Improving fruit anthocyanins in 'Cabernet Sauvignon' by shifting fruit ripening and irrigation reduction post veraison in warmer region

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Summary

In warmer regions, forcing vines to regrow and shifting fruit ripening to the cooler portion of the growing season can increase the concentration of total fruit anthocyanins (TFA) in red winegrapes, but the effect on anthocyanin composition remains unclear. Additionally, irrigation reduction post veraison was reported to improve fruit anthocyanins in cool and temperate regions with low precipitation, whereas this response has not been previously examined in forced vines grown in dry and warm regions. Experiments were conducted with 'Cabernet Sauvignon' (*Vitis vinifera* L.) over two consecutive years in Fresno, California, to investigate the effect of shifting fruit ripening on the concentration and composition of fruit anthocyanins as well as the influence of post-veraison irrigation reduction on fruit anthocyanins of forced vines. Vines under conventional practices (non-forced) were used as the control. Forcing treatment included removing primary leaves, clusters, and laterals, as well as hedging primary shoots, in mid-June. Control vines were irrigated at 80 % crop evapotranspiration (ET_C) post veraison, whereas forced vines were irrigated at 40, 60, 80, or 100 % ET_C post veraison. Results suggest that forcing vines to regrow and shifting fruit ripening led to a significant increase of TFA, primarily non-acylated anthocyanins, during fruit ripening and at harvest over two years. Forcing treatment also altered composition of fruit anthocyanins at harvest, with increased proportions of TFA comprised by the glucosides of delphinidin and petunidin but the decreased proportion of the glucosides of malvidin. This study demonstrates that forcing vines to regrow and shifting fruit ripening in the warmer region can lead to a more balanced profile of fruit anthocyanins, with improved non-acylated derivatives and altered relative abundance of the glucosides of five anthocyanidins. Reducing irrigation post veraison, however, had only a minor effect on fruit anthocyanins in forced vines.

K e y   w o r d s :   anthocyanins; temperature; irrigation reduction; warmer region.

Introduction

The low fruit anthocyanins and poor color profile at harvest is one of the major concerns for red winegrape production in warmer regions. The low fruit anthocyanins at harvest is primarily attributed to the suppressed anthocyanin accumulation during fruit ripening. Indeed, anthocyanins are sensitive to high temperatures. The reduction of fruit anthocyanins was observed previously in a number of red winegrape varieties when berries were exposed to temperatures greater than 30 °C during fruit ripening (Kliewer 1977, Spayd *et al.* 2002, Mori *et al.* 2005 and 2007, Downey *et al.* 2006, Yamane *et al.* 2006, Tarara *et al.* 2008, Azuma *et al.* 2012). On the other hand, fruit ripening often takes place in the hottest period of the growing season. Particularly in the warmer regions, vines are often exposed to temperatures that exceed 30 °C throughout the period of fruit ripening (Winkler *et al.* 1974, Jackson and Lombard 1993, Bergqvist *et al.* 2001). In this regard, high temperatures during fruit ripening is likely the primary contributor for low fruit anthocyanins in red winegrapes produced in warmer regions.

To expose berries to lower temperatures during fruit ripening, vines grown in warmer regions can be forced to regrow. A recent study on 'Cabernet Sauvignon' in the San Joaquin Valley of California indicated that forcing vines to regrow can shift fruit ripening from July and August to September, October, and early November. As a result, compared with vines under conventional practices, forced vines can produce berries smaller in size, with lower pH, higher titratable acid, and greater total anthocyanins and total phenolics (Gu *et al.* 2012). Moreover, when 'Shiraz' vines are double pruned to bear fruit in the summer and in the winter, the fruit ripening in the cool winter has greater total anthocyanins than the fruit ripening in the hot summer (Favero *et al.* 2011). Both studies showed that shifting fruit ripening and exposing berries to a cooler condition during fruit ripening can enhance total fruit anthocyanins in red winegrapes in the warmer region. Little is known, however, about the response of individual fruit anthocyanins when vines are forced to regrow and fruit ripening is shifted from the summer to the autumn. In addition, the influence of forcing treatment on anthocyanin composition at harvest remains unclear.

Anthocyanins are anthocyanidin aglycones that are bound to sugars. Glucosides of five anthocyanidins, including delphinidin, cyanidin, petunidin, peonidin, and malvidin, are identified in red winegrape varieties like 'Merlot' and 'Cabernet Sauvignon'. Those anthocyanins can be acylated (with acetic acid or *p*-coumaric acid) or remain non-acylated (Spayd *et al.* 2002, Mori *et al.* 2007, Tarara *et al.* 2008). The accumulation of individual fruit anthocyanins and their concentration at harvest differ among varieties and are affected by environmental factors, such as temperature. For

example, non-acylated anthocyanins seem to be less stable than their acylated counterparts under high temperatures. In addition, high temperatures tend to suppress the glucosides of delphinidin, cyanidin, petunidin and peonidin more than the glucosides of malvidin (Spayd *et al.* 2002, Mori *et al.* 2007, Tarara *et al.* 2008). Since fruit anthocyanins have varied responses to high temperatures, it is possible that forcing vines to regrow and shifting fruit ripening can influence individual anthocyanins differently and alter anthocyanin composition at harvest.

Moreover, reducing irrigation post veraison is considered another practice to improve fruit anthocyanins in red winegrapes. However, its application in warmer regions is overall limited. It is in part because reducing irrigation under high temperatures may impose vines to excessive water stress and negatively affect vine growth and fruit quality (Hardie and Considine 1976). Additionally, in regions with moderate to warm climate, irrigation reduction post veraison seems to have only a small influence on fruit anthocyanins (Sivilotti *et al.* 2005, Keller *et al.* 2008). On the other hand, in the cool and temperate regions with low precipitation, lowering irrigation rate post veraison can restrain berry growth, enhance anthocyanin accumulation, and thereby improve fruit anthocyanins (Bucchetti *et al.* 2011, Castellarin *et al.* 2007, Ojeda *et al.* 2002, Roby *et al.* 2004). When vines are forced to regrow in the warmer region, berries are subjected to a cool environment during fruit ripening. Thus, reducing irrigation post veraison may improve fruit anthocyanins in the forced vines.

Previously, Gu *et al.* (2012) demonstrated the impacts of forcing vines to regrow and shifting fruit ripening on vine productivity and fruit composition in 'Cabernet Sauvignon' in the warmer region. As a follow-up, this study was conducted in two consecutive years at a commercial vineyard in Fresno, California, to assess the effect of shifting fruit ripening in the warmer region on the accumulation of individual fruit anthocyanins, to understand the influence of shifting fruit ripening on the composition and concentration of fruit anthocyanins at harvest, and to investigate the impact of irrigation reduction post veraison on the concentration and composition of fruit anthocyanins in forced vines.

Material and Methods

Plant materials and experimental conditions: This study was conducted with mature 'Cabernet Sauvignon' grapevines (Clone 7A) grafted on 'Freedom' rootstock in 2013 and 2014 at a commercial vineyard in Fresno, California. The vineyard was established in 1992 on a sandy-loam soil with east-west oriented rows. The row spacing was 3.7 m and vine spacing was 2.1 m. Vines were trained with a single trunk and bilateral cordons and pruned to 16 three-bud spurs per vine. Vines used for this research were of similar size, healthy, and had uniform pest and disease management as well as weed control.

Experiment I was conducted to investigate the effect of shift fruit ripening by forcing treatment on the concentration and composition of fruit anthocyanins during fruit ripening and at harvest. The experiment was set in randomized blocks, consisting of one control and one forcing treatment. Three

replicates with eight vines each were established for each treatment in 2013, while four replicates with six vines each were established for each treatment in 2014. Different vines were used for the experiment in 2013 and 2014, to avoid potential carryover effects. Vines grown under conventional practices (non-forced) were used as controls. Forcing treatment included removing primary clusters, leaves, and laterals, as well as hedging primary shoots to six nodes, in mid of June, as described in Gu *et al.* (2012). Weak shoots were removed prior to the application of forcing treatment. Nitrogen fertilizer (UAN-32) was applied at 28 kg N·ha⁻¹ to forced vines before full bloom. Both control and forced vines were irrigated at 100 % crop evapotranspiration (ET_C) prior to veraison and at 80 % ET_C post veraison.

Experiment II was conducted to evaluate the effect of post-veraison irrigation rates on the fruit anthocyanin in forced vines. The experiment was set in randomized blocks, consisting of four irrigation treatments. Three replicates with eight vines each were established for each treatment in 2013. Four replicates with six vines each were established for each treatment in 2014. The experiment was conducted in different locations of the vineyard in each year. Vines were forced and fertilized, as described in Experiment I, and irrigated at 100 % ET_C prior to veraison. Those forced vines were then irrigated at 40, 60, 80, or 100 % ET_C from veraison to harvest. The two border vines of each replicate were excluded from sampling, to avoid potential interference from the adjacent irrigation treatments. The total irrigation amount post veraison is shown in Tab. 1 for forced vines irrigated at 40, 60, 80, or 100 % ET_C. The total irrigation amount was slightly higher in 2014 than 2013, due in part to a longer duration of fruit ripening.

Table 1

Total irrigation amount post veraison for forced 'Cabernet Sauvignon' vines irrigated at 40, 60, 80, and 100 % crop evapotranspiration post veraison (40, 60, 80, and 100 % ET_C) during 2013 and 2014 growing seasons in Fresno, CA, USA

Irrigation rate (% ET_C)	Total irrigation amount (L·vine⁻¹)	
	2013	2014
40	522	591
60	795	833
80	1060	1090
100	1295	1363

ET_C was estimated weekly based on the reference evapotranspiration (ETo) and crop coefficient (Kc). Weekly ETo was obtained from the California Irrigation Management Information System (CIMIS) weather station No.80 located on the campus of California State University, Fresno (CA, USA), approximately 1 km from the experimental vineyard. The Kc was calculated by multiplying a constant of 1.7 with the ratio between canopy width and row spacing (Williams and Ayars 2005).

Vine phenology: Dates of veraison and harvest were recorded for both control and forced vines. Date of veraison was recorded when approximately 50 % of berries were soft. Fruit was harvested when the content of total soluble solids (TSS) was approximately 25 °Brix. Fruit ripening was defined as the period between veraison and

harvest. The dates of bud break and anthesis for control and forced vines in this study were similar to that reported by Gu *et al.* (2012). For control vines, the dates of veraison were 10 July in 2013 and 3 July in 2014, and the date of harvest were 27 August in 2013 and 13 August in 2014. For forced vines, the dates of veraison and harvest were 15 September in both years, and the harvest dates were 29 October in 2013 and 4 November in 2014. Irrigation treatments had no influence on the harvest date of forced vines. The duration of fruit ripening was approximately 7 weeks for control and forced vines in 2013. In 2014, the duration of fruit ripening was approximately 6 weeks and 8 weeks for control and forced vines.

Analysis of heat accumulation: Hourly temperatures were obtained from CIMIS station No. 80. Heat accumulation was expressed as the number of hours during fruit ripening that vines were exposed to specific temperatures ranging from 0 to 40 °C. Heat accumulation was calculated for both control and forced vines in 2013 and 2014. In addition, daily maximum and minimum air temperature for control and forced vines during fruit ripening were obtained from CIMIS station No. 80 for both years.

Measurements for leaf stomatal conductance: The mid-day leaf stomatal conductance was measured on four fully-exposed expanded leaves per treatment replicate for forced vines receiving different irrigation treatments post veraison in 2014 using leaf porometer (model SC-1, Meter Environment, Pullman, WA, USA). The measurements were taken in the sunny days before irrigation started.

Analysis for fruit anthocyanins. Thirty-berry samples were collected randomly from the fruiting zone of control vines, starting from veraison in 2013 and from when the content of TSS was approximately 23 °Brix in 2014. In forced vines, samples of 30 berries were collected randomly from the fruiting zone starting from veraison in 2013 and 2014. Samples were weighed using a digital scale (model PL 200, Mettler-Toledo, Schwerzenbach, Switzerland) and then stored at -20 °C for analysis of fruit anthocyanins within three months.

At the time of extraction, berry samples were removed from the freezer and thawed to room temperature. Berry skin was separated from the pulp and rinsed with higher purity type 1 water (18 MΩ; Millipore). After drying off the residual water, skin was placed in 30 ml of acetone: water (30:70, v/v) solution and then agitated on a Standard Orbital Shaker (VWR, Radnor, PA, USA) at room temperature in the dark for 24 h. Fifteen ml of the extract were filtrated through #1 filter paper (Whatman, Pittsburgh, PA, USA), and additional five ml of acetone: water (30:70, v/v) solution was used to rinse off the anthocyanins left on the filter paper. For berries collected in 2013 and those from control vines in 2014, acetone was removed from 20 mL of filtrate under -7.5 kPa at 38 °C for 50 min using a parallel vortex evaporator (model Multivapor P-12; Buchi Corporation, New Castle, DE, USA). The aqueous solution was then stored at -20 °C. For berries collected from forced vines in 2014, acetone was removed using a freeze dry system. After 20 mL of the filtrate was collected, one ml was placed in a 1.5 mL centrifuge tube. Acetone in the filtrate was removed by a CentriVap benchtop centrifugal vacuum concentrator (model 7810010; Labconco, Kansas City, MO, USA) with a cold trap (model 7385020; Labconco, Kansas City, MO, USA) at -103 °C. Samples in centrifuge tubes were then saved at -80 °C until the date of analysis.

The analysis of anthocyanins was performed using a high-performance liquid chromatography (HPLC) system (Shimadzu Co. Ltd., Kyoto, Japan). The method for analysis of anthocyanins was described in Lamuela-Raventos and Waterhouse (1994). A C18 analytical column (Novapak, 300 mm x 3.9 mm, 4 μm; Waters Corporation, Milford, MA, USA) with the fitted guard column was used to separate individual anthocyanins. Extracts were thawed at room temperature, diluted 5 times, and filtrated using 0.45 μm PTFE membranes (VWR International, Radnor, PA, USA). Ten μl of each sample were injected for analysis with the flow rate at 0.5 mL·min⁻¹. The temperature of the column oven was maintained at 40 °C during the analysis. Anthocyanin detection occurred at the wavelength of 520 nm and identified based on the retention time and previous report (Lamuela-Raventos and Waterhouse 1994). Malvidin 3-glucoside chloride (Sigma-Aldrich Corporation, St. Louis, MO, USA) was used as the standard. The concentration of anthocyanins was expressed as mg malvidin 3-glucoside equivalent per kg fresh berries basis.

Fifteen anthocyanins, including the non-acylated and acylated glucosides (with acetic acid or *p*-coumaric acid) of delphinidin, cyanidin, petunidin, peonidin, and malvidin, were detected in the berries of control and forced vines during fruit ripening, except that cyanidin 3-coumaoroyl-glucoside was not found in the berries of control vines at harvest in 2013 and in the last two weeks of fruit ripening in 2014.

Data analysis: Data of 2013 and 2014 were analyzed separately. The effect of forcing treatment on fruit anthocyanins at harvest was assessed with *t* test. Diagnostic plots were used to check the homogeneity of variance and normality of the data before *t* test, and no transformation was needed. To investigate the effect of irrigation reduction post veraison on fruit anthocyanins in forced vines, data of fruit anthocyanins were analyzed using analysis of variance (ANOVA) for each sampling date with irrigation rate as the main factor. Where significant difference ($p < 0.05$) was indicated by ANOVA, Tukey's honestly significant difference (HSD) test was followed to separate the means at the significance level of 0.05. Levene's test and Shapiro-Wilk test were used to test homogeneity of variance and normality before data were analyzed using ANOVA. No transformation was needed for data of 2013 or 2014. The statistical analysis was performed in R (veraison 3.3.3; R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Temperature difference between control and forced vines during fruit ripening: The daily maximum and minimum air temperature differed between control and forced vines during fruit ripening in 2013 and 2014 (Fig. 1). Throughout the period of fruit ripening, the daily maximum air temperature ranged between 18 and 37 °C for forced vines, while it fluctuated between 29 and 40 °C for control vines. The daily
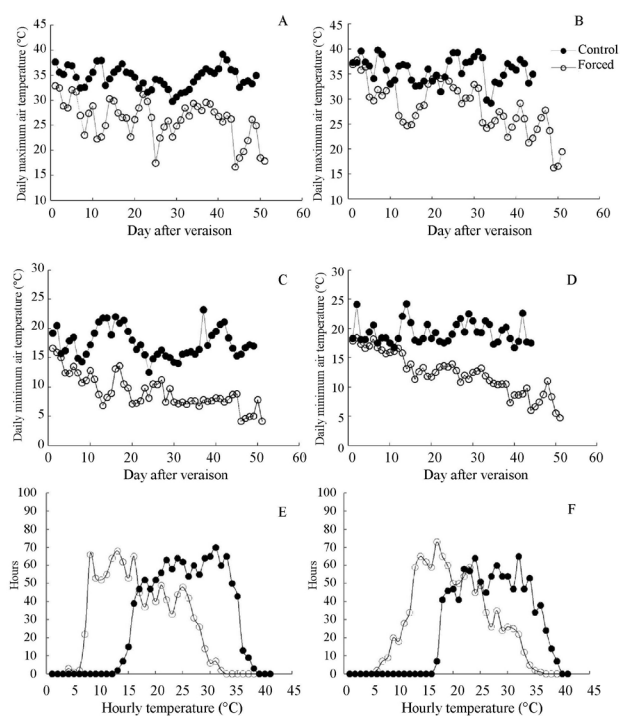
Fig. 1: Daily maximum air temperature (**A** and **B**), daily minimum air temperature(**C** and **D**), and heat accumulation (**E** and **F**) during fruit ripening from veraison to harvest for unforced control and forced 'Cabernet Sauvignon' grapevines (Control and Forced) during 2013 (**A**, **C**, and **E**) and 2014 (**B**, **D**, and **F**) growing seasons in Fresno, CA, USA.

minimum air temperature during fruit ripening was in the range between 4 and 19 °C for forced vines in both years. For control vines, by comparison, the daily minimum air temperature during fruit ripening was respectively between 13 and 24 °C in 2013 and between 18 and 24 °C in 2014. The daily maximum and minimum air temperature tended to decrease as fruit ripening proceeded in forced vines, but they remained fluctuated during fruit ripening for control vines across two years.

Moreover, the pattern of heat accumulation varied between control and forced vines during ripening period in both years (Fig. 1). In terms of vine exposure to low temperatures, forced vines were subjected to air temperature lower than 15 °C for more than 300 h throughout the period of ripening, while control vines were exposed to those low temperatures for less than 20 h. The duration of vine exposure to temperatures between 16 and 30 °C was approximately 600 h, similar for control and forced vines. As regards vine exposure to high temperatures, forced vines were subjected to temperatures greater than 30 °C for less than 70 h, whereas control vines were exposed to those high temperatures for more than 200 h during ripening. The duration of fruit ripening was similar for control and forced vines in 2013, while it was about two weeks shorter for control vines than forced vines in 2014. Nevertheless, forced vines were exposed less frequently to high temperatures and more often to low temperatures during fruit ripening as compared to control vines across two years.

Effect of forcing treatment on fruit anthocyanins during fruit ripening: The accumulation of fruit anthocyanins was altered by forcing treatment, which had a similar effect on total fruit anthocyanins (TFA) in both years. In 2013, the concentration of TFA increased in both control and forced vines, although the increasing rate was greater for forced vines in the first five weeks after veraison. In the following two weeks, TFA increased in forced vines while declined in control vines, resulting a large departure of TFA between control and forced vines in the last two weeks of fruit ripening (Fig. 2A). In 2014, the concentration of TFA increased gradually in forced vines as fruit ripening processed, whereas it decreased slightly in control vines in the last two weeks of fruit ripening (Fig. 2B).

The pattern of total non-acylated anthocyanins accumulated in control and forced vines during fruit ripening was similar to that of TFA, and yet the magnitude of increase in total non-acylated anthocyanins in the forced vines tended to be more substantial than that of TFA (Fig. 2C and 2D). Among non-acylated anthocyanins, the concentration of delphinidin, cyanidin, petunidin, and peonidin 3-glucoside was significantly greater in forced vines than control vines throughout the entire process of fruit ripening in 2013 and between the 5th and 7th week after veraison in 2014 (Fig. 3A, 3D, 3H, 3K, 4A, 4D, 4H, and 4K). Malvidin 3-glucoside, on the other hand, increased at a similar rate in control and forced vines in the first five weeks of ripening in 2013. In the last two weeks of ripening, however, its concentration kept increasing in forced vines while declined in control vines (Fig. 3N). In 2014, the concentration of malvidin 3-glucoside increased in forced vines while remained similar in control vines, between the 5th and 7th week after veraison (Fig. 4N). Consequently, malvidin 3-glucoside was present in a greater concentration in forced vines than control vines in the late stage of ripening in both years.

In comparison to the influence of forcing treatment on non-acylated anthocyanins, its influence on acylated anthocyanins was smaller. The concentration of total anthocyanins acylated with acetic acid was slightly greater in forced vines than control vines in the first five weeks after veraison in 2013, but their concentration became similar in control and forced vines thereafter (Fig. 2E). In 2014, the concentration of total anthocyanins acylated with acetic was similar in control and forced vines between the 5th and 7th week after veraison (Fig. 2F). Among anthocyanins acylated with acetic acid, the response of delphinidin and cyanidin 3-acetyl-glucoside to forcing treatment was most consistent across two years; their concentration was greater in forced vines than control vines during fruit ripening in 2013 and between the 5th and 7th week after veraison in 2014 (Fig. 3B, 3E, 4B and 4E). The response of petunidin, peonidin, and malvidin 3-acetyl-glucoside to forcing treatment differed between 2013 and 2014. The concentration of petunidin and peonidin 3-acetyl-glucoside was generally greater in forced vines than control vines during fruit ripening in 2013, while their concentration was similar in control and forced vines between the 5th and 7th week after veraison in 2014 (Fig. 3I, 3L, 4I, and 4L). In terms of malvidin 3-acetyl-glucoside, its concentration increased throughout the entire period of ripening in forced vines in 2013. In control vines, however, the concentration of malvidin 3-acetyl-glucoside increased, peaked at the 6th week after veraison, and then declined. As a result, although malvidin 3-acetyl-glucoside was greater in
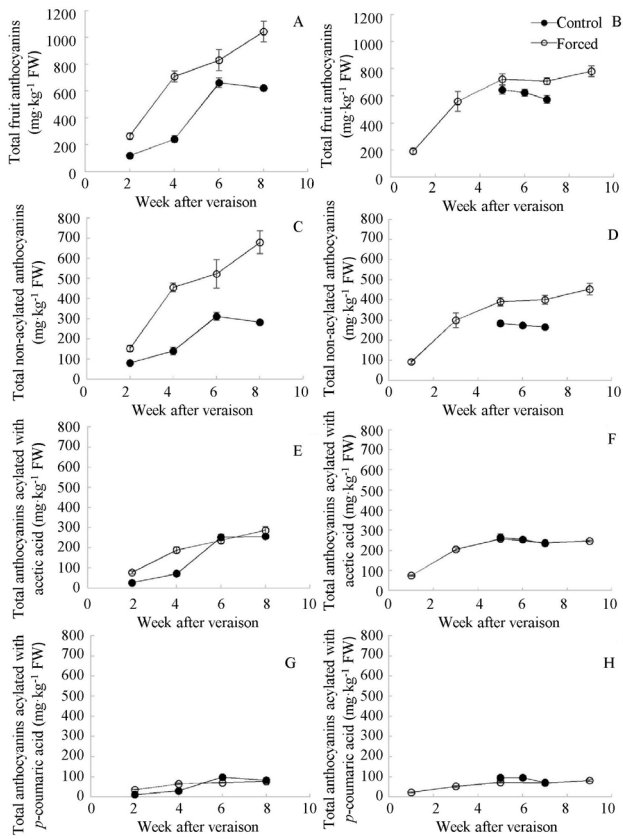
Fig. 2: Accumulation of total fruit anthocyanins (**A** and **B**), total non-acylated anthocyanins (**C** and **D**), total anthocyanins acylated with acetic acid (**E** and **F**), and total anthocyanins acylated with *p*-coumaric acid (**G** and **H**) in unforced control and forced 'Cabernet Sauvignon' vines irrigated at 80 % crop evapotranspiration post veraison (Control and Forced) during 2013 (**A, C, E,** and **G**) and 2014 (**B, D, F,** and **H**) growing seasons in Fresno, CA, USA. Concentration is expressed as mg malvidin-3-glucoside equivalent per kg fresh fruit basis. Values represent means and standard error of three replicates in 2013 and four replicates in 2014. Fruit was harvested at the 8th week after veraison for both control and forced vines in 2013, and at the 7th week after veraison for control vines and at the 9th week after veraison for forced vines in 2014.
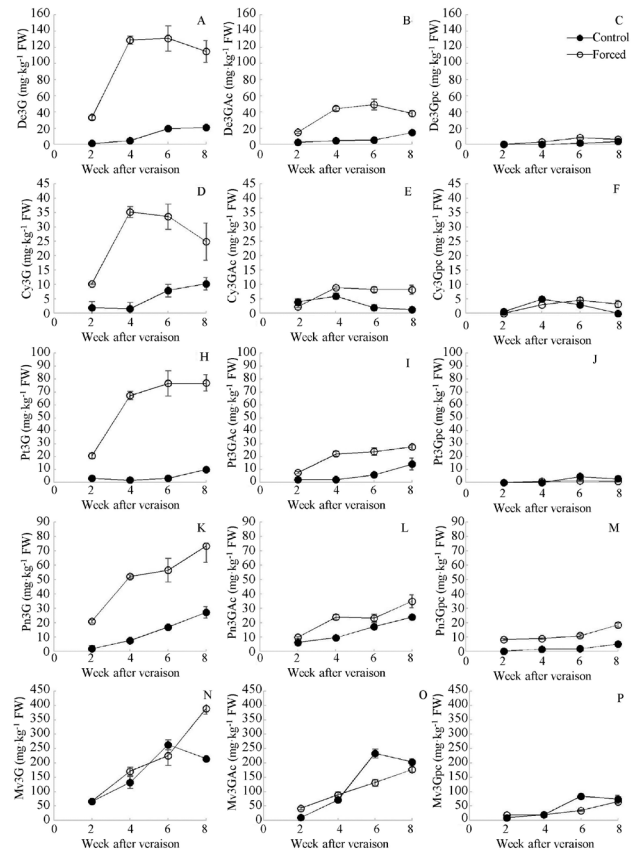
Fig. 3: Accumulation of individual anthocyanins in unforced control and forced 'Cabernet Sauvignon' vines irrigated at 80 % crop evapotranspiration post veraison (Control and Forced) during 2013 growing season in Fresno, CA, USA. Concentration is expressed as mg malvidin-3-glucoside equivalent per kg fresh fruit basis. The abbreviations for individual fruit anthocyanins are shown in Tab. 2. Values represent means and standard error of three replicates. Fruit was harvested at the 8th week after veraison for both control and forced vines.

control vines at the 6th week after veraison, its concentration was similar in control and forced vines in other sampling dates during the period of ripening (Fig. 3O). In 2014, the concentration of malvidin 3-acetyl-glucoside was similar in control and forced vines between the 5th and 7th week after veraison (Fig. 4O). Moreover, the concentration of anthocyanins acylated with *p*-coumaric acid was similar in control and forced vines during fruit ripening across two years, except that peonidin 3-coumaroyl-glucoside had a greater concentration in forced vines than control vines in 2013 (Fig. 2G, 2H, 3, and 4).

Previously, Gu *et al.* (2012) demonstrated that forcing vines to regrow and shifting fruit ripening to the cooler portion of the growing season can enhance TFA. In this study, by investigating individual anthocyanins during fruit ripening in control and forced vines, we found that the increase of TFA with forcing treatment is largely attributed to the marked increase of non-acylated anthocyanins, especially delphinidin, cyanidin, petunidin, and peonidin 3-glucoside. It explains the large difference in accumulation of non-acylated anthocyanins between control and forced

vines. In control vines, the accumulation of non-acylated anthocyanins during fruit ripening reflected mainly the change of malvidin 3-glucoside, owing to the low concentration of delphinidin, cyanidin, petunidin, and peonidin 3-glucoside. When vines are forced to regrow, however, the accumulation of non-acylated anthocyanins mirrored the increase of all the five non-acylated anthocyanins in the early stage of ripening, and then it was primarily influenced by peonidin and malvidin 3-glucoside in the late stage of fruit ripening. Additionally, the accumulation pattern of total acylated anthocyanins seems similar in control and forced vines. It is because the concentration of total acylated anthocyanins during fruit ripening was determined mainly by malvidin 3-acetyl-glucoside in both control and forced vines. Provided that malvidin 3-acetyl-glucoside had a minor response to forcing treatment, the concentration of acylated anthocyanins differs only marginally between control and forced vines, although delphinidin and cyanidin 3-acetyl-glucoside tended to increase with forcing treatment.

Non-acylated and acylated anthocyanins showed a distinct response to forcing treatment, which is likely attributed to the difference in heat sensitivity between non-acylated and acylated anthocyanins and the difference in temperature during fruit ripening between control and forced vines. The difference between non-acylated and acylated anthocyanins
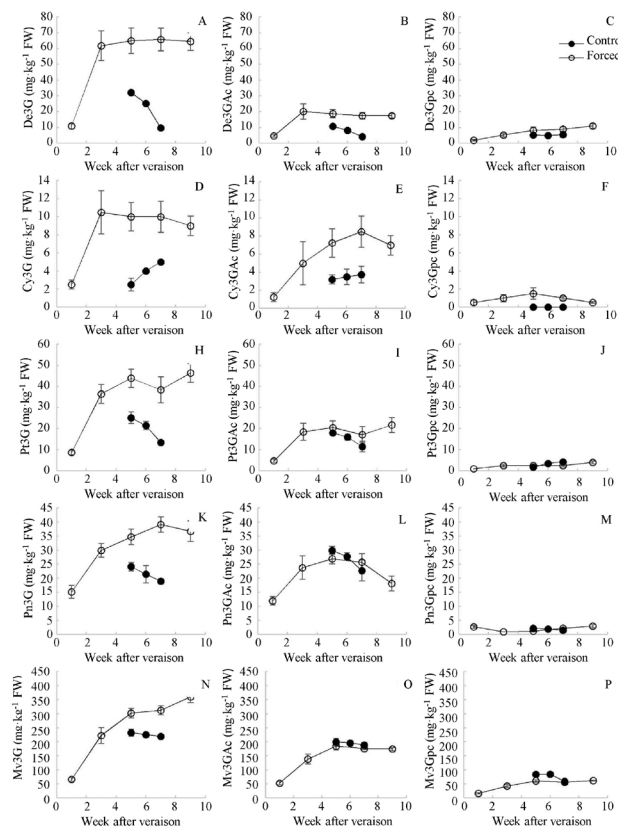
Fig. 4: Accumulation of individual fruit anthocyanins in unforced control and forced 'Cabernet Sauvignon' vines irrigated at 80 % crop evapotranspiration post veraison (Control and Forced) during 2014 growing season in Fresno, CA, USA. Concentration is expressed as mg malvidin-3-glucoside equivalent per kg fresh fruit basis. The abbreviations for individual fruit anthocyanins are shown in Tab. 2. Values represent means and standard error of four replicates. Harvest was in the 7th week after veraison for control vines and in the 9th week after veraison for forced vines.

in the response to high temperatures was well documented in previous studies. Although non-acylated and acylated anthocyanins were both found to decrease under temperatures greater than 30 °C in 'Merlot', 'Cabernet Sauvignon', and 'Pione' (*Vitis vinifera* x *Vitis labruscana*), the reduction of non-acylated anthocyanins under high temperature was more substantial than their acylated counterparts (Mori *et al.* 2007, Tarara *et al.* 2008, Azuma *et al.* 2012). Indeed, in comparison to non-acylated anthocyanins, their acylated derivatives show a better solubility and greater stability under the low pH of vacuoles, which in part explains their relatively lower sensitivity to high temperatures (Bąkowska-Barczak 2005). The difference in the response to high temperatures between non-acylated and acylated anthocyanins can in part explain their varied response to the forcing treatment. Berries of control vines ripened in the hottest month of the growing season (July and August), and thereby control vines were exposed to temperatures above 30 °C for more than 30 % of the time during ripening. Once vines were forced to regrow, fruit ripening took place in the cooler months of the season (September, October, and early November) and berries were subjected to temperatures above 30 °C for less than 10 % of the time during ripening (Fig. 1). Consequently, the accumulation of non-acylated anthocyanins in control vines can be largely suppressed by the high temperature,

resulting in low concentration during fruit ripening. When vines were forced to regrow and fruit ripening was shifted to a cooler period of the season, non-acylated anthocyanins were no longer limited by high temperature, and so their concentration had a remarkable increase during fruit ripening. Acylated anthocyanins, however, seem not much affected by high temperature during fruit ripening in control vines, and therefore shifting fruit ripening to the cooler portion of the season had a small effect on their concentration.

The glucosides of different anthocyanidins tended to have varied response to forcing treatment. The glucosides of delphinidin and cyanidin showed a significant increase as vines were forced to regrow, especially delphinidin and cyanidin 3-glucoside, across two years. The glucosides of petunidin and peonidin responded differently to forcing treatment between years. The concentration of petunidin and peonidin 3-glucoside, petunidin and peonidin 3-acetyl-glucoside, and peonidin 3-coumaroyl-glucoside, increased with forcing treatment in 2013. In contrast, only petunidin and peonidin 3-glucoside showed greater concentration in forced vines than the control in 2014. As to the glucosides of malvidin, malvidin 3-glucoside differed mainly in the last stage of ripening between control and forced vines, and yet, malvidin 3-acetyl-glucoside and malvidin 3-coumaroyl-glucoside were similar in control and forced vines. In general, the glucosides of delphinidin, cyanidin, peonidin, and petunidin are likely more responsive to forcing treatment than the glucosides of malvidin. This observation can be linked to the difference in the tolerance to high temperature between the glucosides of different anthocyanidins. In 'Merlot' and 'Cabernet Sauvignon', the glucosides of delphinidin, cyanidin, petunidin and peonidin were indicated to decrease significantly in berries that were often exposed to temperature greater than 30 °C through the period of ripening, while the glucosides of malvidin were affected by temperatures to a smaller extent (Mori *et al.* 2007, Tarara *et al.* 2008).

Effects of forcing treatment on fruit anthocyanins at harvest: Berries of control and forced vines were harvested when the content of TSS was approximately 25 °Brix. At harvest, berry weight was respectively 1.16 g for control vines and 0.73 g for forced vines in 2013, and 1.13 g for control vines and 0.98 g for forced vines in 2014.

Forcing treatment resulted in great increase of TFA, primarily non-acylated anthocyanins, at harvest (Tab. 2). The concentration of TFA in forced vines was 96 % greater in 2013 and 43 % greater in 2014 as compared to control vines; the concentration of total non-acylated anthocyanins in forced vines was three-fold greater in 2013 and two-fold greater in 2014 than that of control vines. The response of TFA and total non-acylated anthocyanins to forcing treatment tended to be stronger in 2013 than 2014, likely due to smaller berry size at harvest in 2013. This increase in non-acylated anthocyanins with forcing treatment was attributed to the significant increase of delphinidin, cyanidin, petunidin, peonidin, and malvidin 3-glucoside across two years. Furthermore, the concentration of total anthocyanins acylated with acetic acid was slightly higher in forced vines than the control in 2013, but their concentration did not differ in 2014. Although delphinidin 3-acetyl-glucoside was present in greater concentration in forced vines in both years, the

increase in the concentration of cyanidin, petunidin, and peonidin 3-acetyl-glucoside with forcing treatment was observed only in 2013. Malvidin 3-acetyl-glucoside, on the other hand, had a lower concentration in forced vines than the control in 2013, while its concentration was similar in control and forced vines in 2014. Moreover, the concentration of anthocyanins acylated with *p*-coumaric acid was similar in control and forced vines across two years.

As the concentration of individual anthocyanins at harvest was altered by forcing treatment, the composition of fruit anthocyanins differed between control and forced vines in 2013 and 2014 (Tab. 3). The proportions of TFA comprised by the glucosides of delphinidin and petunidin were greater but the proportion of malvidin was smaller in forced vines, in comparison to control vines, across two years. The proportions of TFA comprised by the glucosides of cyanidin and peonidin were greater in forced vines than control vines in 2013, whereas their proportions were similar in control and forced vines in 2014. In addition, with forcing treatment, the partition of TFA between non-acylated and acylated forms was shifted towards non-acylated form in both years. Moreover, the partition of TFA between di-hydroxylated and tri-hydroxylated branches was shifted towards dihydroxylated branch in forced vines in 2013, whereas this partition was unaffected by forcing treatment in 2014.

The difference in fruit anthocyanins at harvest can be carried into wines, since most

Table 2

Concentrations of individual fruit anthocyanins, total non-acylated anthocyanins, total acylated anthocyanins (with acetic acid or p-coumaric acid), and total fruit anthocyanins (TFA) at harvest in unforced control and forced 'Cabernet Sauvignon' grapevines irrigated at 80 % crop evapotranspiration post veraison (Control and Forced) during 2013 and 2014 growing seasons in Fresno, CA, USA

Year/ Treatment	Non-acylated anthocyanins (mg·kg⁻¹ FW)ᶻ					
	De3G	Cy3G	Pt3G	Pn3G	Mv3G	Total
2013						
Control	8 bˣ	2 b	4 b	17 b	215 b	246 b
Forced	122 a	29 a	81 a	78 a	399 a	709 a
2014						
Control	14 b	5 b	13 b	19 b	219 b	270 b
Forced	64 a	9 a	37 a	47 a	361 a	518 a
	Anthocyanins acylated with acetic acid (mg·kg⁻¹ FW)					
	De3GAc	Cy3GAc	Pt3GAc	Pn3GAc	Mv3GAc	Total
2013						
Control	6 b	1 b	2 b	9 b	201 a	219 b
Forced	40 a	9 a	28 a	37 a	180 b	294 a
2014						
Control	8 b	4 a	11 a	23 a	189 a	235 a
Forced	17 a	7 a	16 a	22 a	175 a	230 a

Year/ Treatment	Anthocyanins acylated with *p*-coumaric acid (mg·kg⁻¹ FW)						TFA (mg·kg⁻¹ FW)
	De3Gpc	Cy3Gpc	Pt3Gpc	Pn3Gpc	Mv3Gpc	Total	
2013							
Control	4 a	nd	2 a	3 a	75 a	84 a	549 b
Forced	6 a	3	1 a	4 a	64 a	78 a	1081 a
2014							
Control	9 a	nd	2 a	2 a	60 a	73 a	578 b
Forced	11 a	1	3 a	4 a	63 a	82 a	830 a

ᶻ Concentrations of anthocyanins are expressed as mg malvidin-3-glucoside equivalent per kg fresh berries. De3G, delphinidin 3-glucoside; Cy3G, cyanidin 3-glucoside; Pt3G, petunidin 3-glucoside; Pn3G, peonidin 3-glucoside; Mv3G, malvidin 3-glucoside. Adding the annotation of Ac and pc indicates that anthocyanins are acylated respectively with acetic acid and p-courmaric acid. Cyanidin 3-coumaroyl-glucoside was not detected (nd) in control fruit in both years at harvest.
ˣ Data of 2013 (n = 3) and 2014 (n = 4) are analyzed separately. Means within each column for each year followed by different low-case letters indicate significantly different by t test at the significant level of 0.05.

Table 3

Proportion of total fruit anthocyanins (TFA) grouped by the glucosides of five anthocyanidins (Glucosides), by acylation (Non-acylated vs acylated anthocyanins), and by hydroxylation branches of phenylpropanoid biosynthetic pathway (Di-hydroxylated vs tri-hydroxylated anthocyanins) in control and forced 'Cabernet Sauvignon' grapevines irrigated at 80 % crop evapotranspiration post veraison (Control and Forced) during 2013 and 2014 growing seasons in Fresno, CA, USA

Year/ Treatment	Glucosides (% TFA)ᶻ					Non-acylated vs acylated anthocyanins (% TFA)		Di-hydroxylated vs tri-hydroxylated anthocyanins (% TFA)	
	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin	Non-acylated	Acylated	Di-hydroxylated	Tri-hydroxylated
2013									
Control	3 bˣ	1 b	2 b	5 b	89 a	45 b	55 a	7 b	93 a
Forced	16 a	4 a	10 a	11 a	59 b	66 a	34 b	15 a	85 b
2014									
Control	7 b	2 a	4 b	8 a	79 a	47 b	53 a	10 a	90 a
Forced	11 a	2 a	7 a	9 a	71 b	61 a	39 b	11 a	89 a

ᶻ Delphinidin: all the glucosides of delphinidin; Cyanidin: all the glucosides of cyanidin; Petunidin: all the glucosides of petunidin; Peonidin: all the glucosides of peonidin; Malvidin, all the glucosides of malvidin; Non-acylated, all non-acylated anthocyanins; Acylated, all anthocyanins acylated with acetic acid or *p*-coumaric acid; Dihydroxylated, all the glucosides of cyanidin and peonidin; Trihydroxylated, all the glucosides of delphinidin, petunidin, and malvidin.
ˣ Data of 2013 (n = 3) and 2014 (n = 4) are analyzed separately. Means within each column followed by different low-case letters are significantly different by t test at the significant level of 0.05.

of the anthocyanin in wines are from the harvested berries. Therefore, given the difference in fruit anthocyanins between control and forced vines at harvest, the color of wines made from the fruit of forced vines can differ from wines made from the fruit of control vines. Previously, Gu *et al.* (2012) indicated that the shift of fruit ripening by forcing treatment resulted in greater TFA, tannins and other phenolics in 'Cabernet Sauvignon' at harvest in the warmer region of California. In this study, we found that the shift of fruit ripening can also contribute to a more balanced profile of fruit anthocyanins at harvest. For example, the attributes of anthocyanins to color is dependent on the relative abundance of the glucosides of five anthocyanins. Blueness of the wine can increase with the presence of glucosides of delphinidin, cyanidin, and petunidin, whereas redness improves with the presence of the glucosides of malvidin and peonidin (Keller and Hrazdina 1998). Given that forcing treatment resulted in the increased proportion of TFA comprised by the glucosides of delphinidin and petunidin while the decreased proportion of the glucosides of malvidin, it may shift the color of fruit and wines towards blueness. Moreover, as the shift of fruit ripening can lead to the increase of anthocyanins, tannins, and other phenolics at harvest, anthocyanins in wines made from the fruit of forced vines should have greater chance for polymerization and copigmentation. Considering that polymerization with tannins and copigmentation with other phenolic compounds can stabilize anthocyanins and improve the intensity of wine color during fermentation and aging (Cheynier *et al.* 2006), the color of wines made from fruit of forced vines should be deeper and more stable as compared to wines made from the fruit of control vines. As a result, forcing vines to regrow and shifting fruit ripening to the cooler portion of the growing season can potentially improve the color intensity and balance of red wine produced in warmer regions.

Effects of irrigation reduction post veraison on fruit anthocyanins. Irrigation reduction post veraison had generally limited effect on fruit anthocyanins in forced vines. Although the irrigation rate post veraison was reduced significantly from vines irrigated at 100 % ET_C to those irrigated at 40 % ET_C (Tab. 1), the concentration of individual fruit anthocyanins during fruit ripening was similar in forced vines, regardless of irrigation treatments (data not shown). At harvest, the concentration of the glucosides of delphinidin, petunidin, peonidin and malvidin, as well as the concentration of TFA, were greater in forced vines irrigated at 40, 60, and 80 % ET_C, compared to vines irrigated at 100 % ET_C, in 2013. In 2014, however, irrigation rates post veraison had no effect on anthocyanin concentrations at harvest (Tab. 4). In addition, berry size, the proportion of TFA comprised by five anthocyanidins, and the partition of TFA between non-acylated and acylated forms in forced vines was unaffected by irrigation rates post veraison (data not shown).

Previous studies conducted in regions with cool to moderate climate showed that reducing irrigation post veraison improved the concentration of fruit anthocyanins by restraining berry growth, increasing anthocyanin accumulation, or the combination of both (Ojeda *et al.* 2002, Roby *et al.* 2004, Bucchetti *et al.* 2011). However, as shown in this study, reducing irrigation post veraison overall lacked effect

T a b l e 4

Concentration of glucosides of five anthocyanidins and total fruit anthocyanins (TFA) in forced 'Cabernet Sauvignon' vines irrigated at 40, 60, 80, and 100 % crop evapotranspiration post veraison (40, 60, 80, and 100 % ETC) during 2013 and 2014 growing seasons in Fresno, CA, USA

Year/ Irrigation rate (% ET_C)	Glucosides (mg·kg⁻¹ FW)ᶻ					TFA (mg·kg⁻¹ FW)
	Delphi-nidin	Cyani-din	Petuni-din	Peo-nidin	Mal-vidin	
2013						
40	165 aˣ	39 a	108 b	127 a	645 a	1087 a
60	162 b	31 a	103 b	119 a	636 a	1051 a
80	168 b	41 a	110 a	119 a	643 a	1081 a
100	111 c	25 a	76 c	92 b	517 b	821 b
2014						
40	86 a	19 a	62 a	65 a	618 a	861 a
60	88 a	20 a	67 a	69 a	622 a	872 a
80	92 a	17 a	56 a	73 a	599 a	837 a
100	90 a	21 a	73 a	69 a	600 a	854 a

ᶻ see the footnote of Tab. 3 for abbreviations.
ˣ Data of 2013 (n = 3) and 2014 (n = 4) are analyzed separately. Means within each column followed by different low-case letters are significantly different by Tukey's HSD at the significant level of 0.05.

on fruit anthocyanins and berry growth in forced vines, even though which fruit ripening took place in the cool period of the season. Although the concentration of TFA in forced vines increased with irrigation reduction in 2013, this increase seems too small to influence wine color profile. One possible explanation for the results is that forced vines may not be stressed enough by irrigation reduction post veraison and therefore fruit anthocyanins remained little affected. Indeed, despite the irrigation rate was reduced to 40 % ET_C post veraison, the average mid-day stomatal conductance during fruit ripening in 2014 was 93 mmol· m⁻²·s⁻¹, suggesting that vines seem only moderately stressed with such a low irrigation rate post veraison. Nevertheless, it seems not uncommon that fruit anthocyanins show minor or no response to irrigation reduction post veraison. Previous experiment on 'Merlot' in the temperate region indicated that decreasing mid-day stomatal conductance to below 50 mmol·m⁻²·s⁻¹ with irrigation reduction post veraison did not affect the concentration of fruit anthocyanins at harvest (Sivilotti *et al.* 2005). Additionally, Keller *et al.* (2008) suggested that reducing irrigation post veraison affected the color of fruit in only one of the five experimental years under a moderate climate. Thus, this study indicated that reducing irrigation post veraison can decrease water use for forced vines, while its effect on enhancing fruit anthocyanins was overall small.

Conclusions

As vines were forced to regrow in the warmer region, berries were exposed to a cooler environment throughout the period of ripening and the concentration of TFA increased considerably. The response of fruit anthocyanins to forcing treatment was likely linked with their status of acylation and the anthocyanidin associated. Forcing vines to regrow resulted in a significant increase of non-acylated anthocyanins

while it had a minor influence on their acylated derivatives during fruit ripening. Additionally, forcing treatment had a large impact on the glucosides of delphinidin, whereas it showed a small effect on the glucosides of malvidin. The difference in fruit anthocyanins during fruit ripening between control and forced vines was carried further to the harvested fruit. In comparison to the fruit produced from control vines, the fruit of forced vines had greater concentration of TFA and more balanced anthocyanin profile, which can contribute to a more vibrant color of red wines produced in the warmer region. Moreover, different from the expectation, reducing irrigation post veraison had an overall small effect on fruit anthocyanins in forced vines, suggesting the lower irrigation rate post veraison may not necessarily improve anthocyanins even fruit ripening took place under a cooler condition.

Acknowledgements

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