Impact of diurnal temperature variation on grape berry development, proanthocyanidin accumulation, and the expression of flavonoid pathway genes

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Abstract

Little is known about the impact of temperature on proanthocyanidin (PA) accumulation in grape skins, despite its significance in berry composition and wine quality. Field-grown grapes (cv. Merlot) were cooled during the day or heated at night by +/–8 °C, from fruit set to véraison in three seasons, to determine the effect of temperature on PA accumulation. Total PA content per berry varied only in one year, when PA content was highest in heated berries (1.46 mg berry⁻¹) and lowest in cooled berries (0.97 mg berry⁻¹). In two years, cooling berries resulted in a significant increase in the proportion of (–)-epigallocatechin as an extension subunit. In the third year, rates of berry development, PA accumulation, and the expression levels of several genes involved in flavonoid biosynthesis were assessed. Heating and cooling berries altered the initial rates of PA accumulation, which was correlated strongly with the expression of core genes in the flavonoid pathway. Both heating and cooling altered the rate of berry growth and coloration, and the expression of several structural genes within the flavonoid pathway.

Key words: Biosynthesis, climate, flavonoids, flavonols, proanthocyanidins, tannins, temperature, Vitis vinifera.

Introduction

Plant secondary metabolites have been the focus of much research, warranted by their diversity of structure, function, and occurrence. Of particular interest are products from the phenylpropanoid biosynthetic pathway, compounds resulting from metabolism of phenylalanine and, to a lesser extent, tyrosine. This pathway is integral to the biosynthesis of flavonoids, which include three distinct classes of compounds: flavonols, anthocyanins, and proanthocyanidins (PAs). Production of each class of flavonoid is putatively mediated by a committed enzymatic step to fulfill a specific function within the plant. Flavonols are thought to protect plant tissue from UV radiation whereas anthocyanins are thought to provide some protection from UV radiation, high temperature extremes, and to aid in seed dispersal (Dixon et al., 2002; Winkel-Shirley, 2002; Adams, 2006). Proanthocyanidins, polymers of flavan-3-ol subunits, are thought to deter herbivores and possess antifungal properties (Aerts et al., 1999). These three groups of flavonoids are of interest in wine grapes (Vitis vinifera L.) because of their contribution to perceived wine quality and potential health benefits (i.e. antioxidant properties (Dixon and Paiva, 1995; Robards and Antolovich, 1997; Rice-Evans, 2001). Although anthocyanins are responsible for the colour of red wine, flavonols are thought to contribute to red wine colour via co-pigmentation (Boulton, 2001; Adams, 2006). Proanthocyanidins, found in the skin, seed, and mesocarp, contribute to astringency and
in-mouth tactile sensations associated with wine (Gawel et al., 2001; Vidal et al., 2004; Verries et al., 2008).

One of the more topical concerns relating to grapes and other crops is the effect of climate shifts on yield and fruit quality attributes. With regard to V. vinifera, the relationship to climate is evident in the geographic distribution of cultivars suitable for wine production, likely to be reassessed in subsequent decades (Kenny and Shao, 1992; Jones et al., 2005; White et al., 2006). While there is debate about the anthropogenic influence on climate, there are clearly recorded periods of extreme temperature events (Easterling et al., 2000; Chuine et al., 2004; Mann et al., 2009) that may have implications for grape cultivation and wine quality.

Kliwer and colleagues investigated the effects of various temperatures on the metabolism of sugars, acids, and anthocyanins in controlled environments (Kliwer, 1964, 1977; Buttrose et al., 1971; Kliwer and Torres, 1972; Lukas and Kliwer, 1975). Others have focused on the effects of exposure to solar radiation and temperature on berry composition; however, few have quantified the specific contribution of temperature in these multi-factor studies (Crippen and Morrison, 1986; Bergqvist et al., 2001; Downey et al., 2004; Cortell and Kennedy, 2006). Under field conditions, some studies evaluated anthocyanins and flavonols, but did not assess the impact of temperature on PA biosynthesis and accumulation (Spyd et al., 2002; Tarara et al., 2008).

The purpose of this study was to determine the effect of cluster temperature on PA biosynthesis in the skin of grape berries. Proanthocyanidin biosynthesis occurs during the first phase of berry growth, the focal period of the experiment (Coome and McCarthy, 2000; Robinson and Davies, 2000; Kennedy et al., 2002; Downey et al., 2003a). Berry temperature was modified in the vineyard by two approaches: (i) cooling berries during the day; and (ii) heating berries at night. The effect of temperature on berry development, PA accumulation and composition, and several key genes in the flavonoid pathway was determined.

Materials and methods

Field procedure

The three-year study (2006–2008) was conducted at the Irrigated Agriculture Research and Extension Center near Prosser, WA, USA (46.30° N, 119.75° W). Rows of own-rooted ‘Merlot’ (Vitis vinifera L.) vines (planted in 1999) were orientated north-south. Vines were trained to a bilateral cordon at 1.2 m above ground and spur-pruned. Shoots were loosely trained vertically. Experimental clusters were selected on the east aspect of the vine and were exposed to incident solar radiation by tucking shoots and leaves under a catch wire 1.5 m above ground. Four temperature treatments were applied to individual clusters in replicate (n=4) from developmental stage 27–28 (modified E-L system, berry diameter approximately 2–4 mm) and concluded at the onset of ripening (véraison; Coome, 1995). Temperature treatment classifications used were: (i) untreated control (ambient); (ii) convective control (blower); (iii) night-time heated (heat); (iv) daytime cooled (cool).

Temperature was controlled by a forced-air delivery system (Tarara et al., 2000) modified by replacing the single chilling unit with a pair of units that operated alternately. Treatments were imposed from ∼45 d before véraison (c. 10–12 d after anthesis) to 8°C above (heat) or below (cool) the average temperature of ambient clusters. No chilled air was delivered if berry temperature was below 10°C, a purported low temperature threshold for grapevine growth. Treatments were intended to expand the temperature range and the thermal time accumulated without exceeding detrimental high or low berry temperatures. The temperature and wind velocity around ambient clusters were not manipulated. Convective control refers to ambient air delivered at the same rate that heated or cooled air was delivered to the temperature-controlled clusters, to account for the effects of heat transfer by forced convection. Berry temperature was estimated by fine-wire thermocouple junctions [0.13 mm diameter; Type T (copper-constantan)] each encapsulated in a 4–6 mm diameter bead of silicone. Four junctions were wired in parallel and were positioned between berries along the length of the rachis. Multiplexed signals (AM-25T, Campbell Scientific, Logan, UT, USA) were scanned every 5 s and averaged recorded every 12 min by datalogger (CR-10X, Campbell Scientific). Global irradiance was measured by pyranometer (model 8-48, Eppley Laboratories, Newport, RI, USA). Solar radiation impinging on the fruiting zone was measured by N-S oriented, 1 m long tube solarimeters (n=3; model TSL, Delta-T Devices, Cambridge, UK) parallel to the cordon at same height above ground.

In 2008, clusters were harvested at roughly 10 d intervals (n=4) from the start of the experiment [48 d before véraison (DBV)] to its conclusion (5 DBV), where the last sampling point coincided with the termensus in 2006 and 2007. Véraison was defined as the time at which 50% of berries on clusters from a concurrent experiment had turned colour due to the combined effects of cooling clusters during the day and heating them at night, which advanced their development (Cohen et al., 2008). At harvest, clusters were placed on ice and held under refrigeration. Berries were excised from the rachis (receptacles were cut at the flare of the pedicel and leaf attached), counted, weighed, and snap-frozen in liquid nitrogen the same day. Samples were stored at −80°C prior to analyses.

Metabolite analysis

Analyses of grape skin phenolics were carried out as described previously by Cohen et al. (2008). Mean berry volume (50 or 100 berries) was estimated based on H2O displacement prior to manual dissection and separation of skin and seeds. The percentage coloration for each cluster was determined by counting the berries that exhibited about 80–100% red colour. Skin extracts were prepared by macerating lyophilized skin tissue in an acetone/water solution (2:1 v/v) for 24 h under nitrogen gas as described previously by Kennedy et al. (2000). Following removal of acetone, extracts were brought to volume in ultra-pure water and stored at −30°C prior to chemical analyses.

Analysis of monomeric phenolics was performed following a previously described method extended to 80 min separation time (Lamuela-Raventos and Waterhouse, 1994). Separations were performed on a LiChrospher 100 RP-18 column equipped with a guard column of the same material (EMD Chemicals, Gibbstown, NJ, USA). In 2006, aqueous extracts were filtered using a syringe filter (Acrodisc PTFE (13 mm, 0.45 μm) Pall Corporation, East Hills, NY, USA). In 2007 and 2008, samples were centrifuged at 16 000 g for 15 min to remove solids. Quercetin (Sigma-Aldrich, St Louis, MO, USA) was used as a quantitative standard for flavonols. In 2008, flavonols were determined from 48 DBV to véraison, to assess a potentially competitive branch point in PA biosynthesis.

Compositional analysis of PAs was carried out following acid-catalysed cleavage in the presence of excess chlorogluconol (chlorogluconolysis) (Kennedy and Jones, 2001). Aliquots of aqueous extracts were lyophilized and dissolved in MeOH prior to reacting with chlorogluconol reagent (Kennedy and Taylor, 2003; Cortell et al., 2005). Samples were immediately analysed following the addition of aqueous sodium acetate. Quantification of PA subunits
and estimation of the mean degree of polymerization (mDP) was calculated using (+)-catechin (Sigma-Aldrich) as a quantitative standard. Tannin content, composition, and mDP are shown from the initiation of the experiment (48 DBV) to illustrate changes in metabolites during the course of the study.

The size distribution of intact PAs was analysed by gel permeation chromatography (GPC) following the method of Kennedy and Taylor (2003). Separations were performed on tandem PLGel columns (100 Å and 500 Å) protected by a guard column containing sucrose (200/400 mesh) (200 Å). The instrument was equipped with a diode array detector (Waters, Milford, MA, USA) and a refractive index detector (Waters, Milford, MA, USA). The detection wavelength was 290 nm. The elution was carried out at 30 °C with a flow rate of 1 ml/min. The mobile phase was composed of 0.15 M LiCl in DMF containing 5% and 1% (v/v) water and acetic acid, respectively.

All high performance liquid chromatography (HPLC) analysis was performed on a Hewlett-Packard model 1100 (Palo Alto, CA, USA). The instrument was equipped with a diode array detector and an external column oven when required (Eppendorf CH-430; Westbury, NY, USA). All data were analysed using Agilent Chemstation software (V A.08.03).

Gene expression profiling
The expression levels for several key genes within the flavonoid biosynthetic pathway (Fig. 1) were determined at four times before véraison in 2008. Quantitative real-time PCR was carried out in an ABI PRISM 7700 sequence detector (Applied Biosystems, Carlsbad, CA, USA) as previously described by Castellarin et al. (2007a). Grape skins were collected from 10–12-berry samples maintained in the same material (Polymer labs, Amherst, MA, USA). Aliquots of aqueous extract were lyophilized and dissolved in mobile phase (0.15 M LiCl in DMF containing 5% and 1% (v/v) water and acetic acid, respectively). Samples were DNase-treated and first-strand cDNA was synthesized as outlined by Castellarin et al. (2007b, 2002; Downey et al., 2003b; Bogs et al., 2005; Terrier et al., 2005; Deluc et al., 2006).

Fig. 1. Simplified flavonoid biosynthetic pathway showing steps specific to the biosynthesis of flavonoids (VvFLS), proanthocyanidins (VvLAR and VvANR), and anthocyanins (VvGST); genes included in expression analysis are in bold type.

Statistical analysis
Berry temperature data were summarized over time and by treatment in SAS (version 9.1, SAS Institute, Cary, NC, USA) using the MEANS procedure. Thermal time in degree days (DD, °C) was computed by

$$DD = \frac{1}{n} \sum_{i=0}^{n} (T - T_b)$$

where $T$ is mean temperature over the datalogger averaging interval, $T_b$ the purported base temperature for grapevine growth (10 °C), and $n$ the number of datalogger averaging intervals per day. Statistical analyses of metabolite and temperature data were performed using Statgraphic Plus statistical software (Statpoint Tech. Inc., Warrenton, VA). Differences were determined using one-way ANOVA; Fisher’s LSD was used to determine separation of means ($\alpha=0.05$). Data that were not normally distributed were analysed using Kruskal-Wallis ANOVA.

Results
Berry temperature differed by about 8 °C as designed (Fig. 2) and at the intended times of day. During the experimental period, there was little interannual variation in DD within each treatment ($P<0.10$; Table 1). Ambient and blower berries accumulated equivalent DD. Cooling berries during the day did result in significantly lower DD and duration of exposure to high temperatures (Table 1). By contrast, heating berries at night resulted in a significant increase in DD accumulation from ambient. Weekly thermal time was variable (see Supplementary Table S1 at JXB online). Each year exhibited periods of above- or below-average temperatures (three-year weekly average ≈94 DD) at different stages of development. Temperature differences between years did not reflect differences in solar radiation.

In 2008, the average mass of berries sampled at the initiation of the experiment (48 DBV) was approximately 0.2 g per berry and differed among treatments only early in development. Berry mass followed a similar, two-stage growth curve in 2007b. Expression values are reported as means of biological treatment replicates ($n=4$) and each sample was run in duplicate. Primer pairs were the same as those described by Castellarin et al. (2007a, b). Primers for VvCHS1, VvCHS2, VvCHS3, VvDFR, and VvLDOX were retrieved from Goto-Yamamoto et al. (2002), VvANR, VvMYB5a, and VvMYBD1 primers were acquired from the literature (Kobayashi et al., 2002; Downey et al., 2003b; Bogs et al., 2005; Terrier et al., 2005; Deluc et al., 2006).

Flavonoid accumulation
Three glycosides of quercetin (-3-O-galactoside, -3-O-glucuronide, -3-O-glucoside) were the predominant flavonoids present before véraison. The flavonol content increased during development in all treatments (Fig. 3B); however, by
mass this represents less than 5% of the PAs up to véraison in 2008 (Fig. 3B, C). Temperature control did not affect flavonol accumulation.

There were few differences in PA content at véraison among treatments (Table 2), and these occurred in 2006. The PA accumulation in 2006 was positively correlated with thermal time ($r^2=34.7$; $P<0.05$), but this was not the case in either 2007 or 2008. Despite greater than 30% differences in DD between treatments and mean daily temperature differences of more than 4.0 °C (20.5 °C cool versus 24.9 °C heat) there were no consistent differences in total PAs at véraison.

In 2008, PA content at 48 DBV was 2863±268 nmol berry$^{-1}$ or 0.85±0.081 mg berry$^{-1}$ (Fig. 3C). This illustrates that a 2-fold increase in PA content occurred during the course of the experiment, which reached a maximum at approximately 19 DBV and then decreased slightly. Differences among treatments were detected only at 39 DBV, when cool berries had significantly less PA than either heat or ambient berries and exhibited an apparent lag in PA accumulation following application of the treatment. The timing and order of treatment effects were similar for PA

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**Fig. 2.** Exemplary diurnal variation in berry temperature (°C) over four days (DOY 199–202, 2008). In any 24 h period, the mean difference between ambient and blower berries was <1.0 °C.

**Fig. 3.** Berry mass (A) and flavonol (B) and PA content (C) in Merlot skins during 2008. Letters (a, b, c) denote means separation based on Fisher’s LSD (*$P<0.1$, **$P<0.05$, ***$P<0.01$); nd=not different.

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**Table 1.** Treatment duration, accumulated thermal time, and number of hours berry temperatures exceeded designated thresholds in 2006 through 2008

| Year | Duration (doy to doy) | Treatment | Thermal time (DD) | Number of hours above indicated temperature |
|------|------------------------|-----------|-------------------|---------------------------------------------|
|      |                        |           |                   | Hr $>35$ °C | Hr $>30$ °C |
| 2006 | 179–223 (45)           | Ambient   | 605 b$^a$         | 58 b 257 b |
|      |                        | Blower    | 597 b             | 47 b 236 b |
|      |                        | Cool      | 488 a             | 1.4 a 57 a |
|      |                        | Heat      | 683 c             | 45 b 257 b |
|      |                        |           |                   | $P$-value < 0.001 < 0.001 < 0.001 |
| 2007 | 184–227 (44)           | Ambient   | 595 b             | 37 b 236 b |
|      |                        | Blower    | 597 b             | 37 b 235 b |
|      |                        | Cool      | 494 a             | 0.2 a 48 a |
|      |                        | Heat      | 684 c             | 28 b 231 b |
|      |                        |           |                   | $P$-value < 0.001 0.0034 < 0.001 |
| 2008 | 186–231 (46)           | Ambient   | 590 b             | 38 b 238 b |
|      |                        | Blower    | 589 b             | 29 b 226 b |
|      |                        | Cool      | 471 a             | 0.0 a 20 a |
|      |                        | Heat      | 665 c             | 28 b 215 b |
|      |                        |           |                   | $P$-value < 0.001 0.0085 < 0.001 |

$^a$ Expressed as number of days in experimental period.

$^b$ Values with same letters within column and year are not different; LSD, $a=0.05$. 

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accumulation and the increase in berry mass, suggesting a shift in overall rate of berry development.

Ambient, heat, and cool berries all exhibited similar trends in PA accumulation per DD through development in 2008 (Fig. 4A). Both the increase and subsequent decrease in PA per DD was greater in cool berries, which accumulated equivalent PAs under lower DD. As PAs increased there was a linear relationship ($r^2 = 0.75; \text{DD} < 0.001$) between berry mass and PA content (Fig. 4B), meaning that changes in berry mass explain much of the difference in PA accumulation. A second-order relationship ($r^2 = 0.57; \text{DD} < 0.001$) accounts for the decline in PA content approaching véraison. A linear relationship was also observed between berry mass and DD across the entire experiment (data not shown; $r^2 = 0.64$, $\text{DD} < 0.001$), supporting a direct influence of temperature on metabolic flux as well as specific branch points leading to flavon-3-ols and timing of berry development. The expression levels of three chalcone synthase isogenes ($VvCHS1$, $VvCHS2$, and $VvCHS3$) were followed during berry development coordinating with general flavonoid biosynthesis during berry development (Goto-Yamamoto et al., 2002; Castellarin et al., 2007a). In 2008, the expression patterns of the three genes were similar from 39–19 DBV, declining 4–5-fold to near zero expression (Fig. 6A–C). $VvCHS1$ expression remained near zero whereas, just prior to véraison, $VvCHS2$ and $VvCHS3$ expression increased in heat berries. Analysis of ambient berries (data not shown) harvested 10 d after véraison showed that levels of $VvCHS2$ and $VvCHS3$ continued to increase after véraison, unlike $VvCHS1$.

Expression of downstream structural genes specifically related to flavonoid partitioning showed similar patterns to those of the $CHS$ genes. Core pathway genes involved in general flavonoid biosynthesis are shown in Supplementary Fig. S1 at JXB online) and only subtle changes in the distribution of PA polymer lengths (see Supplementary Fig. S2 at JXB online) during development. The individual subunit contributions also exhibited few significant differences between treatments (see Supplementary Fig. S3 at JXB online).

### Table 2. Proanthocyanidin (PA) content, mDP, and composition in Merlot skins at véraison in 2006 through 2008

| Treatment  | PA content | Terminal subunits | Extension subunits |
|------------|------------|------------------|--------------------|
|            | mg berry$^{-1}$ | mg g$^{-1}$ berry | mDP | C% | EC% | ECG% | EGC% | C% | EC% | C% | EGC% |
| 2006       |             |                  |      |    |    |    |      |    |    |    |      |
| Ambient    | 1.16 a,b,y  | 2.33 b,y**       | 28.0 | 78.2 x*** | 14.5 y** | 7.3 y*** | 43.0 a,x** | 53.6 b,y** | 1.5 | 1.9 x,y* |
| Blower     | 1.14 a,b    | 2.20 b           | 27.3 | 81.1 | 11.6 | 7.3 | 43.8 a | 53.0 b | 1.5 | 1.7 |
| Cool       | 0.97 a      | 1.62 a           | 25.8 | 82.1 | 11.6 | 6.3 | 48.5 b | 48.3 a | 1.6 | 1.6 |
| Heat       | 1.46 b      | 2.45 b           | 28.2 | 86.0 | 7.7 | 6.3 | 41.2 a | 55.8 b | 1.4 | 1.6 |
| P-value    | 0.038       | 0.027            |      | ns | ns | ns | 0.012 | 0.005 | ns | ns |
| 2007       |             |                  |      |    |    |    |      |    |    |    |      |
| Ambient    | 0.96 x      | 1.41 x           | 25.2 | 90.0 y | 8.8 x | 1.2 x | 49.2 a,y | 47.4 b,x | 1.3 | 2.2 y |
| Blower     | 1.18        | 1.98             | 29.5 | 88.1 | 10.2 | 1.7 | 52.4 a,b | 44.5 a,b | 1.1 | 1.9 |
| Cool       | 1.20        | 1.59             | 26.4 | 89.2 | 9.2 | 1.3 | 53.7 b | 43.4 a | 1.1 | 1.8 |
| Heat       | 1.11        | 1.65             | 27.4 | 90.1 | 8.2 | 1.7 | 49.2 a | 47.3 b | 1.2 | 2.2 |
| P-value    | ns          | ns               |      | ns | ns | ns | 0.035 | 0.025 | ns | ns |
| 2008       |             |                  |      |    |    |    |      |    |    |    |      |
| Ambient    | 1.41 y      | 2.24 y           | 27.7 | 89.3 y | 8.6 x | 2.1 x | 42.5 a,b,x | 54.4 a,b,y | 1.4 | 1.6 x |
| Blower     | 1.49        | 2.41             | 28.5 | 88.2 | 9.9 | 1.9 | 44.8 a,b | 52.4 a,b | 1.4 | 1.5 |
| Cool       | 1.43        | 2.08             | 27.3 | 90.2 | 7.9 | 1.8 | 45.9 b | 51.3 a | 1.3 | 1.5 |
| Heat       | 1.53        | 2.24             | 26.2 | 90.5 | 7.9 | 1.6 | 41.6 a | 55.3 b | 1.4 | 1.7 |
| P-value    | ns          | ns               |      | ns | ns | ns | 0.075 | 0.081 | ns | ns |

a. Expressed as mole percentage of total terminal or total extension subunits; C= catechin, EC= epicatechin, ECG= epicatechin-gallate, EGC= epigallocatechin.

b. Values with same letters within grouping are not different. Letters x and y separate means within ambient across years (* p < 0.1, ** p < 0.05, ***p < 0.01); LSD, x=0.05.

c. ANOVA P value within groupings in italics; ns: P > 0.1.

d. Kruskal-Wallis P value.

Regardless of treatment, expression levels of many flavonoid pathway genes correlated with rates of PA accumulation (Fig. 5A–E). In this experiment, relative gene expression was intended to provide information relating to general metabolic flux as well as specific branch points leading to flavan-3-ols and timing of berry development. The expression levels of three chalcone synthase isogenes ($VvCHS1$, $VvCHS2$, and $VvCHS3$) were followed during berry development coordinating with general flavonoid biosynthesis during berry development (Goto-Yamamoto et al., 2002; Castellarin et al., 2007a).
reductase (VvLAR2), leucoanthocyanidin dioxygenase (VvLDOX), anthocyanidin reductase (VvANR), and glutathione transferase (VvGST). Genes involved in the hydroxylation of flavonoids and related metabolite proportions are shown in Supplementary Fig. S5A–C: flavanone-3-hydroxylase (VvF3H), flavonoid-3'-hydroxylase (VvF3'H), and flavonoid-3'-5'-hydroxylase (VvF3'5'H). Most differences manifested at 29 DBV when heat showed a more pronounced decrease in expression of VvF3'H, VvLDOX, and VvANR. Similar trends were observed for other pathway genes; however, no significant differences were observed (VvF3'5'H, VvF3H, VvDFR). Expression of VvLAR2 (putatively related to Ct biosynthesis) and VvFLS (flavonol synthesis) exhibit more divergent patterns compared with other genes. The expression of VvFLS was counter to downstream structural genes related to PA accumulation, likely due to regulation by flavonol specific Myb-type genes (Stracke et al., 2007; Czemmel et al., 2009).

Glutathione transferase (VvGST) is tightly related to anthocyanin biosynthesis (Gomez et al., 2009) and was not detected until 5 DBV, coinciding with berry coloration (see Supplementary Fig. S4 at JXB online). Other genes associated with anthocyanin biosynthesis increased similarly at 5 DBV including VvCHS2, VvCHS3, VvF3'H, VvF3'5'H, VvF3H, VvDFR, and VvLDOX. In most cases, the increases
were more pronounced in heat, intermediate in cool, and least in ambient berries. Expression levels of VvLAR2 and VvANR were near zero at 5 DBV, coinciding with the decline in PAs.

Before véraison, the ratio of VvF3’5’H to VvF3’H expression (Fig. 7A) is in general agreement with the ratio of tri- to di-hydroxylated PA subunits (Fig. 7B). Heat showed a lower ratio between VvF3’5’H and VvF3’H and a lower proportion of tri-hydroxylated PAs. The increase in the expression of VvF3’H was greater in cool berries at 5 DBV, in contrast to

![Fig. 5](https://i.imgur.com/3Q5Q5Q.png)

**Fig. 5.** The relationship between select flavonoid pathway gene’s expression and the rate of PA accumulation across all treatments from 38–19 DBV. VvCHS2 (A), VvCHS3 (B), VvF3H (C), VvF3’H (D), and VvANR (E). The rate of PA accumulation was calculated from the slopes in Fig. 2; $r^2$ and significance values are presented.

![Fig. 6](https://i.imgur.com/5Q5Q5Q.png)

**Fig. 6.** Expression of VvCHS1 (A), VvCHS2 (B), and VvCHS3 (C) in Merlot skins during 2008. Expression levels are relative to the expression of VvUbiquitin. Significant differences are denoted by letters (a, b, c) based on Fisher’s LSD (*$P < 0.1$, **$P < 0.05$, ***$P < 0.01$); nd=not different.
the increase in *VvF3′5′H* in heat berries (see Supplementary Fig. S5 at JXB online). While overall differences in expression were not significant, this may be indicative of a temperature-related partitioning of anthocyanins (Downey et al., 2004; Cohen et al., 2008; Tarara et al., 2008). Expression of three *Myb* genes shown to be involved in flavonoid biosynthesis early in berry development in previous work are shown in Supplementary Fig. S5A–C (Castellarin et al., 2007). Expression of *VvMYB5a* was initially lowest in ambient berries and declined towards véraison in all treatments. *VvMYBD* also declined towards véraison, although expression levels were more sporadic than *VvMYB5a* and were initially highest in ambient. *VvMYBPA1*, determined to be a functional regulator of PA biosynthesis, showed an earlier decline overall followed by an increase approaching véraison (Bogs et al., 2007). Expression patterns of the MYB genes during berry development were as expected based on the published literature (Kobayashi et al., 2002; Deluc et al., 2006) and suggest that *VvMYB5a* and *VvMYBD* are also integral in PA biosynthesis prior to véraison based on similarities to that of *VvLAR* and *VvANR* compared with genes such as *VvFLS*, *VvGST*, and the three hydroxylase genes.

**Discussion**

The goal of this study was to determine the effect of temperature on PA accumulation and composition in field-grown grape berries. Temperature had noticeable effects on berry growth and the timing of véraison. Despite very different berry temperatures (+/-8 °C), there was no consistent relationship between temperature and total PA accumulation across three seasons. Total PA accumulation was not directly related to accumulation of thermal time, and the similar temporal relationships suggest a more likely connection to berry development. However, PA composition was affected, where decreasing DD favoured a shift towards tri-hydroxylated forms. The data indicate a robust feedback response mechanism resulting, in part, from the co-ordinated regulation of flavonoid pathway genes. Differences observed in PA composition may have relevance with respect to PA extractability in a wine system but this requires further investigation (Cortell and Kennedy, 2006; Gagne et al., 2006; Ortega-Regules et al., 2006). Treatment effects in this study appear to be related to shifts in temporal development (indirect effect) and direct temperature effects (e.g. shift in subunit proportions).

Although there has been substantial research pertaining to the environmental impacts on flavonoids, most research on temperature has focused on anthocyanins, which are synthesized after véraison. Some research has determined that moderate temperatures and sun exposure (including visible and UV) encourage anthocyanin accumulation and alter partitioning (Buttrose et al., 1971; Haselgrove et al., 2000; Downey et al., 2004; Cortell et al., 2007; Tarara et al., 2008; Matus et al., 2009) while others determined that high temperatures can be inhibitory to accumulation due to differences in gene expression and chemical degradation of metabolites (Mori et al., 2005, 2007; Yamane et al., 2006). Temperature has been shown to have mixed effects on the relative proportion of di- and tri-substituted anthocyanins in contrast to the current study where lower temperatures resulted in increased proportions of tri-substituted PAs (Mori et al., 2007; Tarara et al., 2008). Although PAs and anthocyanins result from the same core flavonoid pathway there are inherent differences in their regulation and chemical reactivity (Takos et al., 2006; Castellarin et al., 2007b; Czemmel et al., 2009; Mellway et al., 2009).

Due to their photo-protective role in plants, much previous work on grape flavonoids has focused on the effect of UV (Close and McArthur, 2002; Winkel-Shirley, 2002), which leads to significant increases in PAs in grape berry skins at véraison, a higher proportion of PAs in the tri-hydroxylated form, and higher PA mDP (Downey et al., 2004; Cortell and Kennedy, 2006; Fujita et al., 2007; Tarara et al., 2008). Compared with the effects of UV, temperature appears to have little impact on PA biosynthesis and accumulation. However, differences in light environment may contribute to the inter-annual variability encountered in this study (see Supplementary Table S1 at JXB online). The potential driving forces of PA accumulation may be confounded because there is both a period of increase and
decrease in PA concentration prior to véraison. These decreases in PA content could reflect oxidation or reduced extractability due to associations with cell wall material or polymerization (Kennedy et al., 2000; Downey et al., 2003a; Geny et al., 2003; Adams, 2006; Gagne et al., 2006).

Peaks in PA accumulation and related gene expression observed in this and other studies (del Rio and Kennedy, 2006; Takos et al., 2006; Castellarin et al., 2007b; Akagi et al., 2009; Carbone et al., 2009; Gagne et al., 2009; Hanlin and Downey, 2009) illustrate the co-ordination of PA biosynthesis with the early developmental stages of the fruit and an overall reduction in PA biosynthesis after véraison. Therefore, environmental factors that alter the rate of berry development may indirectly affect metabolite accumulation and environmental influences impacting particular metabolites would be time-dependent. In the current study, temperature had significant but temporary effects on growth and PA accumulation. In both cases, berries appear to compensate for these initial temperature effects. For example, cooling berries during the day led to a large initial difference in PA concentration but subsequently, cool berries compensated for this through accumulating PAs at a much more rapid rate. This compensatory mechanism is reflected in patterns of flavonoid pathway gene expression, suggesting a robust feedback response mechanism resulting in part from the co-ordinated regulation of flavonoid pathway genes.

Conclusion
Seasonal variations in air temperatures had limited effect on PA biosynthesis in grape berries. In this study, temperature treatments affected berry development by impacting growth and hastening the inception of véraison, confounding a direct effect of temperature on biosynthesis alone. Additional work should address the relationship between variable light environment, temperature, and PA biosynthesis as previous work has focused on the presence or absence of light. The effect of transient temperature treatments and extended exposure to extreme high (>35 °C) and low (<10 °C) temperatures should also be investigated to determine the thresholds for PA biosynthesis and to further our understanding of temperature influences on crop composition and quality.

Supplementary data
Supplementary data can be found at JXB online.

Supplementary Table S1. Accumulated thermal time for ambient clusters and average daily solar irradiance incident in the fruiting zone by weekly interval.

Supplementary Fig. S1. Pre-véraison PA mDP in Merlot skins as determined by phloroglucinolysis during 2008.

Supplementary Fig. S2. Size distributions of PA polymers in Merlot skins determined by gel permeation chromatography for (A) heat and (B) cool berries during 2008.

Supplementary Fig. S3. Content of PA subunits in Merlot skins during 2008.

Supplementary Fig. S4. Expression of VvFLS (A), VvDFR (B), VvLAR (C), VvLDOX (D), VvANR (E), VvGST (F), VvF3H (G), VvF3′H (H), and VvF3′5′H (I) in Merlot skins during 2008.

Supplementary Fig. S5. Expression of transcription factors VvMYB5a (A), VvMYBPA1 (B), and VvMYBD (C) in Merlot skins during 2008.

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