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# Continued Exploration of Barley Genotype Contribution to Base Malt and Beer Flavor Through the Evaluation of Lines Sharing Maris Otter® Parentage

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**ABSTRACT**

Heirloom barley (*Hordeum vulgare L.*) varieties remain interesting to maltsters and brewers for their perceived unique flavor contributions to beer, despite not meeting contemporary agronomic and malting expectations. This study utilized crosses of the heirloom Maris Otter® and two contemporary genotypes to determine if "updated heirlooms" could be produced that would show improved agronomics and contemporary malt quality, while contributing uniquely to malt and beer sensory and chemical profiles. Using a recently established pipeline of malting; brewing; hot steep and beer sensory; and metabolomics to evaluate barley genotype contributions to malt and beer flavor, four experimental lines were compared to a control. The experimental lines were also assessed for their genomic contribution from their respective parents to further elucidate regions of the Maris Otter genome that may contribute to unique beer flavors. Results show improved agronomic outcomes relative to the heirloom parent and were comparable to the control. Malting quality met current recommendations. However, sensory properties attributable to the unique heirloom parent were not found. Further, chemical profiling did not explain the observed nuanced sensory differences, nor did it reveal unique metabolites not described by the sensory panels.

**KEYWORDS**

barley; heirloom; hot steep; Maris Otter; malt; metabolomics

## Introduction

Malted barley (malt) is the primary source of carbohydrates and assimilable free amino nitrogen required for beer fermentations. It is also the primary source of color in beer, creating a spectrum that ranges from straw-like or golden to dark brown or black. Malt's contribution to the final profile of beer is complemented by hops and yeast fermentation by-products, creating a unique organoleptic experience. However, much of the flavor contributed by barley has been attributed to the malting process and thus breeding programs have focused on malt quality outcomes, rather than positive or unique flavor attributes. Further, within the range of malts available to brewers, pale-colored or "base" malts have not been considered major contributors to a beer's overall malt flavor profile.[1] A recent industry report, however, revealed that a sensory panel can detect differences in research beers brewed with different base malts and that base malt choice impacts the overall flavor profile of finished beers.[2]

The requirements for malting quality barley have been established for many years and targets are regularly evaluated and published by organizations such as the American Malting Barley Association (AMBA). Recommendations are established for both all-malt and adjunct brewing that reflect the requirements of the respective production processes – for example the higher diastatic power (DP) and free amino nitrogen (FAN) values needed for high adjunct brewing.[3] Craft brewers have asked that barley growers and maltsters also focus on malt quality specifications better suited for all-malt brewing, including lower protein, DP, and FAN.[4,5] Craft brewers have also continued to demand "heirloom" varieties (e.g., Barke®, Golden Promise®, and Maris Otter®) because of their reported contributions to beer flavor, despite not meeting contemporary agronomic and malt quality standards.[6]

Developing a deeper understanding of the contributions of barley genotype to malt and beer flavor is an area of active research.[7–10] Previously, research on barley genotype contribution to beer flavor was limited to negative flavor outcomes such as dimethyl sulfide (DMS) and associated precursors[11] or lipoxygenase (LOX) activity.[12,13] Briefly, Herb et al.[9] - using sensory evaluation - found that barley genotype and production environment contribute to beer flavor. Bettenhausen et al.[7] showed that commercially available malts of similar type - but made from different

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genotypes and malted at different locations - had different metabolite profiles that led to different beer sensory outcomes. Metabolomics is a powerful tool for the evaluation of malt and beer flavor because it identifies volatile compounds using various types of chromatography and mass spectrometry.[7,14,15] Bettenhausen et al.[8] applied metabolomic profiling, together with beer sensory, to three selections from the same germplasm used by Herb et al.[9] - derived from the cross of Golden Promise and Full Pint - that were grown at the same location and malted at the same facility. Windes et al. [10] used a novel method for malt sensory analysis (American Society of Brewing Chemists malt hot steep extract method),[16] as well as beer sensory and metabolomics, in order to better trace barley variety contributions to malt and beer flavor in two sets of barley germplasm – one comprised of potential spring habit varieties and the other of contemporary winter malting varieties that did not include Maris Otter. The research described in this report continues using this flavor research pipeline to evaluate the use of an heirloom barley variety as a breeding parent and its impacts on malt and beer flavor and chemical profiles of the resulting progeny.

Maris Otter is considered an heirloom winter two-row barley variety selected from the cross of Proctor and Pioneer and was released in the United Kingdom (U.K.) in 1966. The heirloom designation is not regulated but is generally defined as heritage plant varieties that are, despite their unique properties, no longer widely grown commercially due to agronomic reasons. This designation has been used for foodstuffs ranging from tomatoes to grains and brings a perception of quality and flavor.[17] Maris Otter has a reputation of providing the malty flavors of British beer styles and performing well in a single-infusion mashing and lautering vessel, typical of the traditional British brewhouse and common in the American craft brewery.[18] It was one of the more commonly grown and malted varieties in the U.K. in the 1970s[19] but did not find wider acceptance in continental Europe due to lack of low temperature tolerance.[20] Initially considered high yielding, susceptibility to disease such as barley yellow mosaic virus[21] and scald (*Rhynchosporium commune*)[22] saw newer varieties gain favor and in 1989 it was removed from the U.K.'s list of recommended varieties. In 2002, the intellectual property rights were sold to Robin Appel Ltd, who now grows it solely under license in the U.K.[23]. Despite its low grain yields and high input requirements,[24] the variety has maintained a small, but notable market share due to its reputation for flavor within the craft brewing sector.[25] Maris Otter figures in the pedigrees of more contemporary varieties, such as Puffin and Talisman, that craft maltsters are promoting as flavorful based on the Maris Otter ancestry.[26,27] Despite the notoriety of the variety there is limited peer-reviewed research on the genetic basis behind its contributions to beer flavor. However, other research has shown the importance of heirloom varieties for traditional beer styles, such as Haná barley for decoction mashed Czech lager.[28,29]

The objectives of this study were to investigate (1) if plant breeding involving an heirloom barley variety with desirable flavor contributions to beer, and contemporary germplasm, could produce progeny that meet current agronomic and malt quality specifications; (2) if the resulting "updated heirlooms" would lead to unique and different malt hot steep and beer flavor profiles, which could be identified by a trained sensory panel; and (3) if identifying and quantifying volatile metabolites in hot steeps and beers could explain sensory results and/or provide further insights into differences that were not detectable by sensory evaluation.

## Experimental

### *Barley germplasm*

Four doubled haploids derived from crosses between Maris Otter and Violetta and Maris Otter and 04-028-36 were utilized for this experiment (Table 1). Both Violetta and 04-028-36 are winter-habit, two-row genotypes. Violetta has an AMBA recommendation, while 04-028-26 is an experimental line from Ackermann Saatzucht GMBH & CO, which was selected from the cross of Annicka and Malwinta. These four lines were selected for agronomic performance and malting quality - from an initial set of 47 doubled haploids - over three years of field trials at the Hyslop Crop Science Field Research Laboratory (Corvallis, OR, U.S.A.). Selections were based on agronomic performance superior to Wintmalt and Maris Otter and malting quality performance that approached AMBA recommendations. Maris Otter was planted for selection purposes but, due to intellectual property issues, was not included in the larger planting in the Fall of 2018. Therefore, Maris Otter was not included in the malting, brewing, beer sensory and metabolomics components of this research. Instead, Wintmalt (an AMBA recommended variety and check) was grown with the four doubled haploid selections and served as the control. A commercial source of Maris Otter malt, Crisp Extra Pale (Crisp Malt, Great Ryburgh, U.K.), was used for the hot steep sensory and hot steep metabolomics components. Typically, Maris Otter base malts are kilned to a higher color (2.2-3.5°SRM), but the lower color version was selected as the most apt comparison to the low color research malts used in this study (<2°SRM; Standard Reference Method) and advertised as contributing similar flavors to beer as the standard.[30]

Details on parentage, field trials, selection procedures, and selection outcomes are provided in Supplemental 1 (Tables S1 and S2, and Figures S1 and S2).

**Table 1.** Barley selections used for the malting, brewing, sensory, and metabolomics research described in this report.

| Description | Pedigree | Developer |
|---|---|---|
| Wintmalt[a] | (Opal × 3087 / 96) × 1922 – 23 | KWS Saat SE & Co. KGaA |
| DH141515 | Violetta / Maris Otter | Oregon State University |
| DH141969 | Violetta / Maris Otter | Oregon State University |
| DH150115 | 04-028-36 / Maris Otter | Oregon State University |
| DH142010 | 04-028-36 / Maris Otter | Oregon State University |

[a]Experimental control.

### Haplotyping

Leaf tissue from Maris Otter and the four experimental lines was used for DNA extraction at the seedling stage. The 50k Illumina Infinium iSelect Single Nucleotide Polymorphism (SNP) genotyping array[31] was used for genotyping these lines at the North Central Small Grains Genotyping facility (USDA-ARS, Fargo, ND). Graphical haplotypes were produced using Flapjack (version 1.20.10.07).[32] Genotype information for lines is available upon request.

The chromosome locations and sizes of introgression segments from each parent in each of the four DH lines were estimated by haplotype alignment with Maris Otter. SNP markers that were not in common between a DH line and Maris Otter were assumed to originate from the non-Maris Otter parent. Markers in common between each experimental line and Maris Otter were assumed to define regions inherited from Maris Otter. Monomorphic markers located inside introgression regions were assumed to be part of the introgression. In order to estimate the size of the introgression, cM positions retrieved from Bayer et al.[31] were used.

### Malting

Malting for brewing and subsequent research was performed in the Oregon State University mini-malter using approximately 90 kg batches as described in Windes et al.[10] Micro-scale malting to optimize mini-malter protocols was performed in a Custom Laboratory Products (Milton Keynes, U.K.) steep/germ system and kiln. Malt protocols are provided in Supplemental 2 (Tables S3–S5).

### Barley and malt quality analysis

Barley grain analysis was performed using the American Society of Brewing Chemists Methods of Analysis (Barley-2, Physical Tests; Barley-3, Germination).[33,34] Protein and moisture were measured using a FOSS Infratec-NOVA near-infrared grain analyzer (Hillerød, Denmark).

Malt quality assessments during the selection process were performed by the USDA-ARS Cereal Crop Research Unit (Madison, WI, U.S.A.). Malt samples used for the hot steep, including the commercial example, and brewing reported below were analyzed by the Hartwick College Center for Craft Food & Beverage (Oneonta, NY, U.S.A.) and both used the ASBC Methods of Analysis.

### Brewing and beer analysis

Ales were prepared at the Deschutes Brewery (Bend, OR, U.S.A.) in June of 2020, using an Esau and Hueber (Schrobenhausen, Germany) 2.5 hL brewery. The brewing recipe and protocol were designed with the intention of producing beers that would emphasize malt characteristics. A neutral ale yeast strain (California Ale 001, White Labs, San Diego, CA, U.S.A.) was selected to minimize fermentation metabolite contribution. This yeast was similar to the strain used by Herb et al.[9] and Bettenhausen et al.[7] A single hop addition of Azacca® pellets was used to achieve 20 bitterness units in the final beer. The full brewing protocol is provided in Supplemental 3 (Tables S6 and S7).

Beer analysis was performed at Deschutes Brewery with the following ASBC Methods of Analysis: Beer-2, Specific Gravity; Beer-3, Apparent Extract; Beer-4, Alcohol; Beer-5, Real Extract; Beer-10, Color; Beer-23, Beer Bitterness; Beer-25F, Diacetyl by Gas Chromatography.[35]

### Hot steeps

Hot steep extractions were prepared following the American Society of Brewing Chemists Methods of Analysis (Sensory-14, Hot Steep Malt Sensory Evaluation Method). Extractions for sensory evaluation were prepared at Oregon State University within 2 h of assessment. A unique extraction was prepared for each sensory assessment as well as for metabolomics. For the latter, extractions were frozen at −80 °C, and shipped overnight on dry ice to Colorado State University (Fort Collins, OR, U.S.A.). Upon delivery, they were held at −80 °C until analysis.

### Difference from control (DFC)

DFC sensory testing of hot steeps and beers was performed by evaluating the overall differences between samples and the control (Wintmalt) on a scale from 1 to 10 with increments of 0.1, where 1 represents no difference and 10 represents a very large difference between the sample and the control. The sensory panel consisted of 18 panelists (12 female, six male, ages 22 – 56). Using a panelist-specific random order, samples (50 ml) were presented in 300 ml beer tasting glasses labelled with three-digit blind codes and covered with plastic lids. The samples were evaluated in duplicate, performing separate sessions held on different days for either orthonasal aroma by smelling or for flavor, taste, and mouthfeel by tasting.

### Check all that apply (CATA)

For CATA sensory testing, the same panel was trained to qualitatively describe the aroma, flavor, taste, and mouthfeel of samples prepared as previously described by selecting the presence of a number of descriptors out of 21 potential aroma descriptors or 29 flavor descriptors (21 for flavor and eight for taste and mouthfeel, evaluated together). These descriptors were chosen from the DraughtLab malt flavor map[36] and were defined based on external food references or solutions of aroma compounds (supplementary material 4, Table S8). In addition, to calibrate, panelists were served two different controls representing specific attributes. For evaluating hot steeps, Pilsner Malt (Weyermann, Bamberg, Germany) and Munich Malt (Great Western Malting, Vancouver, WA) served as controls. For evaluating beers, Dead Guy Amber Ale (Rogue Ales, Newport, OR) and Rolling Rock Extra Pale Lager (Latrobe Brewing Company, Latrobe, PA) served as controls. Samples were evaluated for orthonasal aroma by smelling and for flavor, taste, and

mouthfeel by tasting in duplicate on separate days. Panelists had to select at least two attributes for the aroma and flavor and at least two attributes for the taste/mouthfeel of each sample, respectively. DFC and CATA data were collected using tablet computers and tools built in Qualtrics Software (Provo, UT).

### Metabolomics

Volatile metabolites in hot steeps and beer were detected using a non-targeted metabolomics approach. The analysis of volatiles method included the use of headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) as previously described by Windes et al.[10] Briefly, mass spectra from the MS platform were converted to the .cdf file format and processed and annotated using the workflow described in Bettenhausen et al.[8] Each sample resulted in a matrix of molecular features (defined by retention time and mass to charge ratio (m/z)) generated using XCMS software in R v. 4.0.3.[37] Mass spectra were deconvoluted using the RamClust algorithm[38] and normalized to total ion current (TIC); the relative abundance and variance of each molecular feature were determined by the mean area of the pooled quality control (QC) injection. Volatile metabolites were annotated by spectral matching in RamSearch software[39] to an in-house database of ~1,500 compounds and to external and theoretical databases including: NIST v14 (http://www.nist.gov), Metlin;[40] Golm Metabolome Database,[41] MSFinder software (v. 3.26, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa, Japan);[42,43] Human Metabolome Database (HMDB).[44,45] Spectra were also evaluated using the findMAIN function of the interpretMSSpectrum R package[46] and chemical ontologies were established using HMDB and the ClassyFire package in R.[47]

### Statistical analysis

#### Grain and malt analysis

Agronomic and malting data were analyzed using one-way analysis of variance (ANOVA) with the aov function of the R package stats.[48] Further pair-wise comparison of significant results was analyzed with two-sided Dunnett's multiple comparison using the glht function of the R package multcomp.[48]

#### Sensory analysis

Linear regression, Cochran's Q test, agglomerative hierarchical cluster analysis (AHC), two-sided Dunnet's multiple comparison test, one-way analysis of variance (ANOVA) and correspondence analysis (CA) were performed using XLSTAT Premium 2020.4.1 (Addinsoft, Boston, MA).

#### Metabolomics

Volatile metabolite abundances were compared using ANOVA via the aov function in the R statistical environment v. 4.0.3[48] and false discovery rate (FDR) adjustment was performed on the ANOVA p-values using the Benjamini-Hochberg algorithm.[49] Principal Components Analysis (PCA) was conducted on 143 annotated metabolites from the hot steeps with SIMCA software v. 15 (Sartorius Stedim Biotech, Umea, Sweden). Prior to heat-mapping, volatile metabolite abundances were calculated as a fold variation (FV) within and among chemical classes. Within chemical class, mean FV (mFV) was calculated as ratio of the selection mean divided by the check variety (Wintmalt) mean. The resulting fold variation scores were calculated as $\log^2$ FV scores, converted into color code and grouped using hierarchical clustering.[50] These plots were generated in R using the gplots, ggplots2, Reshape2, and stats packages via the heatmap.2, melt, and hclust functions.[51–53]

## Results

### Barley germplasm development and characterization

Over four years at the Corvallis field site, the 47 progeny were culled to the four selections that are the focus of this study, based on assessment of agronomic results, grain quality, and malt quality as compared to Wintmalt and Maris Otter. Wintmalt is one of two national checks used in AMBA trials and is an AMBA-recommended fall-planted variety adapted to Oregon's Willamette Valley.[54]

Using the high SNP density coverage obtained from the 50k Illumina array, we were able to estimate the genome segments inherited from each parent in each of the four selected lines. (Supplemental 1, Figure S3a and S3b). The estimated size of the genome contributions from Maris Otter to each of the four selected lines is shown in Table 2; these contributions range from 32% (DH142010) to 60% (DH150115). Looking at individual chromosomes, there are cases of complete inclusion or exclusion of the Maris Otter genome (Table 3). For example, chromosome 1 in DH141969

**Table 2.** Contribution of Maris Otter to the genome architecture of each of four selected progeny.

| Genotype | cM | % of total genome |
|---|---|---|
| DH141515 | 401 | 44 |
| DH141969 | 423 | 46 |
| DH150115 | 548 | 60 |
| DH142010 | 290 | 32 |

**Table 3.** Percentage contribution of Maris Otter to each chromosome of four progeny.

| | Experimental Line | | | |
|---|---|---|---|---|
| Chromosome | DH150115 | DH141515 | DH142010 | DH141969 |
| 1H | 28% | 28% | 66% | 100% |
| 2H | 62% | 69% | 0% | 31% |
| 3H | 20% | 85% | 11% | 47% |
| 4H | 68% | 32% | 49% | 14% |
| 5H | 93% | 13% | 43% | 48% |
| 6H | 70% | 43% | 57% | 0% |
| 7H | 66% | 44% | 0% | 94% |

Note: Color gradient shows relative inclusion of Maris Otter genome between lines on each chromosome: dark gray – lowest inclusion on each chromosome, light gray – highest inclusion.

traces in its entirety to Maris Otter, whereas 6H entirely to Violetta. There are two instances of greater than 90% inclusion of Maris Otter genome on specific chromosomes: DH150115 with 93% of 5H and DH141969 with 94% of 7H. DH142010 has zero inclusion of chromosome 2H and 7H from Maris Otter. Select lines shared similar inclusions on three chromosomes: 1H (DH141515, DH150115), 2H (DH141515, DH150115), 3H (DH142010, DH150115), and 5H (DH141969, DH142010).

### Agronomics

Averaged over three years, DH141515 and DH141969 were significantly higher yielding than Wintmalt, the agronomic check and all were higher yielding than Maris Otter (Figure 1). Data on other agronomic traits of selections vs. the controls are provided in Supplemental 1 (Tables S1 and S2).

### Malts

Averaged malt quality data of the experimental lines over three station years that were used for selection purposes, and malt profiles used to develop the mini-malter protocols are shown in Supplemental 2 (Tables S4 and S5). Single batches of lines malted using the Oregon State University mini-malter (Table 4) that were used for this research met the recommended parameters for all-malt brewing as defined by AMBA, with the following exceptions: S/T ratio (DH150115, DH142010), FAN (DH141515, DH150115, DH142010), and DP (DH141969). However, these values were in specification for malt for adjunct-brewing. Filtration time and wort clarity were all reported as "normal" and "clear" (data not shown). The mini malter results were similar to the Crisp Extra Pale malt, which met AMBA criteria for all-malt brewing, except for FAN (over specification) and DP (under specification). Lower levels of amylolytic enzymes are to be expected for Maris Otter malts compared to contemporary elite malting lines.[55]

### Beers

The four selections and the Wintmalt control performed as expected within the Deschutes Brewery pilot plant, given the well-modified malts. There was limited variation in brewing outcomes (Table 5). Minor treated brewing water additions were made prior to fermentation in order to reach the targeted original gravity. Fermentation proceeded as normal, producing beers with similar amounts of alcohol by volume (ABV) and a similar real degree of fermentation (RDF) and associated parameters. Realized bitterness units (IBU) were all within a spread of 1.5 IBU. Both sets of parameters showed less variance than in previous studies using similar beers brewed at the Deschutes Brewery[8] and with comparable equipment at Oregon State University.[10] The beers showed some spread in final pH but compared to the fermentation start, the total drop was similar (0.95 – 1.20 pH units). Diacetyl (2,3-butanedione) levels showed some variation, but even the highest value of 17 µg/l (DH150115) was at the lowest threshold value reported in the literature.[56]

### Sensory evaluation of malt hot steeps

#### Difference from control (DFC)

DFC sensory results showed significant differences, according to a two-sided Dunnet's multiple comparison test, between the aroma of three experimental lines and the Wintmalt control; the exception was DH141969. Differences between samples were not as notable for flavor, taste, and mouthfeel; there were significant differences between 3 out

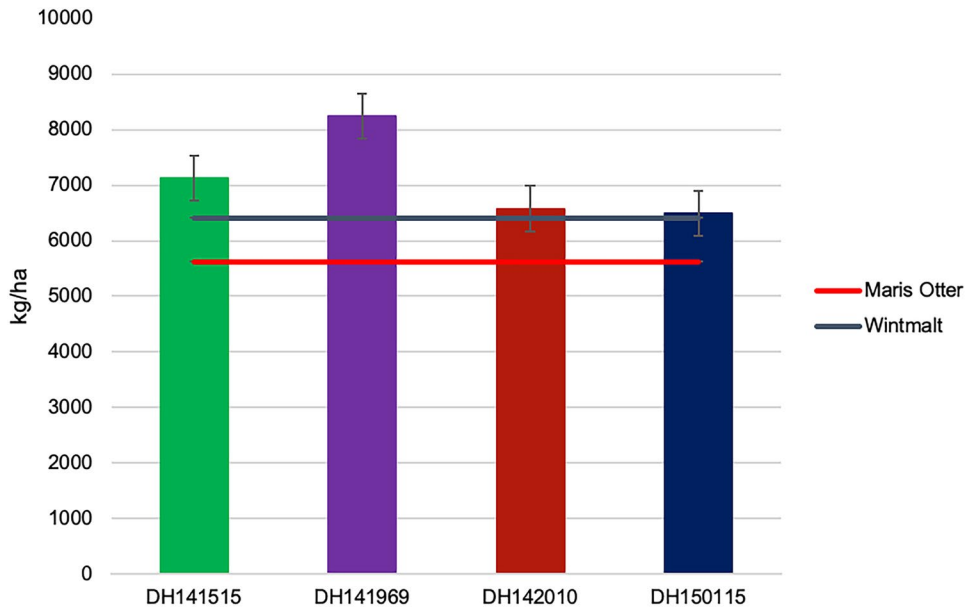


**Figure 1.** Yield of four selected progeny derived from crosses with Maris Otter averaged over three years (2017 – 2019). Color figure available online.

**Table 4.** Malting quality data for four selected progeny derived from crosses with Maris Otter compared to Wintmalt and commercial Crisp Extra Pale.

| Genotype/ Product | Moisture (%) | Friability (%) | Extract (%) | Color (°SRM) | β-Glucan (mg/L) | Protein (%) | Soluble (%) | S/T (%) | FAN (mg/L) | DP (°L) | α-Amylase (20°DU) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Wintmalt | 4.3 | 98.9 | 83.6 | 1.84 | 40.0 | 10.1 | 3.88 | 38.4 | 167 | 115 | 58.8 |
| DH141515 | 4.4 | 98.7 | 83.3 | 1.64 | 61.0 | 10.5 | 4.62 | 44.0 | 197[a] | 139 | 68.4 |
| DH141969 | 4.1 | 95.6 | 81.2 | 1.75 | 73.0 | 10.3 | 4.45 | 43.2 | 177 | 162[a] | 62.3 |
| DH150115 | 4.3 | 98.6 | 83.0 | 1.98 | 73.0 | 9.7 | 4.69 | 48.4[a] | 201[a] | 120 | 57.9 |
| DH142010 | 4.5 | 96.7 | 84.6 | 1.70 | 76.0 | 10.5 | 4.90 | 46.7[a] | 210[a] | 131 | 61.0 |
| Crisp Extra Pale | 5.5 | 92.1 | 82.4 | 1.87 | 69.0 | 10.1 | 4.65 | 46.0 | 198[a] | 107[b] | 59.9 |

S/T, soluble to total protein ratio; FAN – free amino nitrogen; DP – diastatic power.
All malts, except for commercially sourced Crisp Extra Pale, were produced in an OSU mini-malter (90kg) as a single replicate in 2019. All analyses were performed at Hartwick College.
[a]Exceeds AMBA specifications for all-malt brewing.
[b]Below AMBA specifications for all-malt brewing. All other values within acceptable range.

**Table 5.** Brewing process parameters for four selected progeny derived from crosses with Maris Otter compared to Wintmalt.

| Genotype | ABV (%) | OE (°P) | RE (%w/w) | AE (°P) | pH | Color (SRM) | RDF (%) | IBU | Diacetyl (µg/L) |
|---|---|---|---|---|---|---|---|---|---|
| Wintmalt | 5.51 | 11.34 | 2.89 | 0.89 | 4.40 | 2.55 | 75.60 | 20.5 | 5.0 |
| DH141515 | 5.50 | 11.29 | 2.85 | 0.85 | 4.16 | 2.28 | 75.87 | 19.0 | 4.0 |
| DH141969 | 5.40 | 11.25 | 2.98 | 1.01 | 4.13 | 2.78 | 74.70 | 19.0 | 10.0 |
| DH150115 | 5.59 | 11.42 | 2.86 | 0.82 | 4.29 | 2.47 | 76.11 | 20.0 | 17.0 |
| DH142010 | 5.57 | 11.39 | 2.84 | 0.81 | 4.26 | 2.40 | 76.16 | 20.5 | 7.0 |

ABV, alcohol by volume; OE, original extract; RE, real extract; AE, apparent extract; SRM, standard reference method; RDF, real degree of fermentation; IBU, international bitterness units.

of 5 samples compared to the control. The Crisp Extra Pale was significantly different from Wintmalt for aroma, flavor, taste and mouthfeel.

Based on these results, further sensory evaluation was performed using a CATA approach to qualitatively identify differences between malt hot steeps using a predefined set of descriptive attributes.

### *Check all that apply (CATA)*

The attributes grainy, grassy, bread, breakfast cereal, dough, and cracker were most frequently selected to describe the aroma of wort from hot steeped malt, as shown by a percentage of ≥ 30% compared to the maximum total counts among all attributes (Supplemental 5, Table S9). For the attributes of dough, cracker, nutty, and earthy, differences among malts were significant according to Cochran's Q test (CI = 95%). Eight attributes were selected at a percentage ≤ 10%; the attribute spicy was not selected for any of the samples.

The aroma of Wintmalt was strongest for grainy and dough, whereas DH142010 was most dominant for breakfast cereal and sweet bread. In contrast, DH141969 only showed the highest counts among samples for pasta, and DH141515 for grassy, breakfast cereal, vegetal, and stale. DH150115 was most intense for bread, cracker, sweet aromatic, and nutty. Crisp Extra Pale exhibited the strongest aroma in the categories of stale, earthy, and woody.

Compared to aroma evaluation, there were slight deviations in the ranking of attributes and quantitative differences for flavor, taste, and mouthfeel (Supplemental 5, Table S10). However, the overall trends were similar for both data sets. For flavor, taste and mouthfeel, the attributes grainy, grassy, breakfast cereal, bread, sweet bread, sweet aromatic, sweet, cloying, and astringent were most frequently selected based on a percentage of ≥ 30% compared to the maximum total counts among all attributes. The only attributes that were significantly different were butter/diacetyl and bitter according to Cochran's Q test (CI = 95%). Thirteen attributes were only selected at ≤ 10%; the attribute spicy was not selected for any of the samples.

Wintmalt flavor was highest in the categories of grassy, sweet aromatic, cracker, and nutty, and had a cloying mouthfeel. DH142010 was highest for grainy, dough, and fruity and had the greatest frequency of umami taste. In addition to a dominant cloying mouthfeel, DH141969 was high in breakfast cereal and dough flavors. The flavor of DH141515 was ranked the highest for sweet bread, woody, and earthy, whereas DH150115 had the highest breakfast cereal, sweet aromatic, and cracker flavors, as well as a sweeter taste. The flavor of Crisp Extra Pale was highest in the categories vegetal, stale, and earthy and it had a more bitter taste.

To further evaluate the overall differences in aroma, flavor, taste, and mouthfeel between the six malts, an agglomerative hierarchical cluster analysis (AHC) was performed to separate samples into groups. AHC of the aroma data set separated malts into two significantly different groups. DH142010 and DH141515 formed one group and the remaining four malts the other group (Figure 2). Within the second group there were additional, but not significant separations. AHC for flavor, taste and mouthfeel also revealed two significantly different groups of malts: DH141515, DH141969, and DH150115 formed the first group, whereas the second group consisted of Crisp Extra Pale, Wintmalt, and DH142010. Subgroupings were below the significance threshold.

The results of a correspondence analysis (CA) are shown in Figure 3 for flavor, taste, and mouthfeel attributes, excluding attributes that were not significantly different among malts and that were not selected at a percentage of ≥ 30% (flavor) and ≥ 20% (taste and mouthfeel).

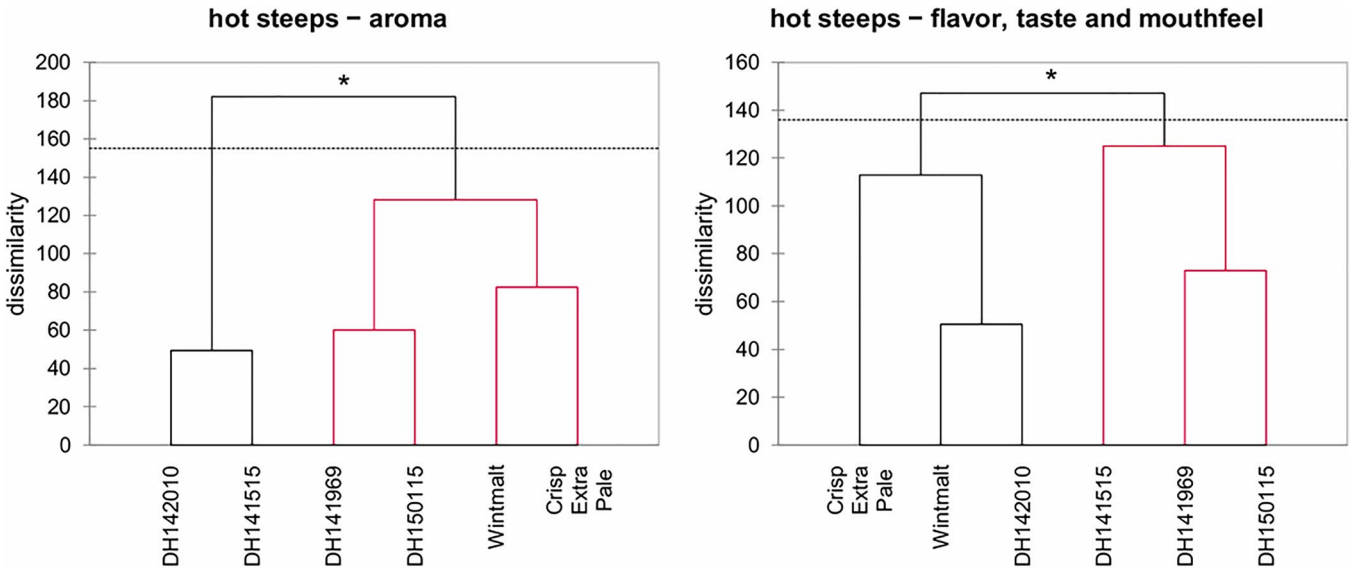


**Figure 2.** AHC analysis of hot steep sensory results for aroma, flavor, taste, and mouthfeel for four selected progeny derived from crosses with Maris Otter, Wintmalt and Crisp Extra Pale. Color figure available online.

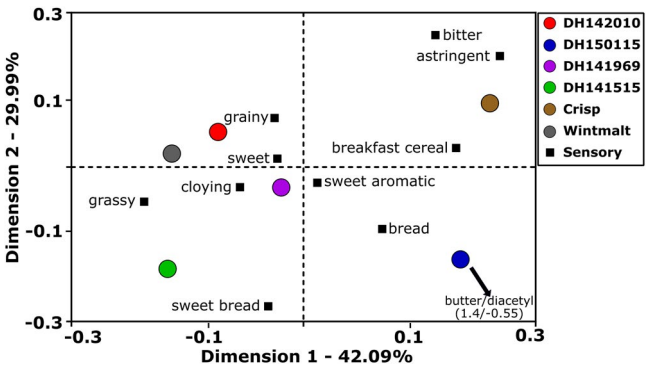


**Figure 3.** Correspondence analysis (CA) of hot steep CATA sensory results for flavor, taste, and mouthfeel of Wintmalt, Crisp Extra Pale, and the four selected progeny derived from crosses with Maris Otter. Excluded from the CA were attributes with no significant differences between malts and a percentage of <30% for flavor and <20% for taste and mouthfeel. Butter/diacetyl plotted far from the main groupings and its true position is noted. Color figure available online.

This two-dimensional plot accounts for 72% of the variation in the data set. As already identified by AHC analysis, Wintmalt and DH142010 were the most similar in flavor, taste, and mouthfeel for the attributes grainy, cloying, and sweet. Crisp Extra Pale was most different from all other malts with a breakfast cereal flavor, a bitter taste, and an astringent mouthfeel. DH150115 was characterized by stronger butter/diacetyl and bread flavors.

## Sensory evaluation of beers

### Difference from control

The DFC analysis showed no significant differences among samples for aroma. However, there was a significant difference between beers made from Wintmalt and DH141969 for flavor, taste, and mouthfeel according to a two-sided Dunnet's multiple comparison test.

### Check all that apply (CATA)

The Wintmalt beer had the most bitter taste and the most astringent mouthfeel; otherwise, it was weaker in most of the categories compared to the beers produced with the Maris Otter progeny. The DH142010 beer had the highest frequency of flavor in terms of sweet aromatic, dough, floral, stale, and fruity; it had the sweetest and saltiest taste; and it had the strongest cloying mouthfeel. In addition to a relatively strong bitterness in taste, the beer produced from DH141969 exhibited a dominant grainy, vegetal, grassy, cracker, rotten, earthy, and burnt flavor compared to the other beers. The flavor of beer made from DH141515 was ranked the highest for sweet bread, nutty, and fruity. The DH150115 beer had the highest frequency for bread, butter/diacetyl, and breakfast cereal; a saltier taste; and a more astringent mouthfeel (Supplement 5, Tables S11 and S12).

The results of the AHC analysis for overall differences in flavor, taste, and mouthfeel are shown in Figure 4. There were three significantly different groups of beers. The first group consisted solely of DH141969, which was also the most different in the CATA analysis. DH141515 and DH150115 formed a subgroup significantly different from the Wintmalt and the DH142010 subgroup.

The results of the CA analysis of flavor, taste, and mouthfeel - excluding attributes that were selected at a percentage of < 30% (flavor) and < 20% (taste and mouthfeel) of the maximum counts, respectively – are shown in Figure 5.

This two-dimensional plot accounts for 84% of the variation. As found with AHC analysis, DH141515 and DH150115 were the most similar in flavor, taste, and mouthfeel; attributes included bread, butter/diacetyl, and sweet bread. DH141969 was the most different, with grassy, vegetal, and grainy flavors. DH142010 was best described by the attributes floral, sweet, sweet aromatic, and dough. The beer made from Wintmalt was the most bitter but otherwise weakest in all flavor categories.

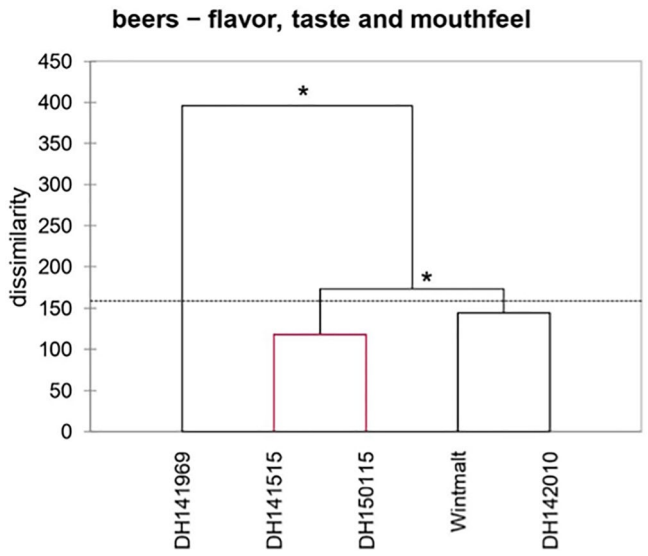


**Figure 4.** AHC analysis of beer CATA sensory results for flavor, taste, and mouthfeel of beers made from four selected progeny derived from crosses with Maris Otter, compared to Wintmalt. Color figure available online.

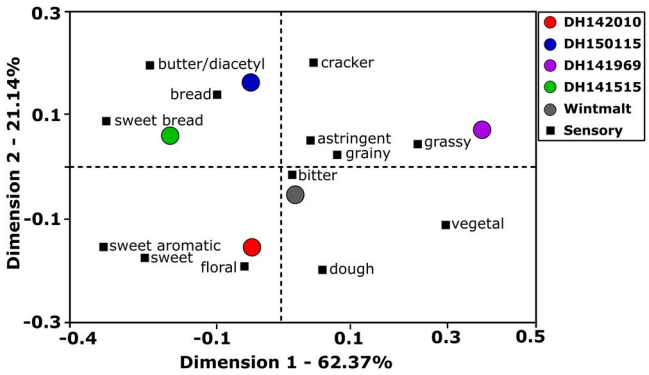


**Figure 5.** Correspondence Analysis (CA) of CATA sensory results for flavor, taste, and mouthfeel of beers made from Wintmalt and the four selected progeny derived from crosses with Maris Otter. Attributes with a percentage <30% for flavor and <20% for taste and mouthfeel of the maximum counts were excluded. Color figure available online.

## Metabolomics

### Malt hot steeps

While attributes describing taste and mouthfeel such as sweet, bitter, or cloying may be driven by non-volatile hot steep chemistry, the aroma attributes are driven by volatile metabolites such as aldehydes, ketones, and fatty acid esters.[14,15] The MS detection and subsequent chemoinformatics identified 143 compounds, which were annotated using spectral matching and classified according to ontological information from public chemical databases (Supplemental 6, Table S13).

Analysis of variance (ANOVA) on the 143 compounds identified 24 volatile metabolites (17%) that differed significantly (FDR adjusted $p < 0.05$) among Wintmalt, the four selections, and Crisp Extra Pale (Table S13, supplementary material). PCA conducted on the 143 volatile compounds resulted in three principal components, which explained 97% of the variation. PC1 (54% of the variation) and PC2 (19%) separated DH150115 and DH141515 from DH142010, DH141969, Wintmalt, and Crisp Extra Pale (Figure 6(a)). PC3 (17%) further separated Wintmalt and Crisp Extra Pale from DH142010 and DH141969 (Figure 6(b)). The loadings plot for PC1 and PC2 was not indicative of any trend associated with metabolite class (Figure 6(c)).

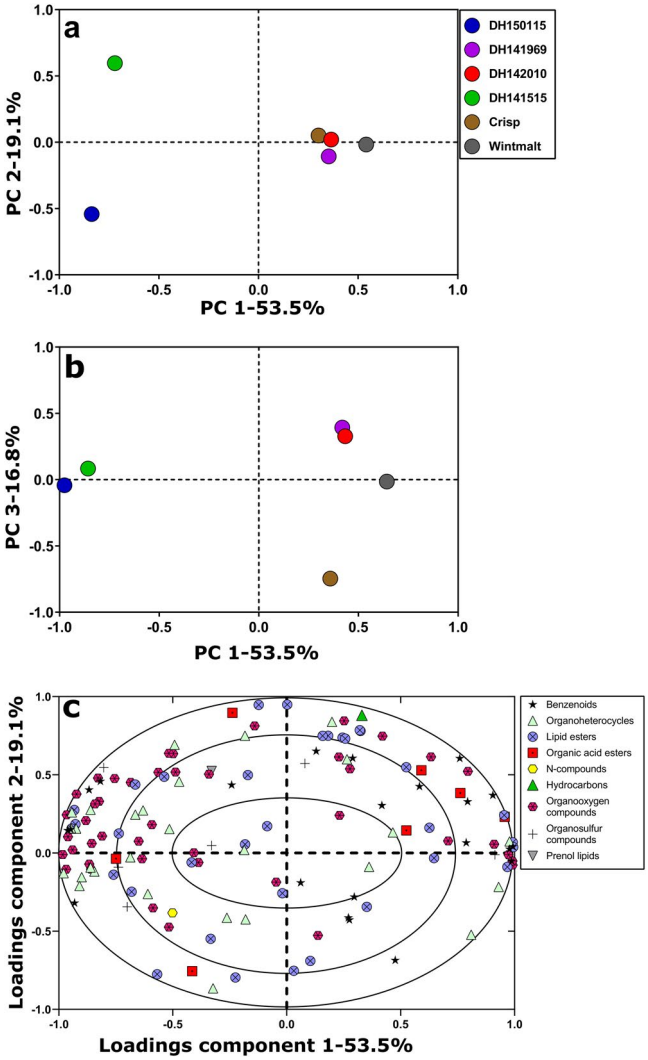


**Figure 6.** Principal Component Analysis (PCA) scores plot of hot steep metabolomics results for Wintmalt, Crisp Extra Pale, and the four selected progenies derived from crosses with Maris Otter. (a) PCA 1 and 2 scores plot (colored circles), (c) PCA 1 and 3 scores plot (colored circles) (c) PCA loadings for PC1 and 2 (symbols represent 9 chemical classes). Color figure available online.

*Trends among chemical classes.* Data on the 143 identified volatiles were used to search for trends in four metabolite classes: lipid esters (to include fatty acid ester formation); organoheterocyclic compounds, which include Maillard reaction products (MRPs); organic acid esters, which are known to be formed during malting and brewing; aldehydes and ketones (organooxygen compounds).[7,15,57]

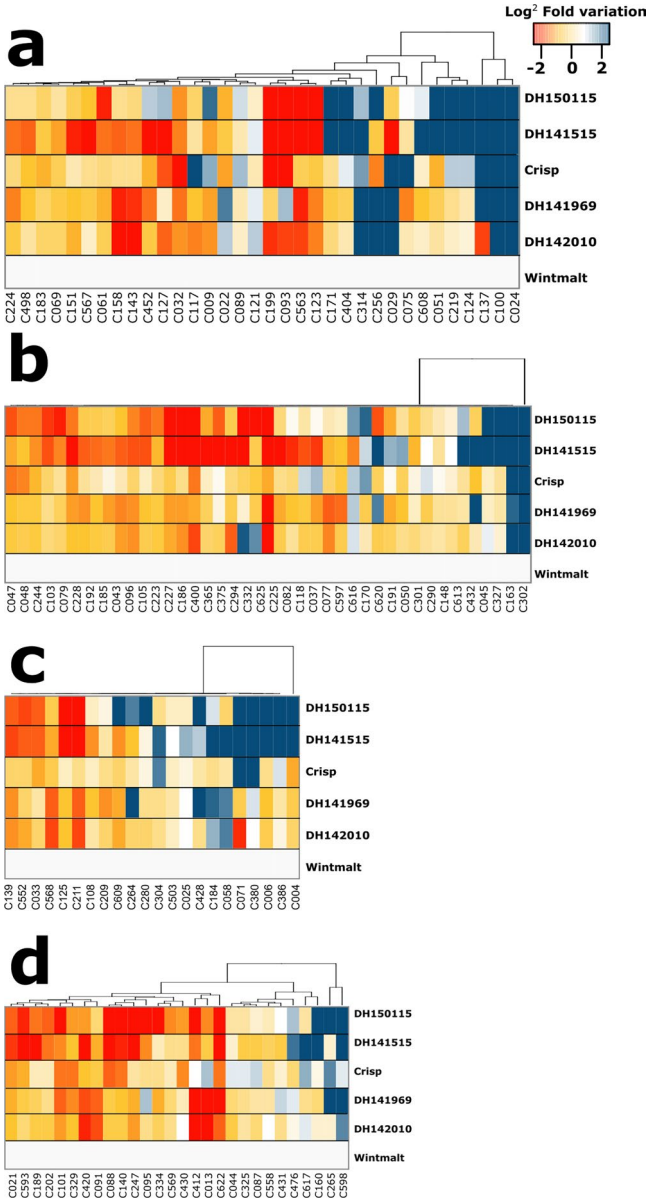


**Figure 7.** Fold variation of volatile metabolites among the six hot steeps within chemical classes. Metabolite abundances were calculated as a fold variation (FV) within and among chemical classes. Subsets of the heatmaps were recreated for (a) lipid esters, (b) organooxygen compounds, (c) benzenoid compounds, and (d) MRPs and organosulfur compounds. The subset heatmaps are colored to indicate metabolites that were lower in abundance compared to Wintmalt, the check (red), more abundant in comparison to Wintmalt, (blue), or metabolites that did not vary (white). Color figure available online.

As shown in Figure 7 – where low/negative FV values are shown in red and blue indicates high/positive FV values – in most cases mean metabolite abundances did not vary among chemical classes. For the entire sample set, there was an FV of 2.5 for all metabolites.

### *Volatile metabolite contributions*

***Lipid esters*** Four fatty acid esters contributed to the DH141515 hot steep profile: methyl (±)-2-methylbutanoate, ethyl decanoate, octadecanoic acid, and 3-methyl-2-butenoic acid. All of these are associated with floral, fruity, and vegetal attributes.[58] The lipids that contributed to the DH150115 profile were two fatty alcohols, (Z)-2-hexen-1-ol and 1-hexanol; and one fatty acid ester, 2-butenoic acid methyl ester. The lipid esters that were present in these two selections were more abundant (indicated by higher fold change) than in Wintmalt. Four fatty alcohols were important for DH141969 and DH142010: (Z)-3-octen-1-ol (fatty, fresh, fruity, and melon), 2-ethyl-1-hexanol (citrus, floral, fresh, green, rose, and sweet), 3-decanol (floral, musty, mushroom, and orange), and decyl propionate (cognac, ether, fruity, and rum).[44] The lipid contribution for Crisp Extra Pale was significantly lower than that for Wintmalt (Figure 7(a)).

*Organooxygen compounds* The alcohols (1), aldehydes (3), and ketones (3) that were more abundant in DH150115 and DH141515 included: 2-buten-1-ol, hexanal, (E)-2-butenal, 5-hydroxymethyl-2-furancarboxaldehyde, methyl acetate, 4-methoxyphenyl)-2-butanone, 3-penten-2-one, 3,4-dimethyl-4-hexen-2-one. These compounds may have been responsible for contributing to the grassy, vegetal, and bready aromas of these samples. DH142010, DH141969, Wintmalt, and Crisp Extra Pale aligned with two ketones: 3-methyl-2-cyclohexen-1-one (caramel, cherry, nutty, and phenolic) and 5-methyl-3-hexen-2-one (berry, cheese, and sweet)[44] (Figure 7(b)).

*Benzenoids* Four benzenoid compounds were much more abundant in DH150115 and DH141515 than in the other hot steeps. These included 3-phenyl-4-pentenal, 4-hydroxy-3,5-dimethoxybenzaldehyde, benzoic acid ester, and 2-phenylethanol. Benzenoids typically contribute bitter taste and floral aroma to beer.[7] DH142010, DH141969, Wintmalt, and Crisp Extra Pale were generally lower in abundance in most benzenoids except 2,4-di-tert-butylphenol, dibenzyl ether, 1-phenylethanol, and hordenine. The prevalence of hordenine in these varieties likely did not contribute to aroma or flavor[8] (Figure 7(c)).

*MRPs and organosulfur compounds* There were many more organoheterocycles/MRPs[18] in higher abundance in DH150115 and DH141515 than in the other hot steeps. These included pyrans, pyrazines, furans, pyrrolidones, oxazoles, and furanones. These compounds are products of the Maillard reaction and contribute a wide range of aromas and flavors, including savory, umami, roasted, and toasted.[44,59] The MRPs with higher abundances in DH142010, DH141969, Wintmalt, and Crisp Extra Pale hot steeps were 1,2-benzisothiazol-3(2H)-one and benzothiazole (contributing more sulfurous aroma/flavors).[60] DH150115 and DH141515 generally had higher abundances of four of the six organosulfur compounds (Figure 7(d)).

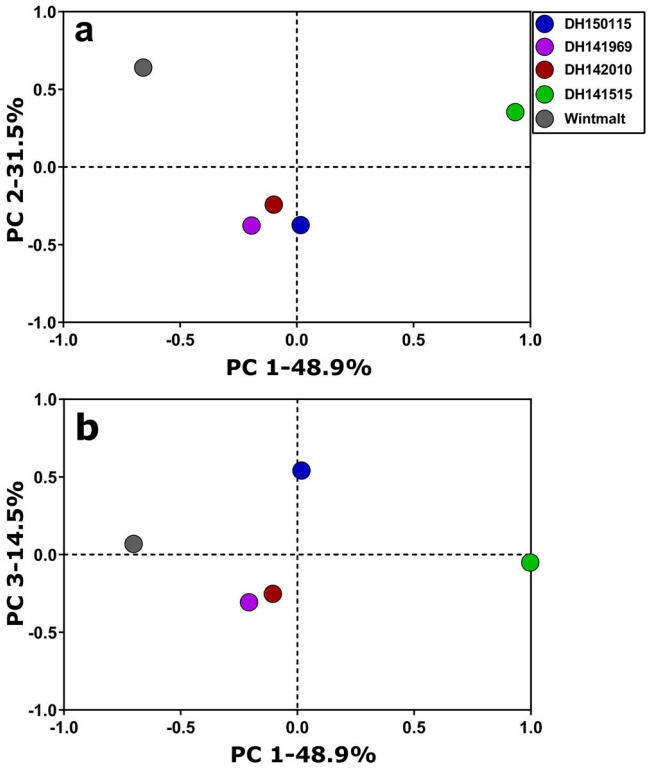


**Figure 8.** Principal Component Analysis (PCA) scores plot of beer metabolomics results for Wintmalt and the four selected progenies derived from crosses with Maris Otter. (a) PCA 1 and 2 scores plot (colored circles), (b) PCA 1 and 3 scores plot (colored circles). Color figure available online.

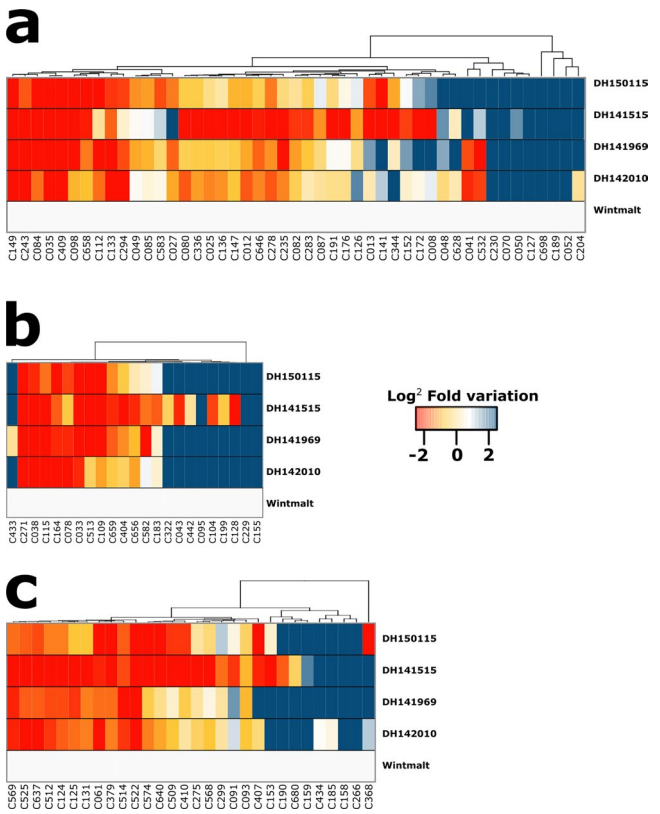


**Figure 9.** Fold variation of volatile metabolites among the five beers within chemical classes. Metabolite abundances were calculated as a fold variation (FV) within and among chemical classes. Subsets of the heatmaps were recreated for (a) lipid esters (b) benzenoid compounds, and (c) MRPs. The subset heatmaps are colored to indicate metabolites which were lower in abundance compared to Wintmalt, the check (red), more abundant in comparison to Wintmalt, (blue), or metabolites that did not vary (white). Color figure available online.

## *Beers*

One hundred seventy-one volatile metabolites were identified via MS analysis and annotated using spectral matching and classified according to ontological information from public chemical databases (Supplemental 6, Table S14). Analysis of variance (ANOVA) of the 171 compounds identified 12 volatile metabolites (14%) that varied significantly (FDR adjusted $p < 0.05$) among the five beers (Table S14). A PCA of the 171 compounds resulted in three principal components which explained 95% of the variation. PC1 (49% of the variation) and PC2 (32%) separated DH150115 and DH141515 from DH142010, DH141969, and Wintmalt (Figure 8(a)). PC3 (15%) further separated Wintmalt and DH141515 from DH142010, DH141969, and DH150115 (Figure 8(b)).

*Trends among chemical classes (beer).* These data were assessed similarly to hot steeps in order to determine if trends of metabolite classes could distinguish the profiles of the beers, specifically, for benzenoid compounds, lipid esters, organoheterocycles/MRPs, organic acid esters, organooxygen compounds, phenylpropanoids, and prenol lipids[61] (Figure 9).

*Volatile metabolite contributions (beer).*

*Lipid esters* DH141515 was the most divergent of the four beers. There were 20 fatty acid esters and fatty alcohols (which are typically high-molecular-weight, straight-chain primary alcohol esters)[62] that were more abundant in this beer. These included nonyl octanoate, ethyl oleate, geranyl formate, ethyl 9-hexadecenoate, 3-methylbutyl decanoate, and others. These fatty acid esters and fatty alcohol esters are associated with a wide-range of beer aroma and flavor properties.[62] The lipids that contributed most to DH150115 were four fatty alcohol esters and fatty acid esters. These included ethyl tetradecanoate, 1-dodecanol, diethyl fumarate, and 3-methylbutyl octanoate. Four fatty acid esters of importance that contributed to DH141969 and DH142010 were ethyl 2-ethylhexanoate and 3-methylbutyl nonanoate (cumin, fresh, fruity, herbal, orris, apricot, fruity, and floral).[44] However, ethyl 9-decenoate and 2-methylbutanoic acid were also abundant in these two selections and their attributes include more pungent notes (acid, cheesy, soapy). These four selections were different from Wintmalt, which was most abundant in seven fatty acid esters/fatty alcohol esters such as citronellyl propionate, methyl sorbate, ethyl octanoate, and ethyl hexanoate (Figure 9(a)).

*Benzenoids* Ten benzenoid compounds were more abundant in DH141969, DH150115, and DH142010,

compared to Wintmalt and DH141515. Notably, these included 2,6-di-tert-butyl-4-methylphenol (camphor, mild, musty, phenolic, and vanilla) (Figure 9(b)).

*MRPs* There were many more organoheterocycles/MRPs[19] associated with DH141515, which likely came from the malting process rather than as a result of brewing.[63] These included pyrans, pyrazines, furans, pyrrolidones, oxazoles, and furanones, as was also observed in the hot steeps. These compounds were less abundant in the other four beers (Figure 9(c)).

## Discussion

### Barley germplasm, agronomics and malt quality

This study utilized four experimental lines with a shared heirloom male parent (Maris Otter). The other two female parents were Violetta and 04-028-036. As shown by the haplotyping analysis, the progeny received between 32% and 60% of the heirloom genome. This is within expectations for F1-derived doubled haploid progeny of a single cross (a 50% overall contribution of total genome from each parent). The differential introgression of complete or portions of chromosomes from specific parents is of particular interest, as it may provide insights into regions of the genome that could be involved in contributions to specific traits, including agronomic performance, malting quality, and beer flavor. The availability of a reference barley genome sequence, and extensive resources documenting genes and quantitative trait loci (QTLs) associated with these phenotypes facilitate this ongoing analysis.[64–66] However, deeper genetic analysis was not a part of this study.

Phenotypic selection was successful: the experimental lines showed improved agronomic performance relative to the heirloom parent and the Wintmalt check when grown in the Willamette Valley of Oregon. All genotypes, including the control, were considered acceptable for all-malt brewing other than the few exceptions noted in the results. Maris Otter is reputed for its quality in single-infusion mashed ales and is associated with lower protein values and subsequent proteolytic modification and it was surprising that the noted high FAN values were comparable to the Crisp Maris Otter Extra Pale reference sample. However, given the introgression of the contemporary parents and the cultural agronomic practices of western North America, it is not unexpected to see higher protein and modification in the experimental lines.[67]

### Hot steep sensory

The malt hot steep is a rapid, repeatable and reproducible preparation of an extract for sensory evaluation of base and specialty malts,[68] but has only recently been utilized in the evaluation of the contribution of barley to beer flavor. Evaluating hot steeps may provide insight into nuanced differences in flavor between lines that may be masked by other steps and raw materials in the brewing process. This research continued the use of this tool to identify flavors and flavor precursors that may be unique to the heirloom parent. There were significant differences among the control, three of the selections (except DH141969), and the commercial sample. However, the CATA panel did not identify many significant descriptors between the genotypes. Only two attributes above the 30% selection threshold – dough and cracker – varied significantly in the aroma assessment and no attributes above the 30% selection threshold varied significantly in the flavor assessment. The attributes used in this study that were most associated with commercial descriptors for Maris Otter malts are sweet bread, bread, cloying, sweet, and sweet aromatic.[19] The most frequent negative characteristic was vegetal, but it did not meet the 30% response threshold nor did it vary significantly across genotypes. DH141969 grouped closely with cloying, sweet, and sweet aromatic. Crisp Extra Pale was unique in the correspondence analysis and grouped with astringent and breakfast cereal. Hierarchical clustering showed the control, experimental lines, and commercial reference forming two distinct groups in the aroma and flavor assessments. For aroma, DH142010 and DH141515 grouped together and the remaining four were in another cluster. In the flavor assessment, DH142010 and DH141515 were separated and moved to new clusters with Crisp Extra Pale/Wintmalt and DH141969/DH150115, respectively. It is notable that DH141515 is the only DH line to not group with either Wintmalt or Crisp Extra Pale for either aroma or flavor, indicating that this selection has unique attributes that were not shared by the control or the commercial Maris Otter malt.

### Beer sensory

The efforts taken to produce an acceptable light ale were successful, and the research beers did not result in strong perceived differences for hop or fermentation associated attributes. Fruity and spicy, common hop and fermentation descriptors, did not meet the 30% response threshold, however, floral did. Despite the muted contributions from other ingredients, there were only nuanced differences for malt flavors. Given the consistency of malt modification and the style of beer produced, the expectation was that more genotype-driven sensory differences would be identified[10] reported differences - albeit nuanced differences - between beers made from Wintmalt as compared to beers made from other winter varieties. The lack of close genetic relationship between Maris Otter progeny and Wintmalt was further grounds for expecting differences in flavor. However, Violetta and the Ackermann selection are closely related to Wintmalt and therefore the regions of the genome they contributed may account for limited differences in beer flavor between the four selections and the Wintmalt control.

None of the selections differed significantly from the control in the aroma assessment and only one line, DH141969, differed from the Wintmalt in the flavor assessment. This selection contributed dominant grainy, vegetal,

grassy, and cracker flavors. Vegetal-like descriptors can be perceived as negative in light beers and are often attributed to DMS and other sulfur containing compounds.[69] However, studies on the contributions of barley genotype to beer DMS have shown mixed results and attribute most variation to environment and malting process.[11,70,71] In this research, the effects of environment and malting are not confounding factors. Three attributes varied among beers for each aroma (vegetal, grassy, and sweet bread) and flavor (vegetal, sweet bread, and sweet aromatic). The lines DH141515, DH150115, and DH142010 came closest to the popular perceptions of commercial Maris Otter, as the first two clustered close to bread and sweet bread in the correspondence analysis while DH142010 plotted close to sweet.

In terms of malt and beer analysis impacts on beer flavor, three of the four selected lines, as well as the commercial Crisp Extra Pale, had higher FAN levels than the all-malt specification. DH141969 and Wintmalt had FAN values within the specifications for all-malt brewing, but also had other potential flavor issues. High FAN can impact beer flavor as a component of higher alcohol synthesis and Strecker aldehyde formation.[72] Higher alcohols are often perceived as alcoholic, solvent-like, fruity, and/or vinous.[73] In contrast, Strecker aldehydes exhibit grainy, varnish-like, almond, floral, and/or cooked potato aromas.[74] Of the attributes used in the CATA evaluation of beers that could be associated with higher alcohols or Strecker aldehydes, grainy, floral, fruity, and nutty were identified. Grainy was the most selected attribute for all beers in both aroma and flavor but was not significantly different between the beers, including those brewed from Wintmalt and DH141969, and is not unexpected in beers brewed only with pale malt. Of the other attributes, only floral met the 30% threshold for response on either assessment and only nutty was found to be significant between the beers in the aroma assessment.

### *Metabolomics*

Chemical profiling paralleled, and in some cases, provided additional insights into the sensory evaluation outcomes. For example, the PCA of hot steeps separated DH150115 and DH141515 from their siblings and the controls. These two DH lines had higher contributions of lipids and fatty acid esters. The volatile metabolites identified within these classes do not, however, contribute to a dough or cracker-like aroma, according to published sensory literature. Separation defined by lipids was notable, as Maris Otter was reported to have a lower lipid fraction relative to its contemporary varieties.[75] This may explain the results for Crisp Extra Pale in this study, as shown in Figure 7a. The analysis of beer revealed that DH141515 separated from the other three DH lines and the control, but DH150115 did not. Notably, DH141515 had the highest abundance of MRPs, which may explain the association with bread and sweet bread in sensory evaluation. Metabolomics also identified variation not found in sensory of hot steeps and beers. Examples include the abundance of benzenoids in DH141969, DH150115, and DH142010. Metabolites not aligning with separation in sensory evaluation may be of interest for future research into brewing practices that may be able to utilize certain compounds to produce unique flavors.

## Conclusion

This research continued the exploration of genetic contributions of barley to beer flavor, building on existing work from this research group. While the four Maris Otter-derived progeny showed strong agronomic outcomes and contemporary malt quality profiles, their flavor and chemical profiles in malt hot steeps and beers were not particularly unique. Despite Maris Otter contributing approximately half of the genome in each of the experimental lines, flavors attributed to the heirloom parent were not observed. The segmental contributions of Maris Otter in specific genomic regions in the progeny could potentially be used to rule out regions where the variety contributes genes that lead to positive beer flavor outcomes. In this way, the search for flavor genes could be focused in the remaining regions (i.e., those tracing to Violetta or the Ackermann selection). The commercial Maris Otter malt used for hot steep sensory and chemical profiling did not show attributes or metabolites associated with the market appeal of the variety. However, the results of this study concur with those of Windes et al.[10] in that hot step sensory may identify malt aromas and flavors that do not readily carry on to finished beer. Therefore, a definitive "dissection" of Maris Otter flavor genes will require beer brewed from the heirloom parent grown and malted at the same location as the progeny. Until specific metabolic compounds and genetic markers for flavor are identified, breeders will continue to select for superior agronomic performance and malting quality profiles. If accelerated brewing and sensory trials can be added to this research pipeline, there may be serendipitous progress in flavor.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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