpopulation | 0.0000 | |
| | | location*Genotype within Subpopulation | 0.0941 | |
| | | Residual | | |
| | | Total | | |

**Table C2**. Random effects for analysis of variance (ANOVA) for multi-location analysis (continued).

| Trait | Locations‡ | Variance Component | Wald p-Value | |
|---|---|---|---|---|
| sqrt(GP Peak Maximum Time)† | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0000 | *** |
| | | Residual | | |
| | | Total | | |
| GP Start-up Energy† | all | Genotype within Subpopulation | 0.1548 | |
| | | location*Genotype within Subpopulation | 0.1676 | |
| | | Residual | | |
| | | Total | | |
| SRC Lactic Acid† | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0933 | * |
| | | Residual | | |
| | | Total | | |
| SRC Sodium Carbonate† | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0163 | ** |
| | | Residual | | |
| | | Total | | |
| SRC Sucrose† | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.2032 | |
| | | Residual | | |
| | | Total | | |
| SRC Water† | all | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.2045 | |
| | | Residual | | |
| | | Total | | |

**Table C2**. Random effects for analysis of variance (ANOVA) for multi-location analysis (continued).

| Trait | Locations‡ | Variance Component | Wald p-Value | |
|---|---|---|---|---|
| Test Weight | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0000 | *** |
| | | Residual | | |
| | | Total | | |
| Protein | CAR, HET | Genotype within Subpopulation | 0.0000 | *** |
| | | location*Genotype within Subpopulation | 0.0119 | ** |
| | | Residual | | |
| | | Total | | |

*, **, *** Significant at P≤0.10, P≤0.05, and P≤0.01, respectively. †GP=GlutoPeak, SRC=Solvent Retention Capacity. ‡CAR = Carrington, HET = Hettinger, and PRO = Prosper.