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Grape Berry Acclimation to Excessive Solar Irradiance Leads to Repartitioning between Major Flavonoid Groups

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Supporting Information

ABSTRACT: Warm viticulture regions are associated with inferior wines, resulting from the interaction between microclimate and fruit biochemistry. Solar irradiance triggers biosynthetic processes in the fruit and dominates its thermal balance. Therefore, deciphering its impact on fruit metabolism is pivotal to develop strategies for fruit protection and ameliorate its quality traits. Here, we modified light quality and intensity in the fruit-zone and integrated micrometeorology with grape and wine metabolomics, allowing a complete assessment, from field to bottle. We analyzed the dynamics of fruit's adaptation to altered conditions during ripening and constructed temporal-based metabolic networks. Micrometeorological modifications shifted the balance between the major flavonoids, associating increased solar exposure with lower levels of anthocyanins and flavan-3-ols, and higher flavonols. Differences were fixed from 2 weeks postveraison until harvest, suggesting a controlled acclimation response rather than external modulation. Differences in grape composition manifested in the wine and resulted in higher color intensity and improved wine hue under partial shading.

KEYWORDS: *metabolomics, fruit quality, wine composition, Vitis vinifera L., microclimate, photoselective shading, climate change*

INTRODUCTION

The tight bond between climate and wine quality is one of the main hallmarks of the wine industry. Climate is regarded as a key factor in shaping the style and quality of the wine and distinguishing between vintages and different growing regions. Temperature, specifically, was found to be a good predictor for wine price and vintage ratings.[1−3] Climate change in the last two decades markedly increased the area of vineyards experiencing extreme events or suboptimal conditions, challenging traditional practices.[2,4,5] Sustaining existing vineyards and mitigating environmentally induced detrimental effects on the quality of wine,[6] thus, requires a deeper understanding of the link between abiotic stresses and wine quality and prompts the adjustment of viticulture practices.

Wine quality is mainly determined by the chemical composition of the grape,[7] and more specifically, the level and composition of specific chemical groups which contribute to the aroma, taste, and color of the wine. The phenylpropanoids are a diverse group of compounds, which are involved in plant response toward biotic and abiotic stimuli.[8] Moreover, their suggested contribution to human nutrition and health and their contribution to the organoleptic features of the grape and wine have been thoroughly investigated.[9−11] The flavonoids constitute the anthocyanins, proanthocyanidins, and flavonols, directly responsible for the color, astringency, bitterness and structure, and the antioxidant capacity of the final product.[12−16] In addition, both proanthocyanidins and flavonols interact with anthocyanins, through molecular associations, leading to color enhancement and stabilization.[17] The majority of these environmental-responsive and sensorial-active compounds, found in the fruit, are synthesized within the fruit tissue.[18−20] This fact emphasizes the role of fruit microclimate as the connecting link between climate and wine quality.

Under vineyard conditions, the surface energy balance of the berry creates a direct positive correlation between solar irradiance and fruit temperature.[21] In warm climate regions, grapes that are exposed to direct sunlight are likely to experience elevated temperatures in addition to the high radiative energy flux.

Sunlight radiative flux is perceived by the plant through the chromoproteins, a family of photoreceptors composed of phytochromes, cryptochromes, phytotropins, and UVR-8, which are red and far-red, UVA and blue, and UVB light receptors, respectively.[22,23] Flavonoid compounds differ in their responsiveness to light induction, among which flavonols glucosides are the most responsive, showing dramatic increase in accumulation upon sunlight exposure.[24−28] Owing to their light-dependent nature and potent radical scavenging properties, they were suggested to act as plant UV protectors.[29] Differently, procyanidins monomers were negatively and not affected by cluster sun exposure,[26,30] while the levels of extension subunits and degree of polymerization were found to be enhanced.[26,31] Anthocyanins content and composition is significantly altered in response to sunlight exposure. A reduction in the proportion of anthocyanins containing acylated and coumarylated forms, and an increase in the proportion of those containing dihydroxyl as well as ortho-diphenol groups was measured.[32−34] While exposure to sunlight generally enhances anthocyanin content in the

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skin,[25,35−37] several works found contradictory results.[32,38] This inconsistency may be attributed to the light/temperature intimate relation in field experiments. Indeed, the responsiveness of anthocyanins to sunlight treatments was suggested to be inferior to their sensitivity to temperature.[39] Elevated grape temperatures, which may be caused by overexposure of the fruit to sunlight has detrimental consequences on fruit coloration,[39,40] and the consequential decrease in anthocyanins level was associated with a decrease in the expression of biosynthesis-related genes.[41−43]

Taken together, these lines of evidence suggest that the interaction between incoming irradiance and fruit temperature may have a synergistic or antagonistic character on grape composition, depending on the ranges of both factors.[42,44] This complex nature hinders attempts to predict the outcome of light versus temperature interaction on fruit quality, emphasizing the importance of precise fruit micrometeorological description in parallel with its corresponding chemical composition.

In this study, we manipulated fruit micrometeorology by applying photoselective shade nets around the cluster-zone, while minimizing any effect on the canopy and whole-vine physiology. We integrated fruit micrometeorology with skin phenylpropanoids profile over a time series, between the onset of coloring (i.e., veraison) to harvest, and through the winemaking process. The high-resolution data set was used to elucidate the regulation of the phenylpropanoid pathway by temperature and light and to test the potential of partial shading to promote the accumulation of quality-related compounds and improve wine quality. Our unique experimental site allowed us to conduct this study under conditions that are currently regarded as extreme, yet represent the future scenario for a large portion of current commercial vineyards.

MATERIALS AND METHODS

Site Description. The experiment was conducted during the 2014 and 2015 growing seasons in a vineyard located in the heart of the Negev Desert, Israel (30°36′55.22″N, 34°45′12.00″E, 800 m altitude). This is an arid region with an average annual precipitation of 70 mm (Israel Meteorological Service), characterized by stable meteorological conditions including high sunlight radiative flux and elevated midday temperatures (Supp. Figure 1). The vineyard was planted in 2007 with *Vitis vinifera* L. cv. Cabernet Sauvignon grafted on 140 Ruggeri rootstock, irrigated using a covered drip-irrigation system. Rows were orientated North−South with a 30° angle to the Northeast\Southwest and trained in vertical shoot positioning. Five adjacent vine rows, each containing approximately 60 vines, were used for the experiment. Three were manipulated and measured, and two were used as border rows, positioned between each pair of experimental rows. In each experimental row, five groups of nine adjacent vines were marked as field repetitions, and the basal leaves in the vicinity of the clusters (up to 30 cm above the cordon) were completely removed at the onset of veraison. Two experimental treatments were established, modulating (i) light incidence and (ii) light quality (only in 2015). In (i), fully exposed clusters, that is, no net (Exposed), were compared with clusters shaded by 30% and 60% neutral grey shading nets. In (ii), clusters from the 30% grey net were compared with those from 30% blue and red colored shading nets. All nets were UV-stabilized woven mesh shading nets for agriculture (Ginegar, Israel). The percent shading of the nets represents the PAR filtering capacity as published by the manufacturer (www.ginegar.com). The nets were placed in a manner that created a shading tunnel with a diameter of about 80 cm around the cluster-zone (Supp. Figure 2) to avoid accumulation of humidity in the vicinity of the fruit by facilitating air flow and to filter the reflected irradiance coming from the soil. Shading was applied from the onset of veraison (the day of basal leaf removal) until harvest date. Treatments were repeated once on each row in a way that represented all locations along the row to minimize effects of spatial differences both between and within the rows. Experimental vines were different in the two seasons to avoid any accumulated differences from year to year.

Meteorological and Cluster Micrometeorological Measurements. During the 2015 growing season, a detailed study of cluster-scale micrometeorological conditions was performed. Global incoming shortwave radiation (R_G), air temperature, relative humidity (RH%), and wind speed and direction were continuously monitored 1 m above the canopy, from veraison to harvest. Measurements were made at 10 s intervals using a multisensor (WS501-UMB, Lufft, Fellbach, Germany), and 15 min averages were logged by a data logger (CR200, Campbell Scientific, Utah, USA). Daily mean, minimum, and maximum values were then computed. In addition, cluster-zone air temperature and RH% were measured continuously at 1 min intervals, and 15 min averages were recorded from veraison to harvest by placing sensors equipped with an internal data logger (Hobo ProV2, Onset, MA, USA) at a shaded spot in the immediate vicinity of the clusters. Two sensors were placed at each treatment site to verify repeatability.

Shortwave radiation flux reaching the grape clusters (R_C) was measured by a pyranometer (LI200R, Li-Core, NE, USA) connected to a data logger (21X, Campbell Scientific, UT, USA). The pyranometer was placed within the cluster-zone at the east side of the canopy facing horizontally to the interrow. This aimed to represent R_C experienced by berries located at the external face (i.e., east-south) of an East-located cluster. This system was rotated between the exposed, and both 30 and 60% grey-shaded treatments, during three rounds of measurements (detailed in Supp. Figure 1). Pyranometer readings were normalized to the R_G measured above the canopy at the same day to allow for a comparison between measurements made on different dates.

Light spectra in the range of 350 to 2500 nm, reaching the cluster-zone in the different treatments, were measured by an ASD pro-FR spectroradiometer with a cosine receptor (Analytics Spectral Devices Inc., CO, USA) on July 24, 2015 between 10:00 and 11:00 am, when East-facing clusters were exposed to direct sunlight. The sensor was positioned as described above for the pyranometer measurements of R_C. The modified light spectra that resulted from the application of colored nets (Supp. Figure 3B) precluded the use of the pyranometer under these treatments. Thus, quantifying the level of modification of both light spectra and intensity by the colored shading nets was examined using the spectroradiometer measurements.

Berry surface temperature (BST) was measured on August 19th (preharvest) 2015, during a diurnal field campaign from 6:00 to 20:00 using an infrared camera (T640, FLIR systems, OR, USA). Every hour, images were taken from two representative clusters, located at the east side of the canopy, in each treatment. The images captured the external face of the cluster (i.e., facing the inter-row) corresponding to the pyranometer measurements. Data analysis included a careful selection of berries located in the middle section of the cluster's vertical axis (i.e., middle height) in each image and the exclusion of pixels representing nongrape background data.

Berry Sampling. In the 2014 season, two sampling dates were determined, at midripening, 28 days after veraison (28 DAV), and at the arrival of the first field repetition to technological maturity, scheduled at 24°Brix (52 DAV). In 2015, this sampling scheme was extended to study the temporal changes of grape composition under the various cluster micrometeorological conditions generated. Since interseasonal variability in meteorological conditions is low (Supp. Figure 4), micrometeorological characterization performed in 2015 can be regarded as representative for both seasons. Sampling was initiated 16 DAV and performed a total of six times until the arrival of the first field repetition to technological maturity, scheduled at 24°Brix (51 DAV) (Supp. Figure 1). Sampling during veraison was avoided due to the large variability within and between clusters, present at that stage. Four vines per field repetition were sampled. For each vine, four to six exterior-located clusters (furthest from the trunk toward the inter-row) were selected from the east side of the canopy to avoid possible shading by the canopy apart from midday hours. Berries were

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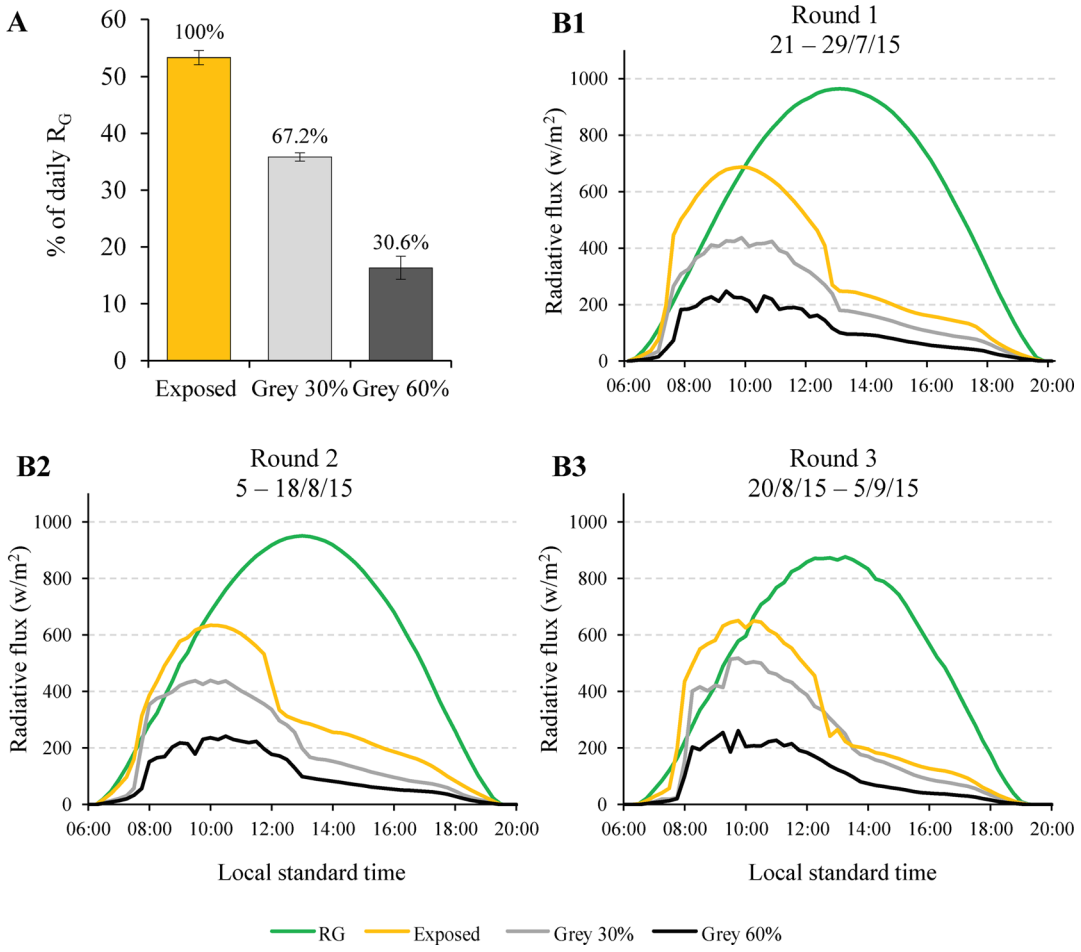

Figure 1. Radiative flux measurements in the cluster-zone of three shading treatments. Fully exposed (yellow), 30% grey shading (light grey), and 60% grey shading (dark grey) during the 2015 season. (A) Given as mean percent of daily global irradiation. (B) Daily temporal data of the three treatments and meteorological station (green line) in three measurement rounds along the season.

collected from the middle height of the external face of the cluster (i.e., facing east-south) to avoid the effect of the spatial location on fruit composition. East-facing berries were the focus of this study since given the row direction, this side received more sunlight hours compared to the West. Berries from vines located in the same field repetition (i.e., under the same net) were pooled together and immediately frozen in liquid nitrogen. In the lab, skin was separated while kept frozen on dry ice, placed in Eppendorf tubes, and stored at −80 °C until further processing. To measure berry weight and analyze the maturity indices, weekly samples of 100 berries were taken from each field repetition as detailed above, put in plastic boxes, and placed on ice until further analysis performed the same day.

Maturity Indices. Berries were counted and weighed using a digital scale (Sartorius TE612, Goettingen, Germany). They were crushed, and the resulting juice transferred to a 50 mL falcon tube, centrifuged at 5692*g* for 3 min at room temperature (Multifuge X3R, Thermo Fisher Scientific, MA, USA). Total soluble solids (TSS) was measured using a digital refractometer (ref-85, MRC, Holon, Israel), expressed as °Brix. Titratable acidity (TA) was measured by mixing 10 mL of juice, 30 mL of distilled water, and 50 μL of phenolphthalein solution (1% v/v phenolphthalein in ethanol), and titrating 0.1 N NaOH until the appearance of color change, corresponding to pH 8. Juice pH was measured by a pH-meter Basic 20+ (Crison, Barcelona, Spain).

Vinification. In the 2015 season, wines were made separately from each field repetition of three treatments: fully exposed, 30% grey, and 60% grey shading. Each field repetition was harvested separately upon the arrival to technical maturity (i.e., 24°Brix). Harvest included all vines, except those located at the edge of each field repetition. For each vine, the number of clusters was counted, and total yield was measured with a digital scale (Shekel, Beit Keshet, Israel). Clusters were destemmed, crushed, placed in 30 L food-grade plastic containers, mixed with the addition of 50 ppm of SO_2, and stored at 20 °C. The following morning, 200 mg/L of dried Montrachet 522 yeast strain was added (Enologica Vason, VR, Italy), following a dehydration protocol, as directed by the manufacturer. Every day, fermentations were monitored for must temperature and density, and skin cap was mixed three times. At the completion of the alcoholic fermentation, wine was transferred to glass demijohns sealed with airlock at 20 °C to allow for the completion of the malolactic fermentation. Upon the depletion of malic acid, measured by malic acid test kit (Accuvin, CA, USA), wines were racked, and 50 ppm of SO_2 was added. One month later, wines were additionally racked, and 15 mL samples were taken and stored at 4 °C until analysis.

Grape and Wine Metabolic Profiling. Grape skin samples were extracted following the protocol for metabolite profiling as described in Weckwerth et al.[45] with adaptation to grape skin as described in Degu et al.[46] Skin tissues were lyophilized, ground (Retsch, Haan, Germany), and weighed (40 mg). Metabolites were extracted in a 1 mL of methanol/chloroform/water extraction solution (2.5:1:1 v/v) containing internal standards (ribitol, ampicillin, and corticosterone). The extraction followed the exact procedure described in Reshef et al.[47] All samples were analyzed using ultra performance liquid chromatography coupled to a quadrupole time-of-flight mass-spectrometer (UPLC-QTOF-MS, Waters, MA, USA).

UPLC-QTOF-MS conditions were exactly as described previously by Hochberg et al.[48] Separation was performed using a C_{18} column (Waters MS Technology, Manchester, UK) maintained at 40 °C.

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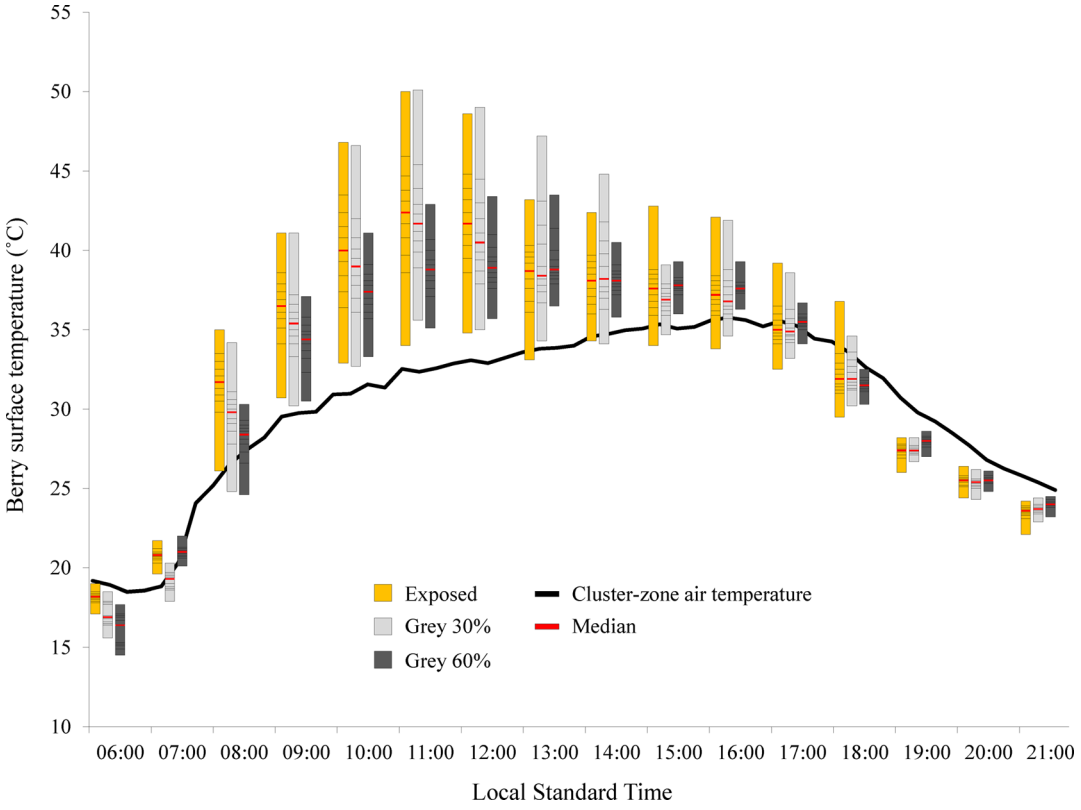

Figure 2. Percentiles graph representing berry surface temperatures (BST) measured on the 19th of August 2015, on East−South orientated berries from three treatments. Fully exposed (yellow bars), 30% grey shading (light grey bars), and 60% grey shading (dark grey bars). Median values of each treatment and time-point are marked by red lines. The black line represents air temperature measurements in the vicinity of the clusters.

Leucine enkephalin was used for lock mass calibration. The mobile phase transitioned from 95% water, 5% acetonitrile, 0.1% formic acid (phase A) to 0.1% formic acid in acetonitrile (phase B), with gradient transitioning from 100 to 60% phase A (0−8 min), 60−0% phase A (1 min), a gradual return to 100% phase A (3.5 min), and conditioning at 100−60% phase A (2.5 min), with a total run time of 15 min.

MassLynxTM software (Waters) version 4.1 was used for system control and data acquisition. Metabolite annotation was validated using the standard libraries described in Arapitsas et al.,[49] based on retention time order, given in Degu et al.[46] Metabolites were also annotated based on fragmentation patterns searched against the Chemspider metabolite database (http://www.chemspider.com/), the consistency of their retention times with those of identified metabolites, and comparison with the data in the current scientific literature.

The levels of detected metabolites referred as relative abundance are based on peak areas normalized to the total ion count and mass of each sample. Values of key metabolites were then quantified using calibration curves of purchased standards.

Chromatic Measurements of Wine Samples. Wine color measurements were based on the modified Somers assay as detailed in Mercurio et al.[50] Samples were diluted 10 times with a model wine solution (0.5% w/v tartaric acid in 12% ethanol adjusted to pH 3.4 with 5 M NaOH). This achieved both the dilution required to avoid saturation of absorbance values and equated the pH levels of the wines. Samples were placed in a 1 cm quartz cuvette, and their absorbance was measured at 420, 520, and 620 nm using a Spectronic 20 Genesys spectrophotometer (Thermo Fisher Scientific, MA, USA).

Statistics and Network Analysis. Statistical analysis was performed using R[51] v3.3.1 in RStudio. Repeated measures analysis of variance (ANOVA) was performed using the *lmer* function ("lme4" package),[52] and *p*-values were extracted using the *anova* function ("pbkrtest" package).[53] Significant differences at each time point were tested using the *aov* function for one-way ANOVA, and post hoc Tukey test ("agricolae" package).[54] The same method was used for comparing both meteorological and wine chromatic data.

The built-in *cor* function using the "pearson" method was used to construct separate Metabolite correlation matrices were constructed with the *cor* function using the "pearson" method for each treatment, using 18 points (i.e., three blocks, and six time-points). The correlation matrices were visualized using the *corrplot* function ("corrplot" package).[55]

Principle component analyses (PCA) were performed in JMP v10, based on correlations, following scaling of the data to metabolite median and log transformation. Correlation networks were constructed based on the Pearson correlation analyses (detailed earlier), using the MetScape application and the NetworkAnalyzer tool, available in Cytoscape v3.4.0. Correlations were incorporated into the network if the r value was $r > 0.5$ or $r < -0.5$.

RESULTS

Photoselective Shading Nets Filtered the Radiative Flux and Affected Light Spectral Characteristics. R_C measurements preformed in the cluster-zone (Figure 1) revealed that fully exposed berries received more than 50% of the total daily R_G measured above the canopy. The measured values under the grey nets of 30 and 60% types corresponded to their stated filtering capacity, transmitting 67.2 and 30.6% of the daily R_C in exposed clusters, respectively. The diurnal patterns showed that these differences were relatively stable throughout the day, except for low transmittance levels found during the first hour of sunlight, and a brief peak at midday, common to both grey-shaded treatments (Supp. Figure 5). This signified near-homogeneous reduction in R_C values. Total R_C in the 30% grey, red, and blue nets, measured by the spectroradiometer, revealed that the red and blue nets

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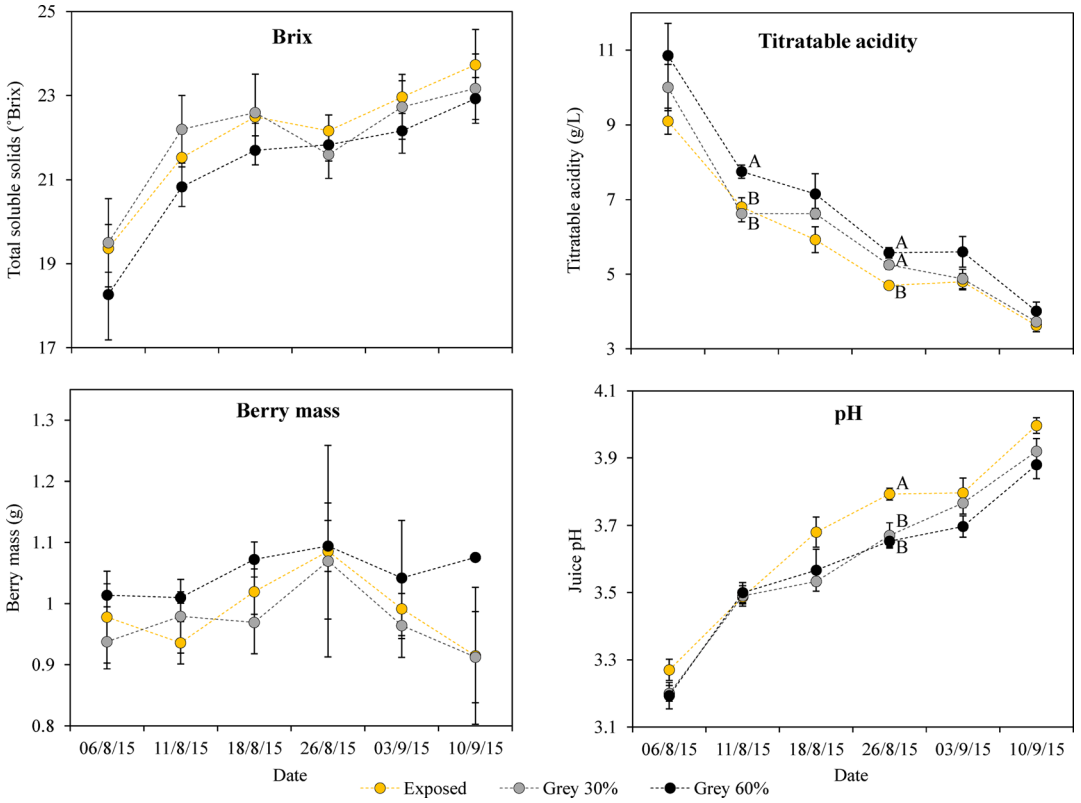

Figure 3. Maturity indices measured weekly throughout the ripening stage, on grapes from three treatments. Fully exposed (yellow), 30% grey shading (light grey), and 60% grey shading (dark grey) during the 2015 season. Points of the same date marked by different letters represent significant differences (ANOVA, $n = 3$, p-value < 0.05). Error bars represent the standard error.

transmitted 17 and 19% more energy compared to the 30% grey net (Supp. Figure 3A), yet this increase was not statistically significant ($p = 0.068$, $n = 3$). Spectroradiometer measurements demonstrated that the visible-light spectrum was indeed modified, yet with minor differences in the overall transmittance. The red net enriched the red light within the cluster-zone by up to 12%, coupled with a decrease of up to 20% in the blue−green wavebands. The blue net caused an enrichment of up to 15% in the blue waveband, peaking around 470 nm, with a minor decrease in the red waveband around 700 nm. Yet, in the shortwave infrared range, transmittance of both red and blue nets was clearly higher compared to the neutral grey nets. This explained the overall increase in transmitted R_C in the 30% blue and red nets compared to the grey net of the same shading factor.

Filtering R_C Mitigated Berry-Surface Temperature Extremes. Berry-surface temperature (BST) from the fully exposed, 30, and 60% grey net treatments were higher than ambient cluster-zone air temperature during day-time (7:00−17:00) and lower otherwise (Figure 2). Note that BST was measured for the berries' East-bound exposure axis; thus, BST was highest during midmorning hours, decreasing thereafter, owing to the shade casted by the overhead canopy. The nets decreased the median and maximum BST between 8:00 and 13:00, with a peak difference of 3.6 and 7.1 °C at 11:00 between the exposed and the 30% and 60% nets, respectively. From midday until 21:00, differences in median BST between fully exposed and 60% shaded clusters were smaller than 0.5 °C. However, minimum BST decreased from midday (13:00) in fully exposed and 30% shaded clusters, yet increased in 60% shaded clusters, leading to up to 3.4 °C (13:00) higher minimum BST compared to the fully exposed clusters. These differences were not related to cluster-zone ambient conditions, that is, air temperature and relative humidity, which were found to be unaffected by the presence and type of shading net applied (Supp. Figure 6).

Loss of Titratable Acidity and Berry Weight Were Minimized by Shading. Fruit maturity indices measured between veraison and harvest in 2015 (Figure 3) and 2014 (Supp. Figure 7) revealed that berries under 60% shading had greater mass, higher TA, and lower pH and TSS levels compared to exposed berries. Differences were found significant for all four indices in both seasons, in a repeated measures analysis, and amounted to an increase of 12.1/17.6% and 10.3/10.7% in berry mass and TA, respectively, and a decrease of 0.4/0.8°Brix in berry TSS, at the final sampling date in 2014/2015 season, respectively.

Principle Component Analysis Highlighted Ripening- versus Shading-Responsive Metabolites. Phenylpropanoid profiles obtained from berries, sampled at six time-points during the 2015 season, were used to construct a seasonal PCA to visualize differences in metabolic profile between time-points and between fully exposed and 60% shaded clusters (Figure 4). The first principle component (PC1) explained 39.8% of the variance and separated samples based on ripening stage, with values becoming more negative as ripening progressed. The eigenvector of PC1 (Supp. Table 1) was largely based on the anthocyanins, apart from malvidin metabolites, the flavan-3-ols, and to a lesser extent, the flavonols, and constructed an axis opposite to the direction of the flow of ripening, decreasing in abundance from SD1 toward harvest. In contrast, the stilbene piceid and coumarate hex. strongly affected PC1 and increased

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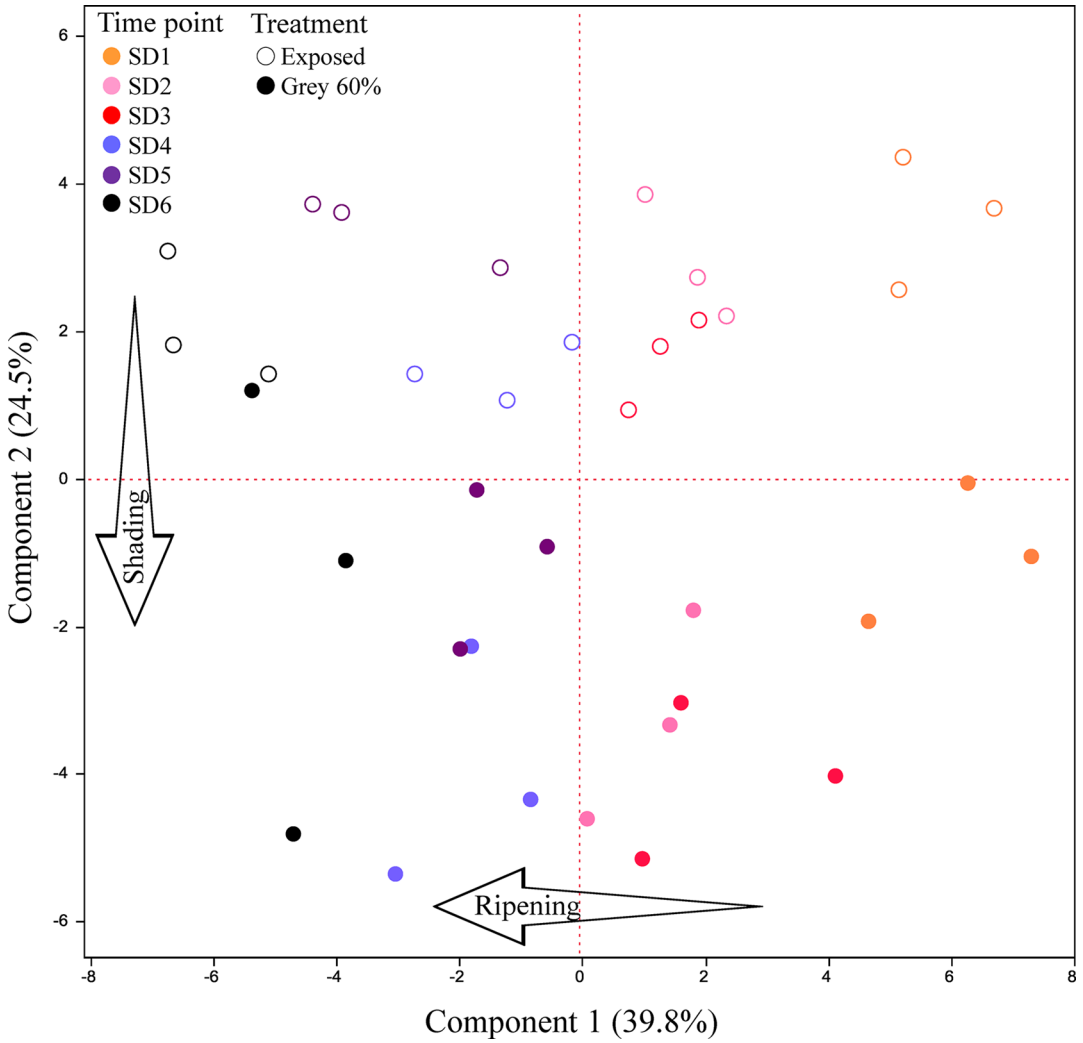

Figure 4. Principle component analysis plot of phenylpropanoid metabolites detected in grape skin samples from the 2015 season. Values used for this analysis are normalized peak areas. Samples are colored according to the sampling date. Hollow and filled circles represent samples of fully exposed and 60% shaded berries, respectively.

with ripening. The second principle component (PC2) explained 24.5% of the variance, reaching a cumulative percent of 64.3 of the total variance in the data set. PC2 separated samples based on the shading treatment, with samples from fully exposed and those from 60% shaded clusters located on its positive and negative scales, respectively. The eigenvector of PC2 (Supp. Table 1) was mainly based on the positive contribution of the anthocyanin cyanidin-3-glu, flavonols, and upstream metabolites of the phenylpropanoid pathway, including phenylalanine, narin-chalc-glu, and hydroxybenzoate hex., and the negative contribution of malvidin, flavan-3-ols metabolites, and coutaric acid.

Fruit Flavonoids Homeostasis Responded to Changes in Radiative Flux. Out of 36 annotated phenylpropanoid metabolites in grape skin tissues, the levels of 17 and 20 significantly differed between fully exposed, 30%, and 60% grey net-shaded berries, in a repeated measures analysis across the season, in 2014 and 2015 seasons, respectively. Of these, 11 metabolites showed consistent response to shading intensity, and similar temporal pattern, in both seasons (Figure 5). Among the anthocyanins, cyanidin-3-glu was negatively affected by shading, and three malvidin metabolites as well as peo-3-coum were positively affected by shading. The latter were 20–30% higher on average during the 2015 season in 60% shaded clusters compared to fully exposed. Differences between treatments were visible from the first time-point, 16 days following veraison, and remained relatively stable until harvest. In addition, all showed a gradual decrease toward harvest point, except for the coumarylated and acetylated forms of malvidin, and vitisin A, that reached their maximum levels 28, 36, and 45 DAV, respectively, and decreased thereafter. A similar trend was found for the flavan-3-ol metabolites. Differences in metabolite levels between treatments were stable from SD1 to harvest, showing a positive response to shading, with overall levels steadily decreasing along the season. Overall, flavan-3-ols levels were 27–42% higher on average during the 2015 season in 60% shaded clusters compared to fully exposed. In the flavonols, differences between treatments diminished during the season, reaching near-equal levels at harvest-point for quer-3-glu and kaemp-3-glr. This was mainly due to a seasonal decrease of up to 40% in the level of quer-3-glu and up to three-fold in the level of kaemp-3-glr in fully exposed and 30% shaded clusters, while non and an 80% decrease were measured for these metabolites in 60% shaded fruits, respectively. In contrast, differences in kamp-3-glu levels persisted throughout the

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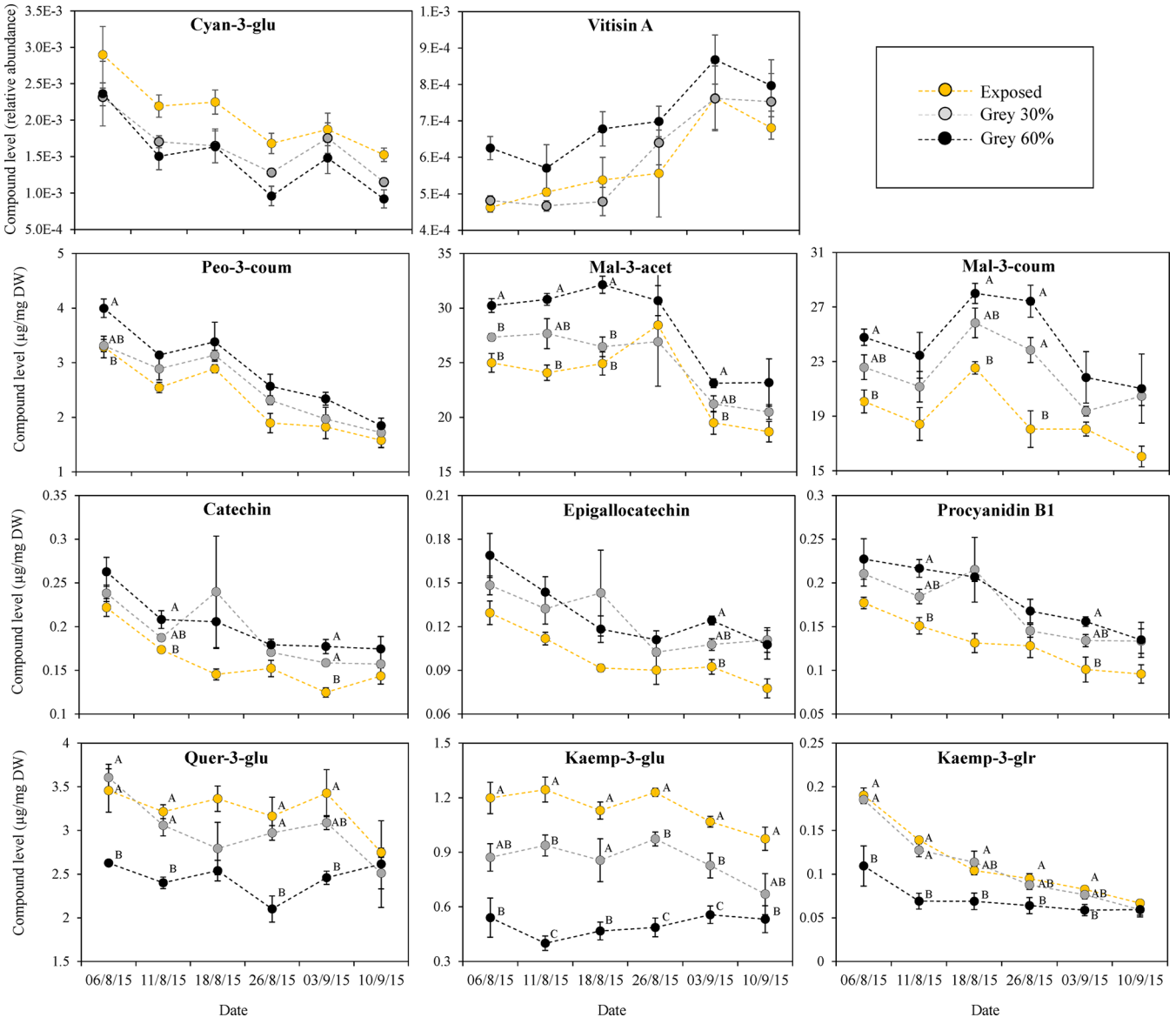

Figure 5. Grape skin flavonoids found to significantly differ between fully exposed (yellow), 30% grey shading (light grey), and 60% grey shading (dark grey) treatments, in a repeated measures analysis, and have similar temporal trend in both studied seasons. The levels represent measured values during the 2015 season. Points of the same date marked by different letters represent significant differences (ANOVA, $n = 3$, p-value < 0.05). Error bars represent the standard error.

season, and were 2.3-fold higher on average in fully exposed compared to 60% shaded clusters.

Modifying Light Spectral Characteristics Had Minor Effect on Flavonoid Composition. Comparison of grape-skin phenylpropanoid profile between the 30% blue, red, and grey nets resulted in small, but significant differences in the levels of four metabolites (Supp. Figure 8). These included the flavonol kaemp-3-glr, the anthocyanins delph-3-glu and pet-3-glu, and the stilbene piceid. Berries under the 30% blue net had on average 12 and 8% lower levels of the anthocyanins delph-3-glu and pet-3-glu, respectively, compared to 30% grey shaded berries, and 13% lower levels of kaemp-3-glr, compared to 30% red shaded berries. In contrast, berries under the blue net had, on average, up to 34% higher levels of piceid, and these differences reached to 55 and 58% compared to grey and red shaded treatments, respectively, at harvest. Taken together, modifying the R_C was found to have clearer and more pronounced effect on a larger number of phenylpropanoid metabolites compared to modifying the light spectra, as achieved by the photoselective shading.

Temporal-Based Correlation Analysis Revealed Positive Association between R_C Levels and Metabolic Coordination. Correlation analysis based on the seasonal time-series of each treatment (Figure 6A) revealed an overall positive coordination among the phenylpropanoid metabolites. A strong positive correlation was found among, and between, most anthocyanins, flavan-3-ols, and flavonols (i.e., flavonoids). Yet, the stilbenes, vitisin A, and upstream phenylpropanoid metabolites, including phenylalanine, coumarate hex, and hydroxybenzoate hex, were negatively correlated with these groups. Shading the clusters had a minor effect on the emerging correlation pattern; however, the correlation strength, both negative and positive, decreased with increasing intensity of shading. The proportion of positive correlations ($r > 0.5$) decreased from 48.3 to 35.8%, and that of the negative correlations ($r < -0.5$) decreased from 10.8 to 6.0% (Figure

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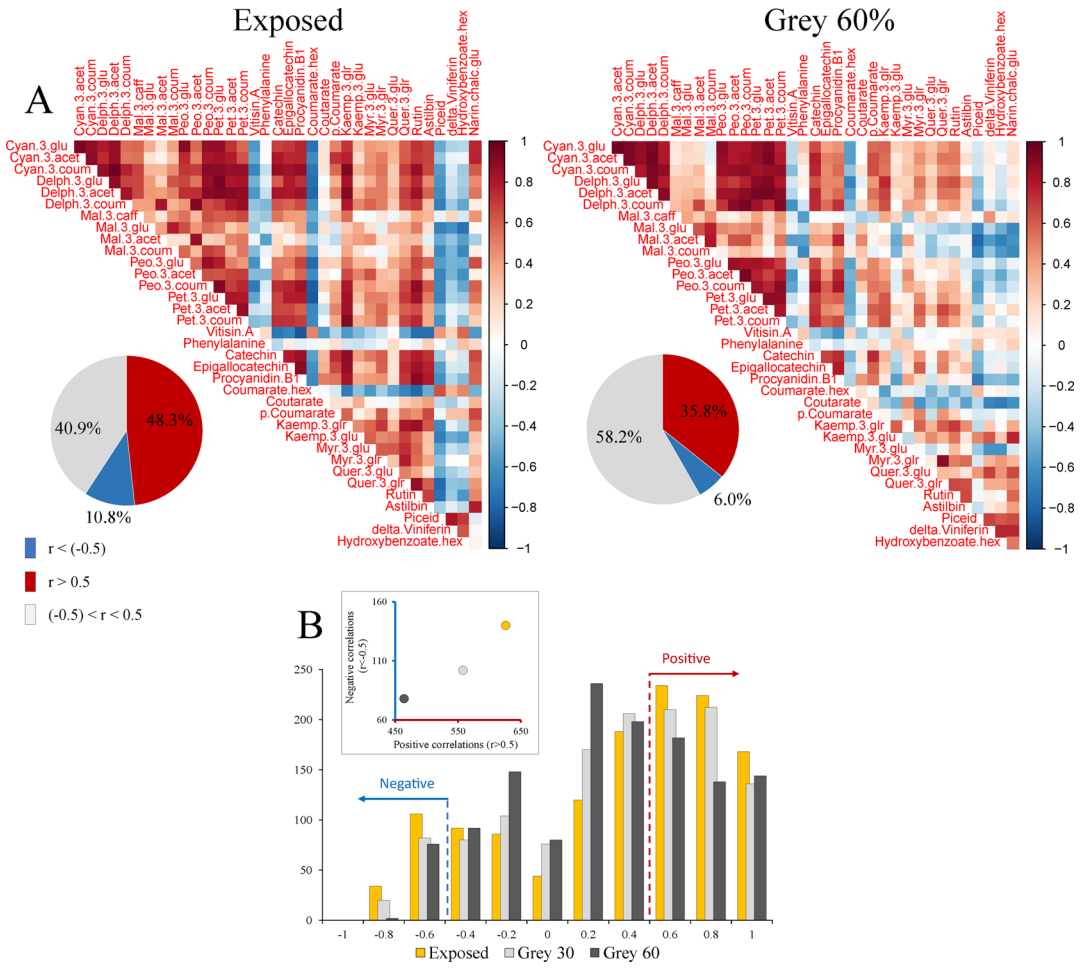

Figure 6. (A) Correlation matrices among grape phenylpropanoids from samples of fully exposed and 60% grey shaded berries based on temporal changes during the ripening stage of the 2015 season. Pie charts represent the distribution of the corresponding Pearson correlation coefficients. (B) Histogram of the Pearson correlation coefficient in the three shading treatments. Fully exposed, 30% grey, and 60% grey shading. In the inset, an $x-y$ scatterplot between the number of negative (r −0.5) and positive (r < −0.5) correlations in the three treatments.

6B) in 60% shaded, compared to fully exposed clusters. The correlation between malvidin metabolites and the rest of the anthocyanins, as well as that among the flavonoids, was impeded by shading. Correlation-based network analysis of fully exposed and 60% shaded clusters (Supp. Figure 9) highlighted the reduction in correlation due to shading, between the flavan-3-ols and flavonols; among the flavonols; between the flavonols and anthocyanins; and between narin-chalc-glu and the flavonoid groups. The decrease in correlation strength by shading led to a decrease in the clustering coefficient, average number of neighbors, and network density, and an increase in network heterogeneity (Supp. Table 2). Extracting the links common in both networks (Supp. Figure 10A) revealed that the strong negative correlation between the stilbene piceid and malvidin anthocyanins was not affected by shading. Finally, constructing a network containing the differences in temporal correlations between the treatments (Supp. Figure 10B) revealed a strong effect of shading on the negative correlations between upstream phenylpropanoids and malvidin anthocyanins, and the former and coutarate.

Wine Metabolic Profile and Chromatic Characteristics Reflected Changes in Fruit Composition and Highlighted the Effect of Shading. PCA of wine samples taken from the fully exposed and 60% shaded treatment (Figure 7) separated the two treatments on both principle components (PCs), with samples from the 30% shaded treatment not fully separated from either. PC1 represented 34.4% of the total variance and was mainly based on the inverse contribution of phenylalanine, and the contribution of the complete set of annotated anthocyanins, p-coumarate, and coutarate, and the flavonols quer-3-glu and myricetin. Samples of the 60% shaded treatment obtained higher values on PC1 compared to those of the fully exposed treatment. PC2 represented 30.6% of the total variance, reaching a cumulative percent of 65 of the total variance in the data set. PC2 mainly separated the treatments (exposed vs 60% shaded) based on the positive contribution of peonidin- and delphinidin-glucoside, narin-chalc-glu, and the complete set of annotated flavonols, apart from quer-3-glu, and myricetin. The Procyanidin B1, coumarate, and stilbenes metabolites, and the coumarylated metabolites of malvidin and peonidin, were the major negative contributors to PC2.

Wine metabolic profiles were characterized by larger within-treatment variability compared to that found in berry samples. As a result, only five metabolites were found to significantly differ in their levels between wines made from fully exposed and 60% shaded clusters (Supp. Table 3). Nevertheless, traces of the descriptors in the fruits were found in the wine. For example, p-coumaric and coutaric acids, and the coumarylated anthocyanins of malvidin and peonidin, were found higher in the wines made from 60% shaded clusters, while the flavonol

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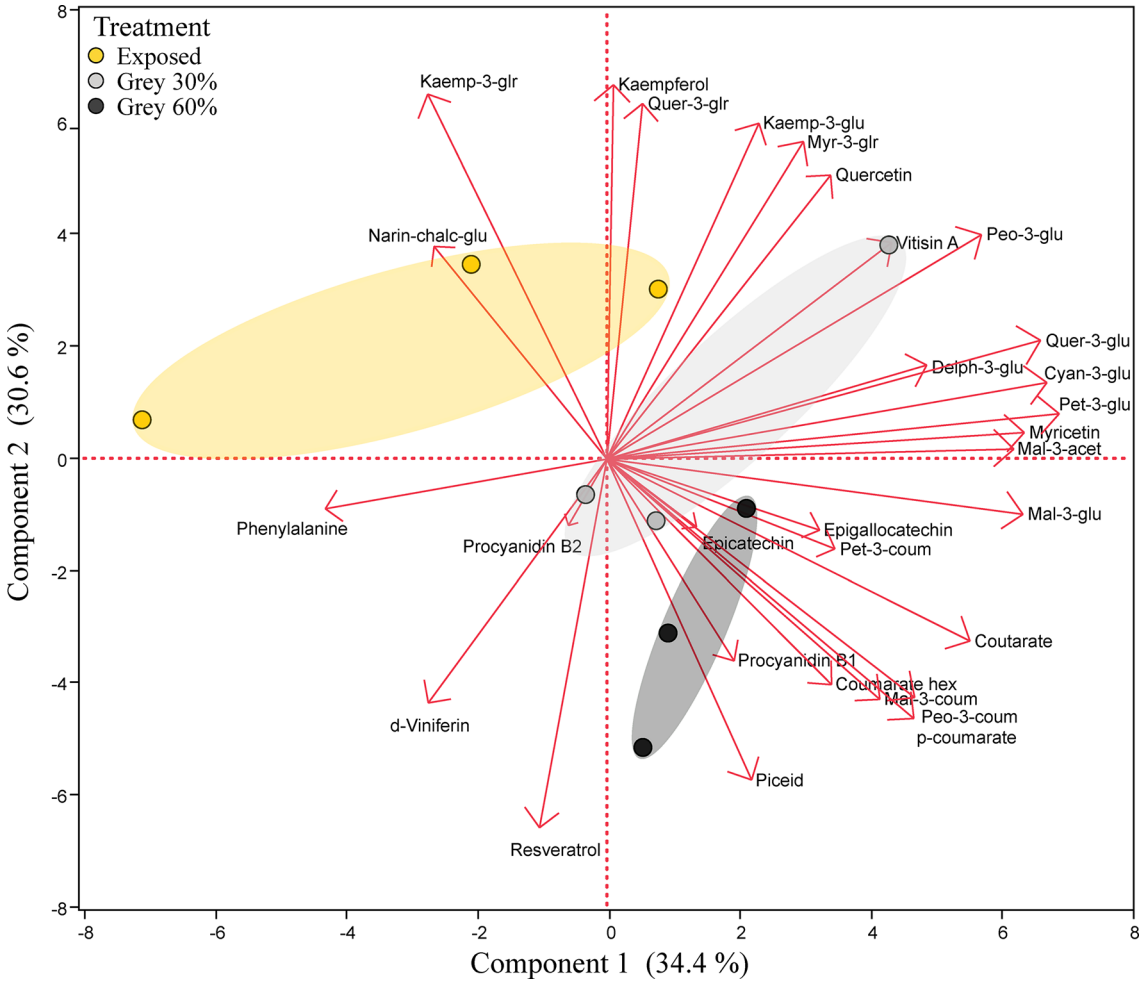

Figure 7. Principle component analysis biplot of phenylpropanoid metabolites detected in wines made from three shading treatments: Fully exposed, 30% grey, and 60% grey shading, in the 2015 season. Values used for this analysis are normalized peak areas. Samples are colored according to the treatment. Arrow length and direction represents the importance of each metabolite to the projected principle components.

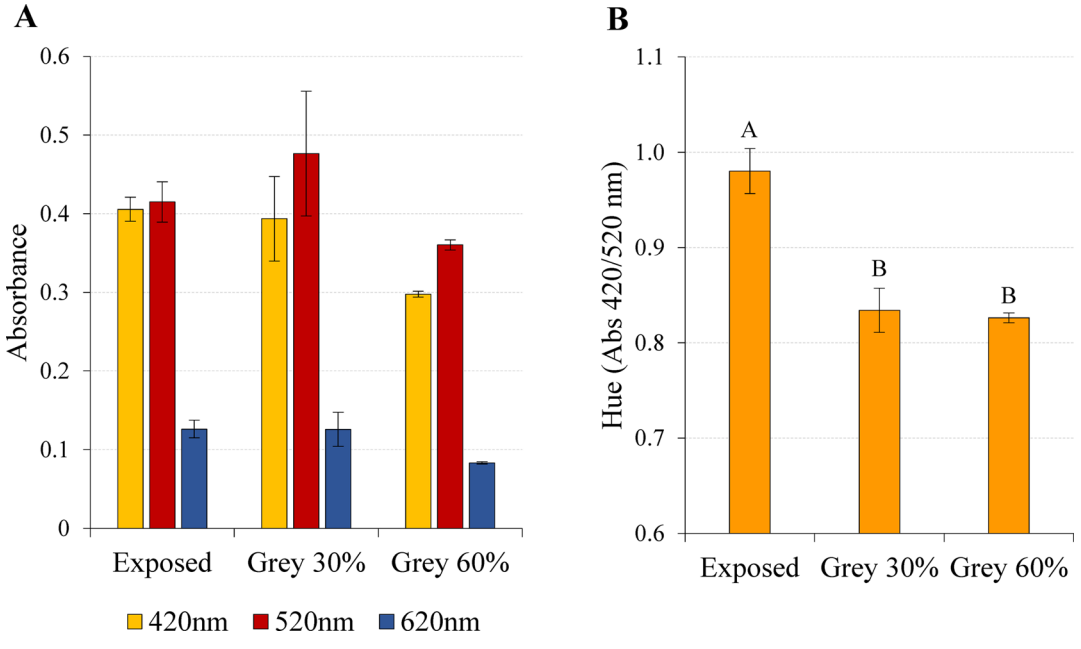

Figure 8. Chromatic measurements of wines made from three shading treatments. Fully exposed, 30% grey, and 60% grey shading, in the 2015 season. (A) Absorbance values as measured by a spectrophotometer. (B) Calculated hue values based on the values presented in panel A.

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kaemp-3-glr was found in higher levels in wines made from fully exposed clusters.

Wine chromatic measurements (Figure 8A) revealed that shading led to a slight, but not significant, decrease in the absorbance at both 420 and 620 nm, while the absorbance at 520 nm, associated with red color, was highest under the partial shading (i.e., 30% grey net). Hue values (Figure 8B), calculated as the ratio of absorbance at 420 nm divided by that measured at 520 nm, significantly decreased with shading. This indicates that wines made from shaded treatments tended more toward red, rather than yellow−orange tones, compared to wines made from the fully exposed treatment. While differences in values measured at each wavelength were found nonsignificant, a three-dimensional representation of the absorbance values (Supp. Figure 11) separated samples by treatment, each having a specific chromatic profile based on the measured wavelengths.

DISCUSSION

Excess R_C and elevated air temperatures are increasingly common in grapevine growing regions. Yet, in depth knowledge regarding the impact of modulating cluster micrometeorology on grape and wine metabolic profile is lacking. Our results demonstrate that restricting the application of photoselective shading nets to the cluster-zone can dramatically decrease fruit temperature during sunlit hours without affecting ambient air temperature and relative humidity, as opposed to the case of overcanopy shading.[56] This can be attributed to the important role played by solar irradiance in the thermal balance of the fruit.[57] Therefore, differences found between shaded and nonshaded treatments in this study are the outcome of changes in irradiance and temperature regimes at the tissue level (i.e., berry).

Fruit shriveling and lack of acidity are typical attributes of grapes grown in warm climates and have been characterized in a large number of studies.[38,44,58−61] Shriveling might be the result of deformation of fruit cuticular wax structure by direct solar irradiance[62] and enhanced transpiration derived by high vapor pressure deficit, common under hot and dry conditions.[63] Low levels of malate were ascribed to the induction of its degradation through the TCA cycle caused by elevated temperatures.[64,65] In this study, shading the fruit-zone was found to restrict berry weight loss and improve the retention of acidity. The observed reduction in berry weight loss is in accordance with Oliveira et al.[66] and could not be related to vapor pressure deficit, which was found unaffected by cluster-zone shading. Instead, it was most probably achieved by limiting the damage to fruit cuticular wax structure through the screening of R_C. The improvement in acidity levels is in accordance with previous works dealing with the effect of shading on fruit composition[67,68] and highlights the importance of daily short-term increases in fruit temperature on the levels of organic acids.

The manipulation of light spectra of biologically active wavebands (i.e., blue and red) was previously found to affect fruit flavonoids and most prominently, anthocyanin composition.[69−71] Nevertheless, in this study, differences were minute. It should be noted that in this trial, light modifications were performed on the clusters, while the canopy was largely excluded. This raises the possibility that in the mentioned works, nonfruit organs were also affected by the treatments, which resulted in a more pronounced metabolic response, compared to that measured here. Conversely, it may also point to the fact that under these experimental conditions, light was a nonlimiting factor, and that, as previously suggested, temperature differences were at the basis of the measured metabolic response.[39]

In contrast to spectral modifications, filtering the R_C coupled by lowering berry surface temperature significantly affected the levels of 11 flavonoids in a stable manner during two consecutive seasons. Shaded berries had lower levels of flavonols, found in accordance with similar works that demonstrated their positive association with fruit R_C.[25,26,28,72] Conversely, shading led to higher accumulation of the major anthocyanins and procyanidins, known to be hampered by elevated temperatures and postveraison sun-exposure, respectively.[26,33,41,42,44,73] However, while this shift in flavonoid composition was visible from the first sampling date, the temporal dynamics in compound accumulation, from that point onward, remained unaffected. Current understanding regarding the interplay between solar irradiance, temperature, and the accumulation of anthocyanins holds that the perception of light exposure by the fruit triggers their biosynthetic pathway, while elevated fruit temperatures inhibits this process and may favor their degradation.[41,42,74] On the basis of this understanding, and since related biosynthetic genes are expressed during the ripening stage and compound levels are dynamic,[11,41,75,76] one could expect the net compound turnover to differ between conditions and result in differential temporal trends, as opposed to the findings here and elsewhere.[25,77] This pattern, in which the fruit responds to environmental perturbation by readily acquiring an altered compositional equilibrium, instead of exhibiting a gradual change, points toward a mechanism of chemical homeostasis rather than a continuous modulation by light and temperature stimuli.

Homeostasis is enabled by the tight biochemical coordination, which characterizes developmental metabolism.[78] While temporal trends of metabolite abundance were found to be similar between treatments, comparing their temporal-based correlation strength highlighted the deviations in metabolite change from the common trends. Our analysis, performed across temporal changes during fruit ripening, revealed that higher radiative flux was associated with enhanced metabolic coordination. This is in agreement with other studies addressing the response of grape composition to water stress.[11,48] In the current study, lower correlation coefficients found within the flavonols, and between them and other phenylpropanoids, in 60% shaded compared to exposed clusters reflected the limited loss of several flavonols during ripening, decoupling their temporal trend from that of other major phenylpropanoid groups.

Wine composition is largely determined by the composition of the grape.[79−81] However, the plethora of metabolites, and the complexity of the winemaking processes, renders any extrapolation from grape to wine so far inaccurate. Indeed, we found that the variability introduced by the process of winemaking masked a large number of differences that were found significant between grape samples, but not between their resulting wines. Nevertheless, our results revealed that shading was associated with higher levels of anthocyanins, coumaric acid derivates, procyanidin B1, and stilbenes, while phenylalanine, narin-chalc-glu, and flavonols were generally lower. The results can be traced back to the differences found between the grape samples, except for the stilbenes. These compositional differences, introduced by shading, created a separable chromatic fingerprint for each treatment. Overall, shading

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resulted in wines with significantly higher red/yellow color tone ratio, generally perceived as superior for red wines.[82]

Our results demonstrate that fruit microclimate and its chemical composition can be modulated by manipulating the penetrating light regime. Under the conditions tested and characterized here, the radiative flux was found as a more prominent factor than light spectral characteristics in exerting fruit compositional response. Filtering R_C led to weaker metabolic associations between the phenylpropanoids across the season. However, this resulted in improved grape and wine parameters generally known to be hampered by warm climate, such as berry weight, retention of acidity, and the accumulation of anthocyanins and flavan-3-ols, and led to improved wine color. Further understanding of the compositional consequence of fruit photoreceptor activation, and quantification of their responsiveness thresholds, is necessary to develop light management strategies that will harness the excess irradiance to optimize fruit and wine quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04881.

Workflow, shading-net layout, background meteorological and micrometeorological measurements, additional figures and tables of grape and wine analysis (PDF)

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Author Contributions

N.R., N.A., and A.F. conceived and planned the study. N.R. applied the viticultural treatments, collected the berry samples, and performed all field measurements. N.R. performed the sample extraction and analysis using the UPLC-QTOF-MS device. N.R. integrated and analyzed the data. N.R. wrote the body of the paper with A.F. and N.A. All authors reviewed and approved the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Cyan-3-glu, Cyanidin-3-O-glucoside; Pet-3-glu, Petunidin-3-O-glucoside; Peo-3-glu, Peonidin-3-O-glucoside; Mal-3-glu, Malvidin-3-O-glucoside; Delph-3-glu, Delphindin-3-O-glucoside; Delph-3-acet, Delphinidin-3-O-(6″-acetyl-glucoside); Cyan-3-acet, Cyanidin-3-O-(6″-acetyl-glucoside); Pet-3-acet, Petundin-3-O-(6″-acetyl- glucoside); Mal-3-acet, Malvindin-3-O-(6″-acetyl-glucoside); Peo-3-acet, Peonidin-3-O-(6″-acetyl-glucoside); Delph-3-coum, Delphinidin-3-O-(6″-p-coumaroyl-glucoside); Mal-3-caffe, Malvidin-3-O-(6″-caffeoyl-glucoside); Cyan-3-coum, Cyanidin-3-O-(6″-p-coumaroyl-glucoside); Pet-3-coum, Petunidin-3-O-(6″-p-coumaroyl-glucoside); Peo-3-coum, Peonidin-3-O-(6″-p-coumaroyl-glucoside); Mal-3-coum, Malvidin-3-O-(6″-p-coumaroyl-glucoside); Myr-3-glr, Myricetin-3-O-glucuronide; Rutin, Quercetin-3-O-rutinoside; Myr-3-glu, Myricetin-3-O-glucoside; Quer-3-glr, Quercetin-3-O-glucuronide; Quer-3-glu, Quercetin-3-O-glucoside; Kaemp-3-glr, Kaempferol-3-O-glucuronide; Kaemp-3-glu, Kaempferol-3-O-glucoside; Narin-chalc-glu, Naringenin-chalcone-4-O-glucoside; Hex, Hexoside

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