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Timing of Cluster Light Environment Manipulation during Grape Development Affects C_{13} Norisoprenoid and Carotenoid Concentrations in Riesling

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Sunlight exposure of winegrape clusters is frequently reported to increase C_{13}-norisoprenoids in resulting wines, but the timing and mechanism of this influence is not well understood. Fruit zone leaf removal was applied to *Vitis vinifera* cv. Riesling at three timings: 2, 33 and 68 days past berry set (PBS), and compared to an untreated control. Free and total 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), vitispirane and β-damascenone were measured in juice and wines, and carotenoid profiles were determined in grapes at midseason and maturity. Significantly higher total TDN was observed in grapes from the 33-day PBS treatment compared to the control and other treatments (195 μg/L vs 54−87 μg/L). Total vitispirane in juice was also significantly increased in the 33-day PBS treatment, while total β-damascenone was reduced in the 68-day PBS treatment compared to the control. Existing HPLC protocols were modified to allow for quantification of zeaxanthin in *V. vinifera* berries, and zeaxanthin was determined to be significantly higher in the 33-day PBS treatment than the control or other treatments ($p < 0.05$). Total TDN in juice correlated with free TDN in wine, with 11.0% ± 2.5% of total juice TDN converted to free TDN in wine. In contrast, total vitispirane increased significantly during fermentation, and was not correlated with vitispirane in juice. In summary, leaf removal at 33 days PBS significantly increased zeaxanthin in Riesling grapes midseason, total TDN and vitispirane in the juice of mature Riesling grapes, and free and total TDN in finished wine, while earlier or later leaf removal had no effect.

KEYWORDS: Vitispirane; damascenone; leaf removal; TDN; *Vitis vinifera*; lutein; zeaxanthin; xanthophyll cycle; winegrape

INTRODUCTION

The C_{13}-norisoprenoids are one of several classes of grape-derived odor-active compounds associated with wine aroma quality (*1*). While trace levels of free C_{13}-norisoprenoids are detectable in juice, the majority of C_{13}-norisoprenoids in wine appear to derive from precursors, including nonvolatile C_{13}-norisoprenoid glycosides derived from carotenoid cleavage (*2*), and can be released during winemaking or storage by enzymatic and non-enzymatic mechanisms (*3*). The best studied C_{13}-norisoprenoid in wine and grapes is arguably TDN (l,l,6-trimethyl-1,2-dihydronaphthalene), which is associated with "kerosene" or "petrol" aromas and has an orthonasal sensory threshold of 20 μg/L in wine (*4*). TDN has been detected in several varietal wines, but its presence is most closely associated with the aroma of bottle-aged Riesling (*5*). While TDN concentrations around sensory threshold are generally acceptable to consumers, excessive levels are considered undesirable, especially in young Riesling (*5*).

Free TDN in Riesling juice is generally below detection threshold, but TDN concentrations in excess of 200 μg/L in Riesling wine are reported to occur following prolonged storage (*4*, *6*). TDN precursors, e.g. C_{13}-norisoprenoid glycosides, have been reported in grapes, and the concentration of TDN in a finished wine is proportional to the concentration of acid-releasable TDN precursors in must (*1*, *5*). Warmer growing conditions and greater cluster exposure to sunlight are associated with higher TDN concentrations in finished wines, due to a larger concentration of precursors in the juice (*5*). Conversely, lower TDN concentrations in wine are associated with shaded fruit, either through direct means like canopy management (*7*) and indirectly through increased vine fertilization (*8*) or irrigation (*9*) resulting in increased vine canopy. A similar decrease in the concentration of several other volatile C_{13}-norisoprenoid precursors has been observed in shaded clusters, including vitispirane and the actinidols (*10*). One possible exception to this trend is β-damascenone, which has been implicated in enhancing fruity aromas in wines. Some authors have reported an increase in β-damascenone in response to cluster shading (*5*, *7*), while others have reported either no change or a decrease in shaded grapes (*11*, *12*).

Because of the clear link between TDN precursor production and cluster light exposure, and assuming lower TDN concentrations were desirable, a superficially obvious solution to reducing

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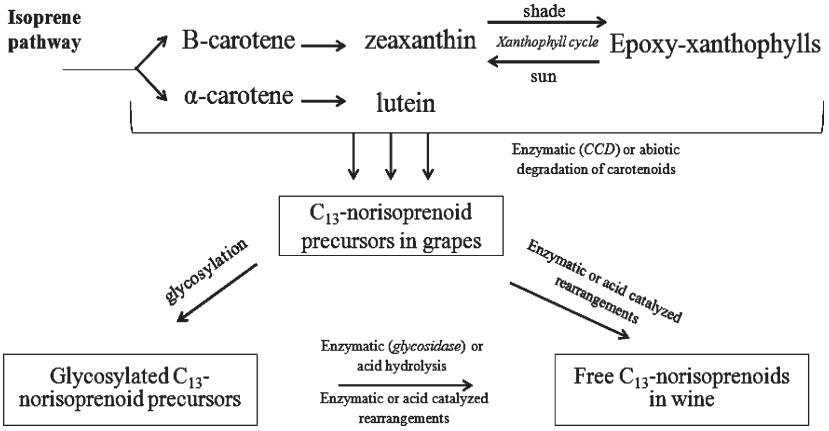

Figure 1. (Top) Simplified carotenoid biosynthetic pathway in flowering plants (*23*). (Bottom) General mechanism by which norisoprenoid aroma compounds are formed from carotenoids in grapes and wine (*15*).

the TDN potential of Riesling or other winegrapes would be to avoid cluster exposure. However, increasing berry sun exposure is often desirable for reducing disease pressure (*13*), decreasing titratable acidity, and potentially for increasing production of other desirable compounds like monoterpenes (*14*). Therefore, it is advantageous to identify canopy management practices that will produce desirable outcomes independently of C_{13}-norisoprenoid concentrations, especially TDN. A better understanding of the key period(s) during the growing season in which cluster sun exposure increases C_{13}-norisoprenoid precursors could assist winegrape growers in making appropriate canopy management decisions for targeting specific wine flavor profiles.

The (bio)chemical mechanisms underlying C_{13}-norisoprenoid precursor formation in grapes have been subject to considerable study (*1*, *15*). TDN and other C_{13}-norisoprenoids show structural similarities to carotenoids, and there is strong evidence that C_{13}-norisoprenoid precursors in mature grapes are derived via oxidative degradation of carotenoids (*16*). The major carotenoids in grapes, β-carotene and lutein, begin to decrease at or just prior to veraison (*17*). C_{13}-Norisoprenoid precursor formation commences within 1−2 weeks after veraison and may reach a maximum within 30 days postveraison, although some studies report a late spike in concentration near maturity (*5*, *16*). Grape C_{13}-norisoprenoids were originally proposed to be formed by abiotic carotenoid degradation, e.g., TDN can be formed from lutein under acidic conditions (*18*). Alternatively, a family of carotenoid cleavage dioxygenase (CCD) enzymes has been implicated in production of plant apocarotenoids, e.g. C_{13}-norisoprenoids (*19*), and a CCD capable of producing C_{13}-norisoprenoids from lutein and zeaxanthin (*VvCCD1*) was recently cloned from grapes (*2*). Expression of *VvCCD1* increases at veraison, although a 1−2 week lag is reported to occur between increased transcript expression and a significant increase in glycosylated C_{13}-norisoprenoids. Following enzymatic or nonenzymatic biogenesis, part of the pool of C_{13}-norisoprenoids is proposed to undergo *in vivo* glycosylation, potentially after further transformations (e.g., hydration, oxidation) within the grape berry (*15*, *20*) (**Figure 1**). Grape-derived C_{13}-norisoprenoid glycosides can be hydrolyzed during fermentation and storage, and both native and glycoside-derived C_{13}-norisoprenoid aglycones can be further transformed enzymatically or nonenzymatically to odor active forms, e.g. TDN and β-damascenone (*21*, *22*).

Carotenoids are expressed in photosynthetically active tissues of plants as part of photosystem II (PSII). The major carotenoid species in grapes (β-carotene, lutein) act as light harvesting antennae pigments, while other oxygenated carotenoid species (e.g., neoxanthin, zeaxanthin) participate in photoprotection of the plant via the xanthophyll cycle (*23*). Total carotenoid concentrations are believed to be primarily developmentally regulated (*23*), but environmental factors such as cluster light exposure also influence concentrations (*24*−*26*). Since preveraison berries are photosynthetically active, higher concentrations of carotenoids, and thus higher substrate availability, are one potential explanation for higher concentrations of C_{13}-norisoprenoid precursors in sun-exposed grapes (*24*). However, cluster exposure does not consistently yield higher concentrations of carotenoids preveraison (*27*). A second explanation is that postveraison cluster exposure may accelerate carotenoid degradation, possibly by increasing *VvCCD1* expression (*28*), although the effect of sun exposure on increasing carotenoid degradation rates has also been disputed (*9*). A third potential explanation is that sun exposure results in conversion of epoxyxanthophylls (e.g., violaxanthin) to de-epoxidized xanthophylls (e.g., zeaxanthin). Since the putative starting point for the precursors of TDN, vitispirane, and related compounds may be de-epoxidized xanthophylls (*15*), sun exposure may alter the proportion of de-epoxidized vs epoxidixed forms of xanthophylls, and these different substrates could yield different C_{13}-norisoprenoid precursors postveraison (*16*). Berries exposed to sun preveraison are reported to have a higher proportion of de-epoxidized xanthophylls (*26*) than shaded berries, but a clear correlation between a specific carotenoid or carotenoids in preveraison grapes and eventual concentrations of TDN or other C_{13}-norisoprenoids in mature fruit has not been conclusively demonstrated.

In summary, increased cluster exposure may increase concentrations of TDN precursors and related compounds through one or more mechanisms, including greater accumulation of carotenoids, faster carotenoid degradation, or increased availability of specific carotenoid substrates. This lack of understanding of the relationship between C_{13}-norisoprenoids and light is inadequate for designing viticultural management strategies to avoid TDN precursor production while ensuring an open canopy to reduce disease and improve fruit composition. Although many reports have studied the relation of TDN precursor concentrations to cluster light exposure, none have considered altering the timing of the cluster exposure treatment. Our current study aimed to elucidate these relationships by observing the effects of cluster exposure timing on carotenoid profiles and eventual C_{13}-norisoprenoid concentrations.

MATERIALS AND METHODS

Chemicals. Astaxanthin was obtained from ChromaDex (Irvine, CA). Zeaxanthin, α-carotene, β-carotene, β-damascenone, and 2-octanol were

obtained from Sigma-Aldrich (St. Louis, MO). Lutein was provided as a gift from the Institute for Genomic Diversity, Cornell University. All carotenoids were ≥95% purity, and the other standards were >97% purity. NaCl and butylhydroxytoluene (BHT) were reagent grade (Fisher-Scientific, Pittsburgh, PA). Methanol, ethanol, dichloromethane, tetrahydrofuran, and petroleum were HPLC grade (Fisher-Scientific). TDN was synthesized from α-ionone (Sigma-Aldrich, 99%) via ionene using the protocol of Miginiac (*29*), and the purity of the TDN standard was estimated to be >99% by NMR.

Vineyard. The field experiment was conducted during the 2008 growing season with established Riesling vines (clone 90) within a commercial vineyard. Vines had been planted on 3309 rootstock and were located on the west side of Seneca Lake in the Finger Lakes region of New York State (latitude 42.54° N, longitude 76.87° W). Vines were trained to a cane-pruned Scott−Henry system with 2 m spacing between vines and 3 m spacing between rows, with rows oriented north−south on a western facing slope. Other than the treatments described below, the test panels were managed by the commercial cooperator in the same manner as the rest of the vineyard, according to typical practices for the region.

An experimental unit consisted of an interior vineyard panel of four contiguous vines between trellis posts. A randomized complete block design was employed with four panel replicates per treatment. Test panels were inspected prior to bud-break and chosen for consistency. One 2-day PBS (past berry set) experimental unit was removed from the study early in the season after exhibiting chlorosis and a loss of vigor. This removal resulted in each treatment consisting of four panels with a total of 16 vines except for the 2-day PBS which consisted of 3 panels with a total of 12 vines for a total of 60 vines in the study. To achieve consistency among vines in the study, shoots were thinned at 20 days before berry set to 17.5 shoots/meter, which was the lowest density found before thinning, when shoot density had varied from 17.5 to 21.5 shoots/meter.

Treatments and Canopy Assessment. Three leaf removal treatments (75% of leaves in the fruiting zone removed by hand) were applied at 2 days (June 24), 33 days (July 25), and 68 days PBS (August 30), with a control where no leaf removal was administered. Berry set was defined as when swelling had initiated and flower senescence (nearly 100%) was obvious from visual inspection. The 68-day PBS treatment was applied at approximately 3 days postveraison, with veraison defined as the point when at least 50% of the berries had softened. Leaf removal was conducted by hand in a manner similar to common vineyard production practices. Following leaf removal treatment, vines were allowed to refoliate, i.e. vines were not maintained at 75% leaf removal following treatment. The day following leaf removal, the canopy density was quantified for all panels using enhanced point quadrat analysis (EPQA) and analyzed using EPQA-CEM Toolkit version 1.6 (*30*). EPQA was also administered at approximately 30 day intervals following leaf removal, which coincided with the day following the next leaf removal treatment, for a total of 3 EPQA sampling points. EPQA was utilized to describe canopy architecture and quantify cluster exposure, with canopy data collected at 20 cm intervals. No attempt was made in this study to separate the effects of temperature and cluster exposure, so it should be assumed that increased exposure coincided with increased cluster temperature (*7*, *31*). The calculated EPQA metric cluster exposure layer (CEL) was used. A lower value of CEL indicates greater cluster light exposure.

Sampling and Harvest. Carotenoid analysis was conducted on whole berry samples taken at two different periods, 52 days after berry set (midseason) and at harvest. The midseason sampling was prior to the final 68-day PBS treatment. Samples for C_{13}-norisoprenoid precursors were taken at harvest, with juice used for analysis. Berries were sampled from all experimental units for carotenoid analysis at 56 days after berry set (August 17) which was 23 days after the 33-day PBS treatment, and at harvest (October 8), with 100 randomly collected berries collected from each experimental unit in duplicate. The first carotenoid samples were taken before the final leaf removal treatment. The harvest date of October 8 was dictated by the vineyard manager. Each vine was hand-harvested separately, with the number of clusters per vine and yield per vine determined. Yield and pruning weight (see below) were measured on a per vine basis using a hanging scale. Samples for analysis were frozen and held at −40 °C until pressing, while the rest of the harvested fruit was pressed, vinified and bottled.

Duplicate 200 mL juice samples from each experimental unit were frozen at −40 °C until C_{13}-norisoprenoid analysis was conducted. Pruning was conducted on February 11, 2009, leaving four canes of approximately 15 nodes each (approximately 60 buds per vine). Weight of removed prunings was recorded on a per vine basis. Crop load was calculated on a per vine basis by calculating yield/pruning weight.

Winemaking. Fruit from all experimental units from a treatment were combined and pressed using a hydraulic basket press on the day of harvest. Wines were vinified with two replicates for each vineyard treatment, for a total of 8 fermentations. The collected juice was treated with 50 mg/L SO_2 and allowed to settle for 12 h. Juice was racked into 19 L carboys. Juice was inoculated with *Saccharomyces cerevisiae* strain R-HST yeast (Lallemand, Inc. Toulouse, France) previously rehydrated in GoFerm (Lallemand) according to manufacturer's instructions. FermAid K (Lallemand) was added (0.13 g/L) at inoculation and when wines had reached approximately 10 °Brix. Wines were fermented to dryness as determined by Clinitest (Bayer, West Haven, CT), racked, cold stabilized and bottled about four months after the grapes had been pressed. No pH or sugar adjustments were performed during vinification, and wines did not undergo malolactic fermentation. C_{13}-norisoprenoid analysis was conducted on wines 6 months after the grapes had been pressed and two months after bottling.

Juice Soluble Solids. The juice soluble solids content was analyzed from pressed, previously frozen samples. Samples for each experimental unit were analyzed separately, with duplicate analytical replicates. Soluble solids were measured using a Leica temperature compensating Brix scale (0−30) refractometer (Leica Inc., Buffalo, NY).

Carotenoid Analysis of Grapes. The carotenoid extraction method was adapted from a previously published method (*32*). Briefly, 100 frozen berries (~60 g) were homogenized with a Waring blender divided into 25 g aliquots, and astaxanthin added as an internal standard (final concentration = 100 μg/kg). Carotenoids were extracted with 25 mL of 50:50 methanol/tetrahydrofuran with 0.1% BHT. Extracts were centrifuged, and the precipitate was re-extracted with methanol/tetrahydrofuran. The two supernatant fractions were pooled and combined in a separatory funnel with 50 mL of petroleum ether + 0.2% BHT (w/v) and 25 mL of aqueous NaCl (20% w/v). The organic phase was dried under a vacuum to approximately 0.5 mL, with drying finished under nitrogen, and redissolved in 2 mL of ethanol. The extraction protocol was performed in duplicate. Carotenoid extracts were not saponified prior to analysis since during methods development zeaxanthin and the internal standard astaxanthin were not detected following saponification (data not shown). The basic conditions of saponification have been previously reported to result in oxidation of astaxanthin (*33*). Additionally, stereomutation of the *all-trans* native forms of lutein and zeaxanthin to *cis* forms is accelerated at higher temperatures resulting in chromatographic peak broadening, and making it impossible to discern zeaxanthin from the lutein coelution (*33*).

HPLC analysis of carotenoids was conducted using an Agilent (Santa Clara, CA) Zorbax XDB-C18 column (150 mm ×4.6 mm, 5 μm) fitted with a Zorbax XDB-C18 guard column (20 mm × 4 mm, 5 μm) on a Hewlett-Packard 1100 series HPLC system equipped with a UV/vis diode array detector, set to record λ: 350−600 nm. The absorbance at 450 nm was used for quantification of grape carotenoids (*17*). Two different HPLC gradients were employed to achieve baseline resolution of all carotenoids of interest. β-Carotene, neochrome, neoxanthin and violaxanthin were analyzed by an acetone:water solvent system (gradient I): 0 to 20 min 70:30 (v/v) to 100% acetone; 20 to 30 min constant 100% acetone. The flow rate was 1 mL/min (*17*). β-Carotene was identified and quantified with respect to an authentic standard. Neochrome, neoxanthin and violaxanthin were identified by comparison of spectra and retention times to previous reports using the same solvent system (*17*), and reported as lutein equivalents.

The zeaxanthin and lutein peaks were not adequately separated by the first gradient, so an alternative gradient (gradient II) was developed with the same acetone:water solvent system: 0 to 5 min 50:30 (v/v); 5 to 10 min 70:30 to 76.5:23.5, and held until 16 min; then 76.5:23.5 to 78:22 from 16 to 18 min; 78:22 to 100% acetone from 18 to 24 min; then held at 100% acetone from 24 to 35 min. The flow rate was 1 mL/min. An average resolution of 1.5 was obtained between lutein and zeaxanthin in samples. Zeaxanthin and lutein were identified and quantified with respect to authentic standards.

Analysis of Free and Total C_{13}-Norisoprenoids. For measurements of free C_{13}-norisoprenoids in wines and juices, a solid-phase extraction (SPE) protocol was adopted from conditions used in previous studies (*34*). Wine and juice samples were centrifuged and filtered through #1 Whatman filter paper. The internal standard (2-octanol) was added to 50 mL of sample to yield a final concentration of 50 μg/L (*34*). Samples were loaded onto SPE cartridges (Merck, Darmstadt, Germany) containing 200 mg of LiChrolut EN sorbent preconditioned with 5 mL of dichloromethane, 5 mL of methanol and 10 mL of H_2O. Solvent elution was facilitated by use of a Varian (Walnut Creek, CA) Cerex SPE processor and N_2 headpressure (10 psi). Following sample loading, cartridges were rinsed with 4 mL of H_2O prior to elution of the analytes with 2 mL of dichloromethane, and the eluent was dried under N_2 gas to a final volume of 100 μL.

Measurements of total C_{13}-norisoprenoids utilized an acid hydrolysis step prior to SPE (*6*, *18*). Following filtration and addition of the internal standard, samples were acidified to pH = 2.0 with 2 M HCl and heated (100 °C, 60 min). Due to the formation of a haze after cooling the samples, juice samples were refiltered prior to the subsequent SPE analyses.

GC−MS analysis was conducted on a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 ion trap MS (Walnut Creek, CA). Separation was performed on a Varian CP-Wax 58 column (40 m × 0.25 mm × 0.5 μm). The initial oven temperature was 40 °C and held for 6 min; then ramped to 140 °C @ 10 °C/min.; then to 170 °C @ 5 °C/min; then to 250 at 10 °C/min and held at 250 °C for 20 min. The GC was operated at a constant flow rate of 1 mL/min. Three microliters of extract was injected splitless, with a purge time of 0.75 min. The temperatures for the transfer line, manifold, and ion trap were 250 °C, 50 °C, and 170 °C respectively. The ion trap MS was operated over the range $m/z = 25-220$. Data processing and quantification was performed using the native Varian Saturn GC−MS software (version 5.52). Calibration curves for β-damascenone and TDN were generated in model juice and wine against the 2-octanol internal standard over a range of 1−300 μg/L for TDN and 0.1−30 μg/L for β-damascenone. Since standards were not available, vitispirane A and B were identified by retention index and MS library spectra (NIST Mass Spectral Library version 1.7a) and the sum of the isomers was reported as TDN equivalents. The following ions were used for quantification and identification: β-damascenone (quantification ion m/z = 121, qualifier ion m/z = 69 and 175), TDN (157, 172 and 142), vitispirane A (192, 177 and 93), vitispirane B (177, 192 and 121). Peak definition and quantification was based on the selected ion chromatograms from the full mass spectral data set.

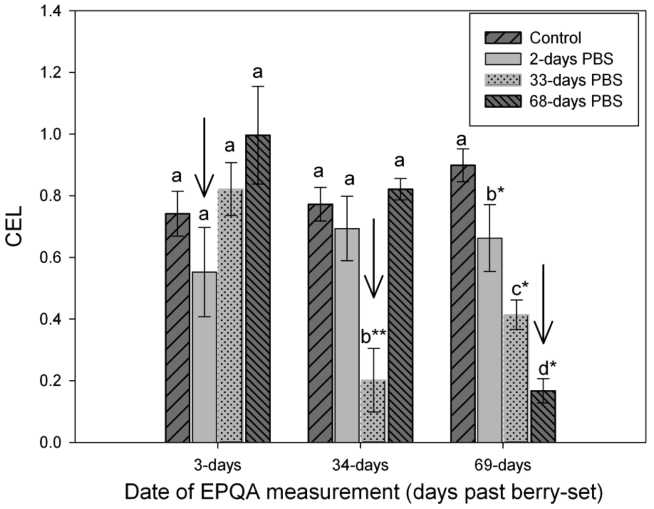

Figure 2. Effect of leaf removal treatment on cluster exposure layer (CEL) measured one day after each treatment application. Measurements were taken 3 days (June 25), 34 days (July 26) and 69 days (August 31) after berry set, corresponding to the days following each leaf removal treatment. Veraison was approximately 65 days after berry set. The control received no leaf removal. Different lowercase letters at same date of measurement (a, b, c, d) indicate difference in means by Tukey HSD at a significance level of $*p < 0.05$, $**p < 0.01$. Error bars indicate one standard deviation. An arrow indicates the treatment that received leaf removal the previous day.

Statistical Analysis. Statistical analysis was conducted using SAS JMP version 8.0 (SAS Cary, NC) for standard deviation, and Tukey−Kramer HSD, and linear regression. Welch's t test (*35*) was conducted in Microsoft Excel 2007 (Redmond, WA). A p-value less than 0.05 was necessary for results to be reported as significant.

RESULTS AND DISCUSSION

Impact of Leaf Removal Treatments on Canopy Microclimate and Fruit Maturity. EPQA was performed on the canopies of all experimental units, one day after each leaf removal treatment was applied, to assess the effects of the treatments on canopy architecture and cluster light environment (**Figure 2**). This also allowed assessment of canopy regrowth in those treatments that had undergone leaf removal earlier in the season. CEL measures the average number of occlusions experienced by clusters, and thus is an indicator of cluster light exposure, where lower CEL indicates greater cluster exposure. Berry temperature was not measured, but previous reports have observed an increase in berry temperature with increased cluster light exposure (*7*, *36*) and it is difficult to decouple these parameters experimentally (*31*). As expected, significantly lower CEL was observed for 33-day and 68-day PBS treatments immediately following their respective leaf removal events compared to the other treatments ($p < 0.01$). The 2-day PBS treatment did not result in significantly lower CEL than the control (0.55 vs 0.72) when quantified at 3 days after berry set, however a significantly lower CEL was observed than the control for the 2-day PBS treatment following the 68-day PBS leaf removal (0.66 vs 0.90, $p < 0.01$). The treatments had little effect on yield and yield components (**Table 1**). There were no significant differences among treatments for yield per vine or average cluster weight, however the pruning weight for the 2-day PBS treatment (1.59 kg/vine) was higher than the control (1.31 kg/vine) and 33-day PBS (1.28 kg/vine, $p < 0.05$). These results suggest the 2-day PBS treatment may have induced vegetative growth outside of the fruiting zone, since the CEL was significantly lower than the control at the final EPQA (69 days after

Table 1. Mean Vine Growth and Crop Measurements for Leaf Removal Treatments[a]

	treatment (leaf removal timing)							
	control	SD	2 days PBS	SD	33 days PBS	SD	68 days PBS	SD
cluster count/vine	92.3 a	4.2	90.6 a	6.7	98.6 a	3.9	88.0 a	4.5
fruit wt (kg/vine)	11.0 a	0.6	11.0 a	0.4	11.4 a	0.9	11.6 a	0.5
mean cluster wt (kg)	0.12 a	0.00	0.12 a	0.00	0.12 a	0.01	0.13 a	0.00
pruning wt (kg/vine)	1.3 a	0.1	1.6 b*	0.1	1.3 a	0.0	1.4 a,b	0.1
crop load index[b]	8.7 a,b	0.9	7.1 a	0.4	9.1 b*	0.7	8.8 a,b	0.2
total soluble solids	19.1 a,b	0.8	20.3 a	0.6	19.1 a,b	0.2	18.7 b*	1.1

[a] Treatment timings refer to the days past berry set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. Using Tukey HSD significance of means among all experimental units was compared. Different lowercase letters within a row (a, b), with significance level of $*p < 0.05$. [b] Crop load index was calculated by fruit weight/pruning weight.

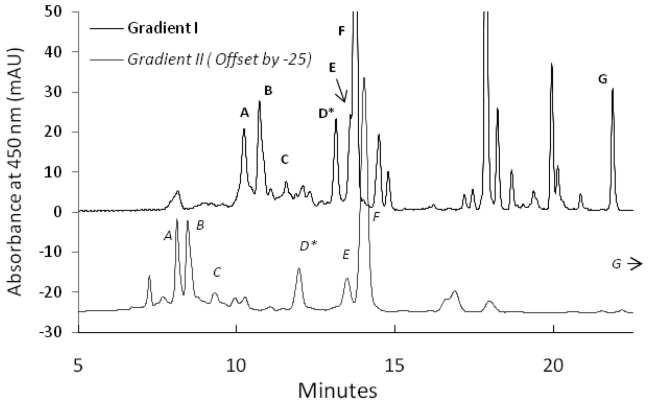

Figure 3. HPLC chromatogram (450 nm) of carotenoids in a harvest sample using gradient I and gradient II (signal offset by −25 on plot). (A) neoxanthin; (B) neochrome; (C) violaxanthin; (D*) astaxanthin, internal standard; (E) zeaxanthin; (F) lutein; (G) β-carotene. Gradient I labeled in bold, gradient II in italics.

berry set). Crop load (vine yield/pruning weight) was significantly lower for the 2-day PBS treatment than the 33-day PBS treatment (9.05 vs 7.07, $p < 0.05$), due to the higher pruning weight values for the 2-day PBS treatment. Soluble solids of the 2-day PBS treatment were significantly higher than that of 68-day PBS (20.3 vs 18.7 °Brix), but no difference was observed between any of the treatments compared to the control. Early leaf removal has been previously reported to increase Brix and advance fruit maturity (*13*, *14*).

Berry Carotenoids. Quantification of four carotenoids (β-carotene, neochrome, neoxanthin and violaxanthin) was performed by a previously described C_{18}-HPLC protocol (gradient I, **Figure 3**) (*28*, *37*). Using HPLC gradient I, quantification of zeaxanthin was not possible due to its coelution with lutein. Previous reports investigating the impact of sun exposure on berry carotenoids have observed a similar coelution using this protocol, which accounts for the limited reporting of zeaxanthin in grape related literature (*16*, *26*, *28*, *37*). Quantification of the total de-epoxidized xanthophyll pool in grapes, including zeaxanthin, has been previously reported using a non-end-capped reverse phase HPLC column (*26*). However, non-end-capped columns are not widely used due to increased peak tailing from free silanol groups. Using a conventional end-capped column, we modified the solvent gradient (gradient II) to yield acceptable baseline resolution between lutein and zeaxanthin ($R_s = 1.5$) (peaks E and F, respectively, **Figure 3**). Using gradient II, we also observed that the β-carotene peak obscured a small α-carotene peak (< 10% β-carotene peak area), confirmed by comparison to an authentic standard (data not shown). The separation was not adequate for quantification of α-carotene. To our knowledge, α-carotene has not been previously reported in grapes.

Total berry carotenoid concentrations decreased during maturation from 1,500 to 2,700 μg/kg at midseason to 330−880 μg/kg at harvest (**Table 2**), similar to previous reports (*7*, *15*). The major carotenoid species were β-carotene and lutein, and these two compounds summed to account for 69% (midseason) and 60% (harvest) of the total measured carotenoids. This is lower proportionally than values around 85% reported previously for other varieties of grapes (*28*). We observed no significant differences among treatments for total carotenoids at either sampling time point. The existing literature on the impact of preveraison cluster light environment on carotenoids is inconsistent (*25*, *27*). Preveraison cluster shading has been reported to lead to lower carotenoid content midseason (*25*), as would be expected from the

Table 2. Berry Carotenoid Concentration (Mean and Standard Deviation, SD) for Mid-Season (52 Days after Berry Set) and Harvest (107 Days after Berry Set) Samples[a]

	berry carotenoid concn, mean and SD (μg/kg or % of total)							
	control[b]		2 days PBS[b]		33 days PBS[b]		68 days PBS[b]	
	mean	SD	mean	SD	mean	SD	mean	SD
Lutein								
midseason								
μg/kg	971 a	145	737 a	86	902 a	58	939 a	46
% of total	50 a	3	46 a	3	47 a	1	46 a	2
harvest								
μg/kg	280 a	60	301 a	27	300 a	43	189 a	32
% of total	53 a,b	3	59 a	3	47 a,b	1	43 b*	5
β-Carotene								
midseason								
μg/kg	405 a	137	348 a	153	391 a	90	497 a	62
% of total	21 a	5	19 a	6	19 a	4	24 a	2
harvest								
μg/kg	45 a	14	49 a	28	84 a	27	27 a	8
% of total	9 a	2	9 a	4	12 a	3	6 a	1
Zeaxanthin								
midseason								
μg/kg	88 a	13	63 a	2	142 b*	14	93 a	4
% of total	5 a	0	4 a	1	7 b***	1	5 a	0
harvest								
μg/kg	24 a	2	39 a	3	39 a	2	36 a	6
% of total	5 a	1	8 a,b	1	6 a,b	1	8 b*	1
Neoxanthin								
midseason								
μg/kg	159 a	24	175 a	20	164 a	17	158 a	9
% of total	8 a,b	0	11 a	1	9 a,b	1	8 b*	0
harvest								
μg/kg	58 a	12	44 a	3	77 a	11	65 a	10
% of total	11 a,b	2	9 a	1	12 a,b	1	15 b*	2
Neochrome								
midseason								
μg/kg	232 a	25	243 a	20	254 a	19	261 a	33
% of total	12 a	1	15 a	1	13 a	1.9	13 a	1
harvest								
μg/kg	87 a	24	52 a	13	85 a	3	77 a	16
% of total	16 a,b	2	10 a	1	14 a,b	2	18 b*	4
Violaxanthin								
midseason								
μg/kg	75 a	10	76 a	11	87 a	6	85 a	4
% of total	4 a	0	5 a	0	5 a	0	4 a	0
harvest								
μg/kg	39 a	9	27 a	1	47 a	8	41 a	5
% of total	7 a,b	1	5 a	1	7 a,b	0	9 b*	1
Total								
midseason								
μg/kg	1932 a	297	1641 a	289	1941 a	143	2034 a	150
harvest								
μg/kg	532 a	104	512 a	66	632 a	84	434 a	46

[a] Treatment timings refer to the days past berry set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. At the mid-season time point, leaf removal had not been performed on the 68-day PBS treatment, and was thus similar to the control. Differences among treatments were determined by Tukey HSD. Different lowercase letters within a row (a, b), with significance level of $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. [b] Treatment (leaf removal timing).

general observation that sunlight can stimulate photosystem II activity in plants, and consequentially carotenoid biosynthesis (*38*). However, at least one study has observed higher concentrations of total carotenoids in shaded grapes during development (*39*), which has been observed in other higher plants, especially those grown in extreme shade (*40*). It was suggested in these studies that the increased carotenoids in shade-grown plants are used as light harvesting antennae, or for protection from brief periods of direct sun. Similarly, some previous studies have observed higher total carotenoid concentrations in mature shaded fruit (*24*, *28*), putatively because light exposure accelerates photo- or enzymatic degradation *in vivo*, but this result was not observed in another study (*27*), and no change in carotenoid degradation rate was observed in vines exposed to partial root zone drying, despite increased cluster sun exposure (*9*).

Of the six individual carotenoids quantified in our study, only zeaxanthin showed a significant response to any treatment when considering absolute concentrations, though treatment effects were observed on other carotenoids when expressed as a percentage of the total carotenoid pool (**Table 2**). In the 33-day PBS treatment samples, zeaxanthin at midseason had a mean concentration of 142 μg/kg, significantly greater ($p < 0.05$) than the control, 2-day PBS and 68-day PBS treatment samples (range = 63−93 μg/kg). In plants, zeaxanthin is formed either from β-carotene or via de-epoxidation of epoxyxanthophylls (e.g., violaxanthin) as part of the xanthophyll cycle to dissipate excess energy during photosynthesis (*23*, *41*). The midseason samples also showed a significant increase ($p < 0.005$) in the proportion of zeaxanthin relative to total carotenoids, with zeaxanthin constituting 7% of total carotenoids in 33-day PBS treatment versus 4−5% for other treatments. A significant correlation of zeaxanthin as a percentage of total carotenoids (% zeaxanthin) at midseason (56 days after berry set) vs CEL taken 34 days after berry set was found ($R^2 = 0.83$, $p < 0.0001$, data not shown). This correlation was particularly strong when considering only the four experimental units of the 33-day PBS treatment ($R^2 = 0.99$, $p = 0.005$, plot not shown), but significant correlations were not observed within the other treatments, possibly because of a narrower range of zeaxanthin concentrations (**Table 2**). The increase in zeaxanthin in midseason berries in the 33-day PBS treatment is expected, since sun exposure and resulting PSII overexcitation is reported to increase the total xanthophyll pool (*26*) as well as the ratio of de-epoxidized xanthophylls to epoxidized xanthophyll forms (*41*). At harvest, no difference in the absolute zeaxanthin concentration was observed between the 33-day PBS treatment and the control. This may indicate greater enzymatic degradation of zeaxanthin via *VvCCD* in the 33-day PBS treatment after veraison, although it is also possible that zeaxanthin is recycled to an epoxy form prior to carotenoid degradation.

No difference was observed among the 68-day PBS treatment and other treatments at either time point. However, a significantly higher zeaxanthin proportion was observed at harvest in the 68-day PBS treatment compared to the control (8% vs 5%). As mentioned previously, sun exposure is reported to increase the proportion of zeaxanthin in the carotenoid pool. The lack of a significant impact of the 68-day PBS treatment on absolute zeaxanthin concentrations at harvest as compared to the 33-day PBS treatment at midseason may have been due to the larger gap between the treatment timing and the carotenoid sampling point, and the resultant change in light environments likely caused by canopy growth; 39 days elapsed between the 68-day PBS treatment and the harvest date, as compared to the 22-day difference between the 33-day PBS treatment and the midseason sampling time point. In comparison to the % zeaxanthin values, absolute zeaxanthin concentration will be more influenced by other factors regulating total carotenoid concentration, e.g. berry size, and as a result suffer from more biological variability. Additionally, carotenoid production in postveraison grapes has been demonstrated to be minimal (*16*).

Sun exposure has been reported to deplete epoxyxanthophylls in some plants (*42*), but no significant difference was observed for neoxanthin among treatments in our work with respect to the control at either time point. Interestingly, the 2-day PBS treatment had a higher proportion of lutein and a lower proportion of neoxanthin in midseason samples than the 68-day PBS treatment, although no difference was observed compared to the control. The 2-day PBS treatment also had a lower violaxanthin and neochrome proportion in mature fruit samples. The reason for these differences is not apparent but may be related to the increased fruit maturity observed in the 2-day PBS over the 68-day PBS treatment as measured by soluble solids.

Free and Total C_{13}-Norisoprenoids in Juice. Concentrations of free and total (free + bound) C_{13}-norisoprenoids were quantified in both juice and wine. Although glycosylated precursors can be liberated either enzymatically or by acid hydrolysis during winemaking, we selected acid hydrolysis for determining total C_{13}-norisoprenoids because the species of interest (TDN, β-damascenone, vitispirane) are not observed under enzymatic hydrolysis conditions (*16*). Acid hydrolysis under heated conditions has the additional benefit of evolving potential nonglycosylated precursors (*18*). Additionally, TDN and vitispirane concentrations are observed to increase dramatically during storage (*6*), so acid hydrolysis would be expected to better reflect the total potential concentrations of these compounds in particular.

Mean concentrations of free and total C_{13}-norisoprenoids in juice and wine are shown in **Table 3**. In the juice samples, free TDN and vitispirane were below the method's detection threshold (< 0.1 μg/L as TDN equivalents), and only trace levels (below quantification limit, < 0.3 μg/L) of free β-damascenone were detectable in the juice samples. This is in concordance with previous reports which have observed undetectable or trace levels of C_{13}-norisoprenoids in juice (*7*).

Significantly higher concentrations of total, acid-liberated TDN and vitispirane were observed in the 33-day PBS treatment juice samples compared to the control and the other treatments ($p < 0.05$). The mean total TDN concentration was 195 μg/L for the 33-day PBS treatment, vs 54−81 μg/L for the other treatments (**Table 3**). Cluster light exposure has been previously linked to increased concentrations of TDN/vitispirane precursors in harvested fruit (*5*), but the critical time period during which exposure impacts precursor formation has not been established. In our current work, the 33-day PBS treatment timing increased acid-hydrolyzable TDN/vitispirane precursors in juice. However, neither 2-day PBS nor 68-day PBS treatments increased TDN/vitispirane precursors in juice, suggesting that the critical time during the growing season for forming TDN/vitispirane precursors is ~33 days after berry set. The lack of a significant effect by the 68-day PBS treatment also indicates that cluster light exposure does not induce formation of TDN/vitispirane precursors by increasing the rate of carotenoid degradation and C_{13}-norisoprenoid formation postveraison.

β-Damascenone concentrations in juice samples were lower in the 68-day PBS treatment than the control and 33-day PBS treatment (**Table 3**). No differences were observed among the 2-day and 33-day PBS treatments and the control ($p < 0.05$). Unlike TDN, total β-damascenone concentrations in wines have been reported to decrease in response to cluster exposure (*5*, *7*).

Free and Total C_{13}-Norisoprenoids in Wine and Correlation with Juice. In finished wines, total TDN was significantly higher in the 33-day PBS compared to the control and free levels were higher

Table 3. C_{13}-Norisoprenoid Concentrations for Juice and Wine Samples from Each Leaf Removal Treatment[a]

	C_{13}-norisoprenoid concn, mean and SD (μg/L)							
	control[b]		2 days PBS[b]		33 days PBS[b]		68 days PBS[b]	
	mean	SD	mean	SD	mean	SD	mean	SD
TDN								
juice								
free	nd[c]		nd		nd		nd	
total	71 a	12	54 a	9	195 b**	26	87 a	22
wine								
free	9 a	0	7 a	1	20 b*	1	11 a	2
total	52 a	16	61 a,b	2	138 b*	31	81 a,b	12
Vitispirane A + B (as TDN Equivalents)								
juice								
free	nd		nd		nd		nd	
total	37 a	5	30 a	3	56 b*	10	32 a	5
wine								
free	10 a	2	7 a	1	9 a	0	9 a	1
total	122 a	4	109 a	11	132 a	8	129 a	13
β-Damascenone								
juice								
free	nq[d]		nq		nq		nq	
total	10 a	1	7 a,b	1	10 a	1	7 b*	1
wine								
free	6 a	1	8 a	1	5 a	0	9 a	0
total	4 a	0	9 a	1	4 a	2	8 a	1

[a] *Total* refers to the concentration following acid hydrolysis. Standard deviations (SD) were calculated from treatment replicates for juices (n = 3 or 4). The juices were pooled prior to winemaking, and mean and SD were calculated for the winemaking replicates (n = 2). Treatment timings refer to the days past berry set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. Different lowercase letters within a row (a, b) indicate difference in means by Tukey HSD at a significance level of *p < 0.05, **p < 0.01. [b] Treatment (leaf removal timing). [c] Not detected (<1 μg/L for TDN, <1 μg/L for vitispirane). [d] Not quantifiable (0.1 < damascenone < 0.3 μg/L).

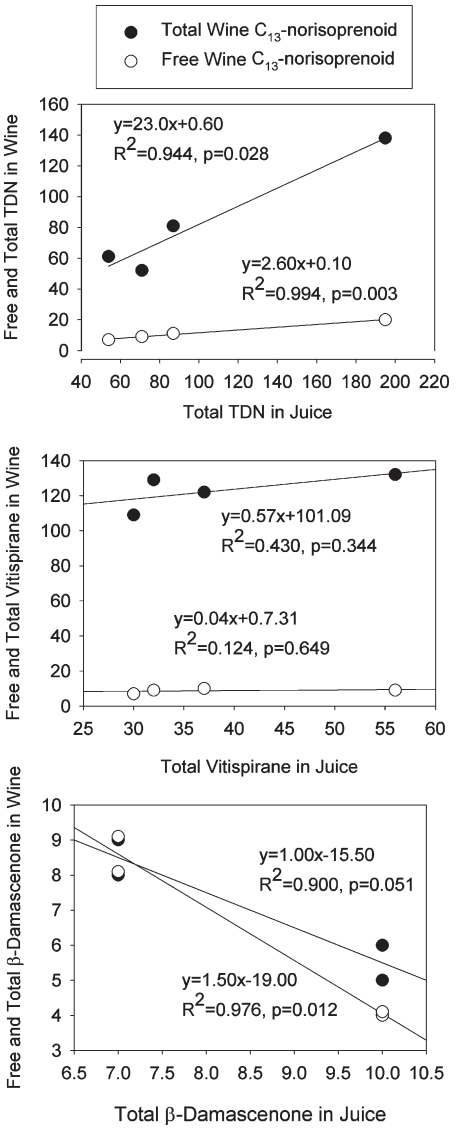

Figure 4. Comparison of the relationship between the means of total, acid-hydrolyzable C_{13}-norisoprenoids in juice to the free and total concentrations found in wine for TDN, vitisipirane and β-damascenone. All values are given in μg/L.

than the other treatments and control (**Table 3**). No differences in the concentrations of vitispirane or β-damascenone were observed between treatments in wine (**Table 3**). Free TDN in wine was > 2-fold higher in the 33-day PBS treatment than the control. The 20 μg/L concentration of TDN in the 33-day PBS treatment is equal to the reported sensory threshold in wine (*4*). Mean concentrations of total TDN in grapes were significantly correlated with free TDN and total TDN concentrations in wine (**Figure 4**). The mean conversion rate of total grape TDN to free TDN in wine was 11% ± 2.5%. Across treatments, we observed no significant change in total TDN in juice vs wine, i.e. the fermentation did not result in a significant change in the total, TDN pool (**Figure 5**). These findings suggest that total TDN in juice is a good indicator of total TDN in wine.

The β-damascenone pool in wine existed entirely in the free form, as no increase was observed in β-damascenone following acid hydrolysis (**Table 3**). The mean conversion rate of total grape β-damascenone to free β-damascenone in wine was quantitative, 81% ± 35% (**Figure 5**). In contrast, the majority of TDN still existed in the form of bound precursors following fermentation. The faster kinetics of β-damascenone formation may be due to different rates of acid or enzymatic hydrolysis or rearrangement on precursors during winemaking. Interestingly, we observed a significant negative correlation of total β-damascenone in juice with free β-damascenone in wine (**Figure 4**). The reason for this phenomenon is unclear, but a potential explanation is that the fruit with low total β-damascenone may have had other precursors that were transformed to β-damascenone precursors during fermentation.

Free and total concentrations of vitispirane in wine were not correlated with total vitispirane in juice (**Figure 4**). We observed a significant increase (mean = 91 ± 10 μg/kg) in total vitispirane following fermentation compared with juice samples, or a 330% increase in total vitispirane (**Figure 5**). To our knowledge, an increase in total vitispirane in wine with respect to grape juice has not been previously reported, since direct or indirect measurement of potential volatiles in wine is rarely reported. An increase in total vitispirane was observed previously when apple leaf glycoside extract was fermented with baker's yeast (*43*). The authors proposed this resulted from enzymatic reduction of glycosylated TDN precursors to glycosylated vitispirane precursors. While this could explain the increase of total vitispirane in our study, it does not explain why total TDN did not show a corresponding decrease. Regardless, these results show that predicting vitispirane concentration in finished wines based on acid hydrolysis is not advisible.

Relation of C_{13}-Norisoprenoids to Carotenoids and Cluster Exposure. Our current work indicates that preveraison cluster

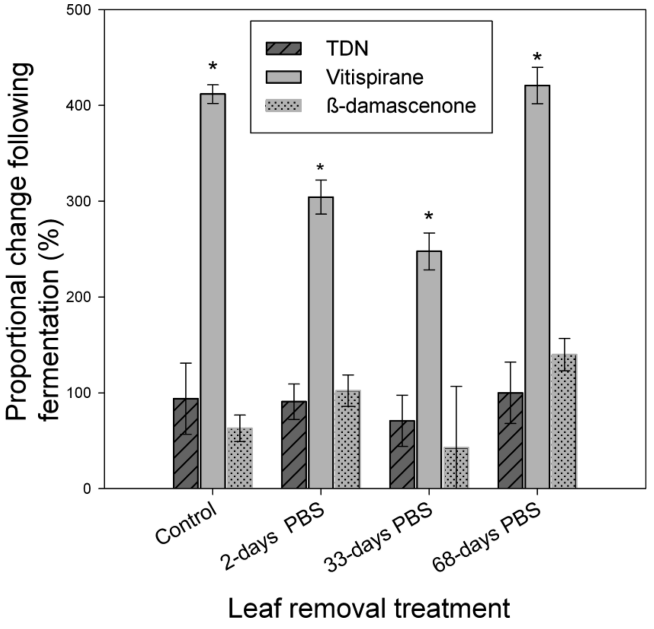

Figure 5. Change in total (free + potential) C_{13}-norisoprenoids resulting from winemaking, calculated as the ratio of C_{13}-norisoprenoids to C_{13}-norisoprenoids in must × 100%. By definition, 100% indicates identical concentrations in juice and wine. Error bars indicate one standard deviation. * indicates that C_{13}-norisoprenoid concentration in wine was significantly different than C_{13}-norisoprenoid concentration in must ($p < 0.0001$, Welch's *t* test, $n = 3$ or 4 for juice, $n = 2$ for wine).

exposure (33 days PBS) by leaf removal will significantly increase TDN and vitispirane precursors in juice compared to exposure at berry set or postveraison. The preveraison leaf removal timing also results in significantly higher free and total TDN in wine. Though total carotenoid content was not affected by any treatment, an increase in zeaxanthin in the 33-day PBS treatment was observed in midseason berry samples.

The observation that both zeaxanthin and total TDN increase in the 33-day PBS treatment is intriguing as zeaxanthin has been demonstrated to generate two putative precursors of TDN *in vitro* via photo-oxidation to yield 3-hydroxy-5,6-epoxy-β-ionone (*44*), or enzymatic degradation to yield 3-hydroxy-β-ionone (*2*). Thus, it is possible that zeaxanthin may be a precursor for grape-derived glycosylated TDN precursors *in vivo* as well. We observe a significant correlation between midseason zeaxanthin and total juice TDN ($R^2 = 0.59$, $p = 0.0009$, plot not shown) and % zeaxanthin and total juice TDN ($R^2 = 0.68$, $p = 0.0003$, plot not shown). However, within treatments, a significant correlation was only observed for the four 33-day PBS treatment replicates ($R^2 = 0.96$, $p = 0.02$, plot not shown). This may be because of the narrower range of TDN in the treatments other than 33 days PBS and the greater proportional importance of noise, or that the zeaxanthin−TDN relationship is correlative rather than causal. The peak concentration of zeaxanthin in grapes is unknown, so it is also possible that we did not capture the maximum zeaxanthin concentration. Since other carotenoids have also been reported to yield TDN *in vitro*, e.g. lutein following acid hydrolysis (*2*, *18*), cell culture or labeling studies with putative precursors may be necessary to distinguish the critical pathways *in vivo*.

Implications with Respect to Cultural Practices. Our findings could have important implications for selecting cultural practices to target specific flavor profiles. Excessive concentrations of TDN in young wines are sometimes reported to be undesirable (*24*), likely because strong "petrol" aromas would mask other Riesling aroma attributes. Reducing cluster light exposure during the growing season is one strategy for growers interested in reducing the eventual concentration of TDN in wines. However, as mentioned in the Introduction, increasing berry sun exposure is often desirable for reducing disease pressure, decreasing titratable acidity, and effecting other desirable changes to fruit qualities. The results of our study indicate that the key period during the growing season associated with production of acid-releasable TDN precursors is preveraison (33-day PBS treatment). Growers could implement leaf removal at berry set or postveraison for disease control, etc., without a resulting increase in TDN. Conversely, preveraison leaf removal could be employed if higher TDN concentrations were desired in wine.

In summary, we have demonstrated that the timing of leaf removal can alter the midseason carotenoid profile, as well as TDN and vitispirane precursors in mature Riesling grapes. Leaf removal at 33 days PBS resulted in elevated midseason zeaxanthin concentrations, elevated total TDN/vitispirane in juice, and elevated free TDN in wine as compared to other treatments and the control. Therefore, our results suggest that leaf removal can be practiced at berry set and postveraison without a significant effect on TDN or vitispirane potential. However, the implication that zeaxanthin is the source of TDN/vitispirane precursors still needs to be evaluated in future studies. β-Damascenone in wine was unaffected by the leaf removal treatments, in concordance with previous reports indicating differential regulation of β-damascenone and TDN/vitispirane precursors. Finally, total vitispirane increased by up to 4-fold after fermentation, indicating that the conditions associated with fermentation may transform glycosylated precursors, and that potential vitispirane in grapes is a poor predictor of vitispirane postfermentation.

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