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RESEARCH PAPER

# 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 fulfil 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.

Kliewer and colleagues investigated the effects of various temperatures on the metabolism of sugars, acids, and anthocyanins in controlled environments (Kliewer, 1964, 1977; Buttrose *et al.*, 1971; Kliewer and Torres, 1972; Lakso and Kliewer, 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 (Spayd *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 (Coombe and McCarthy, 2000; Robinson and Davies, 2000; Kennedy *et al.*, 2002; Downey *et al.*, 2003*a*). 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 oriented 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 treatment regimens 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; Coombe, 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) Target temperature differences were 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 averages 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 terminus 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 left 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 $H_2O$ 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-Alrich, 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 phloroglucinol (phloroglucinolysis) (Kennedy and Jones, 2001). Aliquots of aqueous extracts were lyophilized and dissolved in MeOH prior to reacting with phloroglucinol 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 to 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 $PL_{Gel}$ columns (100 Å and 500 Å) protected by a guard column containing 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).

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 (DAD) 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.* (2007*a*). Grape skins were collected from 10–12-berry samples maintained in liquid nitrogen during dissection. Extraction of RNA from approximately 0.3 g tissue followed the protocol by Iandolino *et al.* (2004). Samples were DNase-treated and first-strand cDNA was synthesized as outlined by Castellarin *et al.* (2007*b*). Reaction mixtures (20 µl) contained 10 Ml Power SYBR Green Master Mix (Applied Biosystems), 5 µl of 1:100 diluted cDNA, and 250 nM of each primer. Thermal cycling was at 95 °C for 10 min followed by 40 amplification cycles (95 °C, 30 s; 58 °C, 30 s; 65 °C, 60 s), and a melting cycle from 65 °C to 95 °C. Relative quantitation of transcripts was determined by comparing the cycle threshold ($C_T$) of the target gene to that of *VvUbiquitin1* (TC32075, TIGR database) as in previous studies (Bogs *et al.*, 2005; Castellarin *et al.*, 2007*b*). 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.* (2007*a*, *b*). Primers for *VvCHS1*, *VvCHS2*, *VvCHS3*, *VvDFR*, and *VvLDOX* were retrieved from Goto-Yamamoto *et al.* (2002). *VvF3H*, *VvANR*, *VvF3′H* (coded *VvF3-1*), *VvF3′5′H* (coded *VvF35-1*), and *VvMYBPA1* (coded *VvMYBC* and subsequently re-assigned the designation of VvMYBPA1 based on sequence homology by Bogs *et al.*, 2007) were designed by Castellarin *et al.* (2007a) based on published sequence information. *VvFLS* (*VvFLS1*), *VvLAR* (*VvLAR2*), *VvGST*, *VvMYB5a*, and *VvMYBD* primer sequences were acquired from the literature (Kobayashi *et al.*, 2002; Downey *et al.*, 2003*b*; Bogs *et al.*, 2005; Terrier *et al.*, 2005; Deluc *et al.*, 2006).

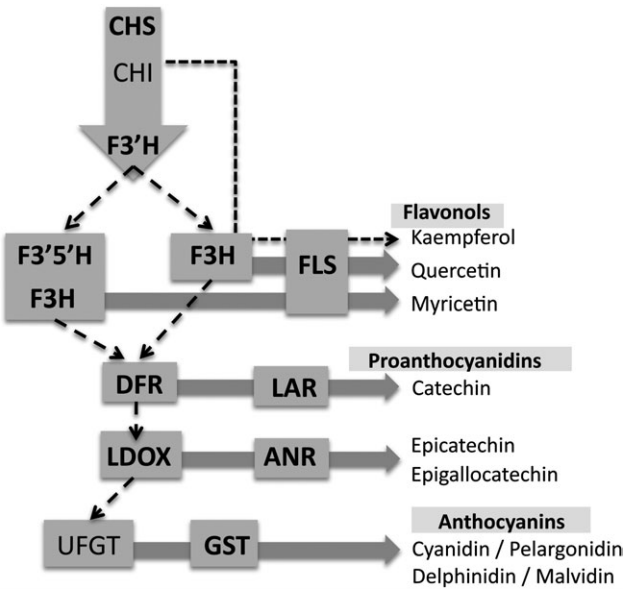


**Fig. 1.** Simplified flavonoid biosynthetic pathway showing steps specific to the biosynthesis of flavonols (*VvFLS*), proanthocyanidins (*VvLAR* and *VvANR*), and anthocyanins (*VvGST*); genes included in expression analysis are in bold type.

### *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

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

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 (39 DBV; Fig. 3A). Berry mass followed a similar, two-stage growth curve in *ambient* and *heat* berries with little increase in mass between 29 DBV and 19 DBV, while *cool* berries increased in mass linearly.

### *Flavonoid accumulation*

Three glycosides of quercetin (-3-*O*-galactoside, -3-*O*-glucuronide, -3-*O*-glucoside) were the predominant flavonols present before véraison. The flavonol content increased during development in all treatments (Fig. 3B); however, by

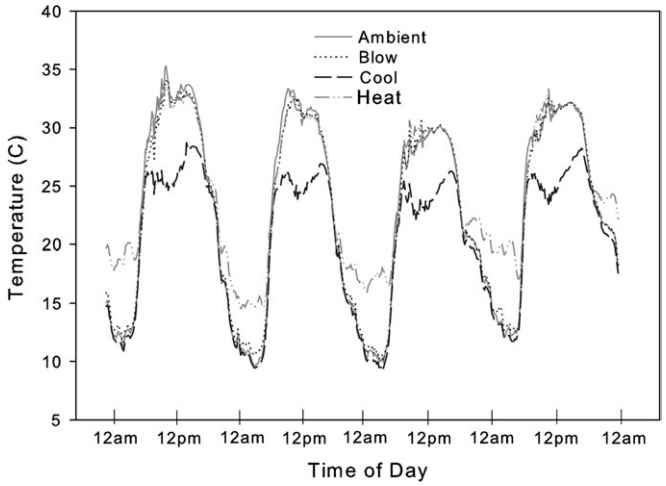


**Fig. 2.** Exemplary diurnal variation in berry temperature (°C) over four days (DOY 199–202, 2008). In any 24 h period, the mean difference between *ambient* and *blower* berries was <1.0 °C.

**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 | Ambient | 605 b[b] | 58 b | 257 b |
| | (45[a]) | 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 | Ambient | 595 b | 37 b | 236 b |
| | (44) | 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 | Ambient | 590 b | 38 b | 238 b |
| | (46) | 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, α=0.05.

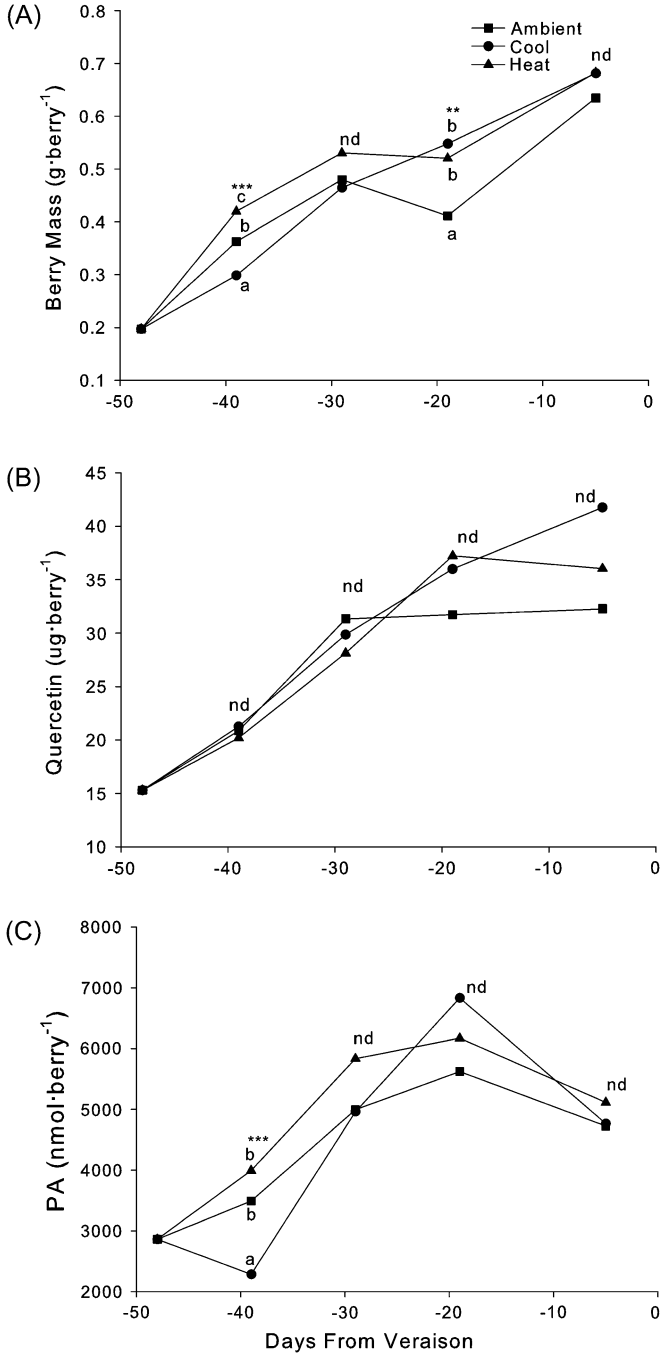


**Fig. 3.** Berry mass (A) and flavonol (B) and PA content (C) in Merlot skins during 2008. Letters (a, b, c) denote means separation based on Fisher's LSD (*$P \leq 0.1$, **$P \leq 0.05$, ***$P \leq 0.01$); nd=not different.

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

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

| | | PA content | | | Terminal subunits[a] | | | Extension subunits | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment | mg·berry$^{-1}$ | mg·g$^{-1}$ berry | mDP | C% | EC% | ECG% | EGC% | EC% | C% | ECG% |
| 2006 | Ambient | [b]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* | [c]*ns* | *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 | 9.4 | 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* | *ns* | *0.035* | [d]*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* | *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, $\alpha$=0.05.
[c] ANOVA *P* value within groupings in italics; ns: *P* >0.1.
[d] Kruskal–Wallis *P* value.

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; $P$ <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; $P$ <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, $P$ <0.001), supporting a direct influence of temperature on berry growth.

There were few treatment effects on composition of skin PAs (Table 2). For example, average PA polymer size (mDP) did not differ significantly among years. The proportions of (−)-epicatechin (EC) and (−)-epicatechin-3-*O*-gallate (ECG) as terminal subunits (ECt and ECGt, respectively) were significantly greater in 2006 than in 2007 or 2008. However, collectively they represent a relatively small contribution to the total pool of terminal subunits (9.8–21.8% across years). Conversely, (+)-catechin (C) as a terminal subunit (Ct) was lowest in 2006 (78.2–90% of total terminal subunits across years). Together, (−)-epigallocatechin (EGCx) and EC extension subunits (ECx) constituted over 95% of the total PA pool in berry skins. The proportion of these subunits relative to each other was consistent between years and treatments. In 2008, there were no significant differences between treatments in mDP of PAs (see 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).

### *Temperature effects on flavonoid biosynthetic genes*

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.*, 2007*a*). 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. S4A–F at *JXB* online: flavonol synthase (*VvFLS*), dihydroflavonol-4-reductase (*VvDFR*), leucoanthcyanidin

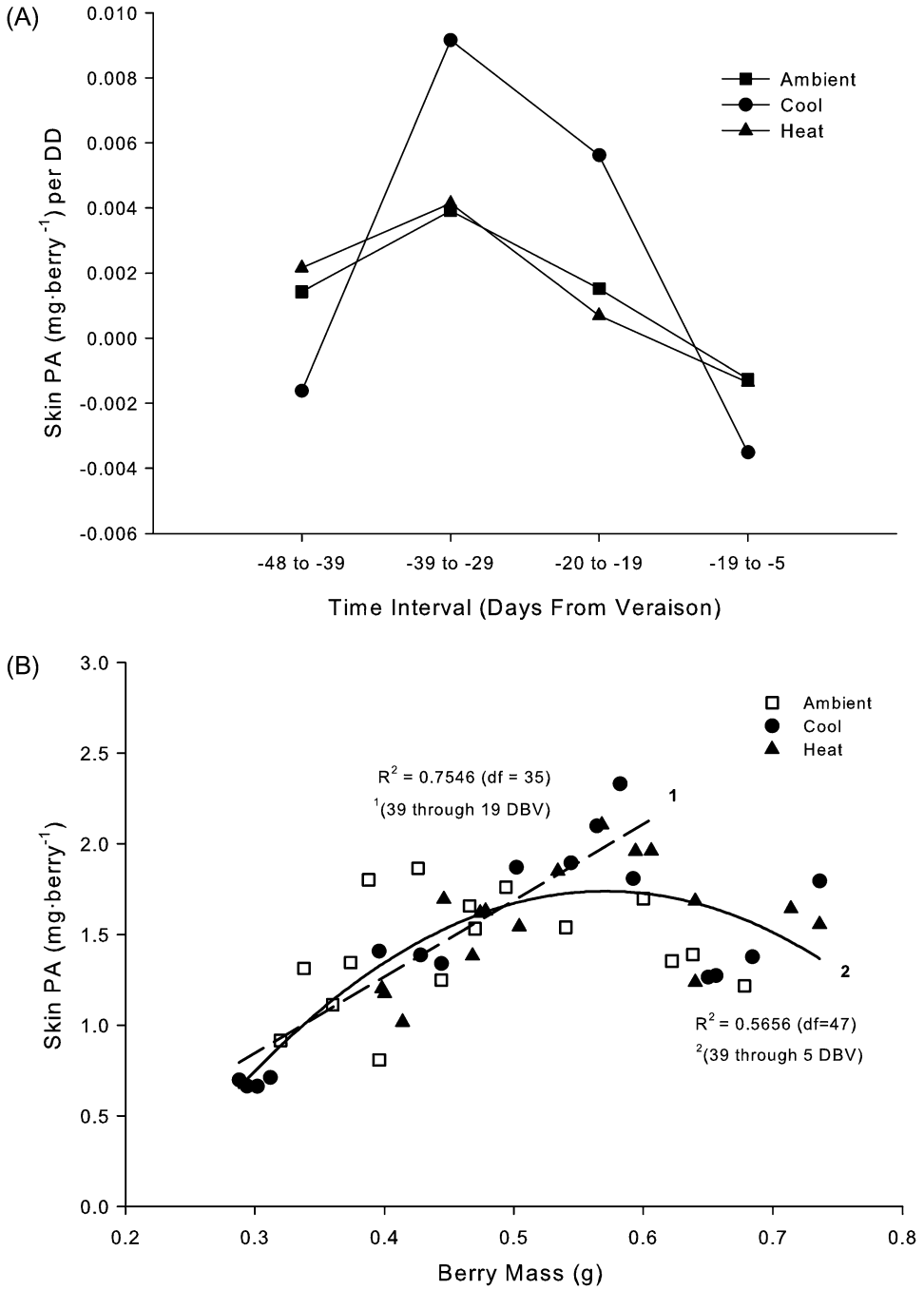


**Fig. 4.** Skin PA content as a function of DD accumulation (A) and the relationship between Skin PA content and berry mass (B) of Merlot berries during 2008.

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

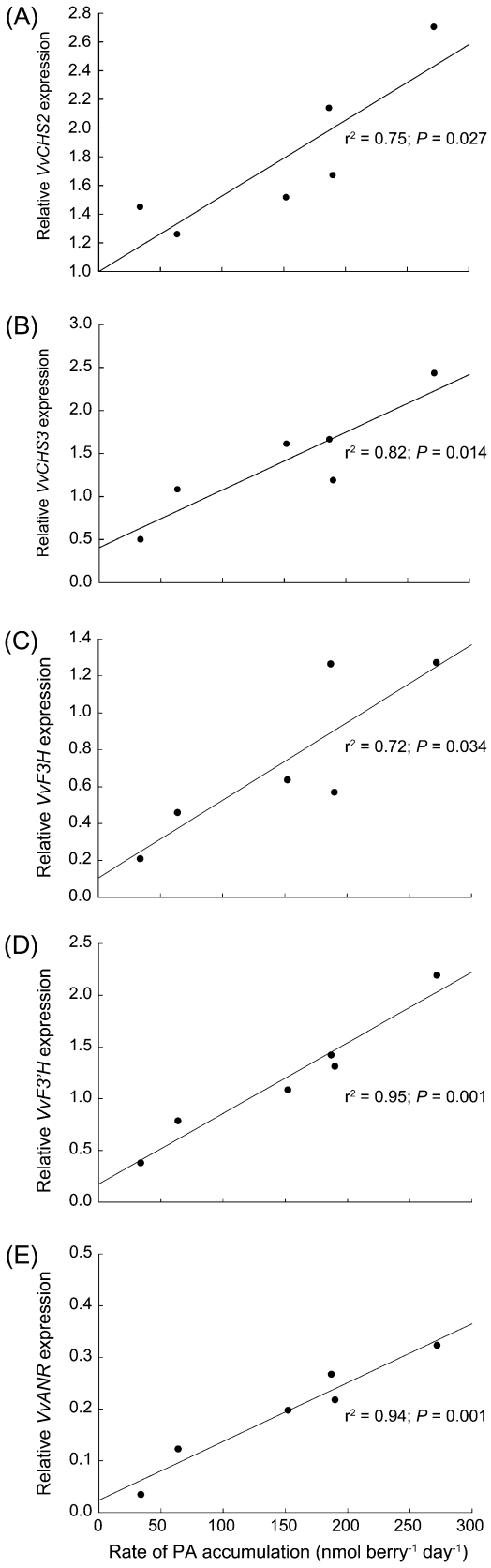


**Fig. 5.** The relationship between select flavonoid pathway gene's expression and the rate of PA accumulation across all treatments from 38–19 DBV. *VvCHS2* (A), *VvCHS3* (B), *VvF3H* (C), *VvF3′H* (D), and *VvANR* (E). The rate of PA accumulation was calculated from the slopes in Fig. 2; $r^2$ and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

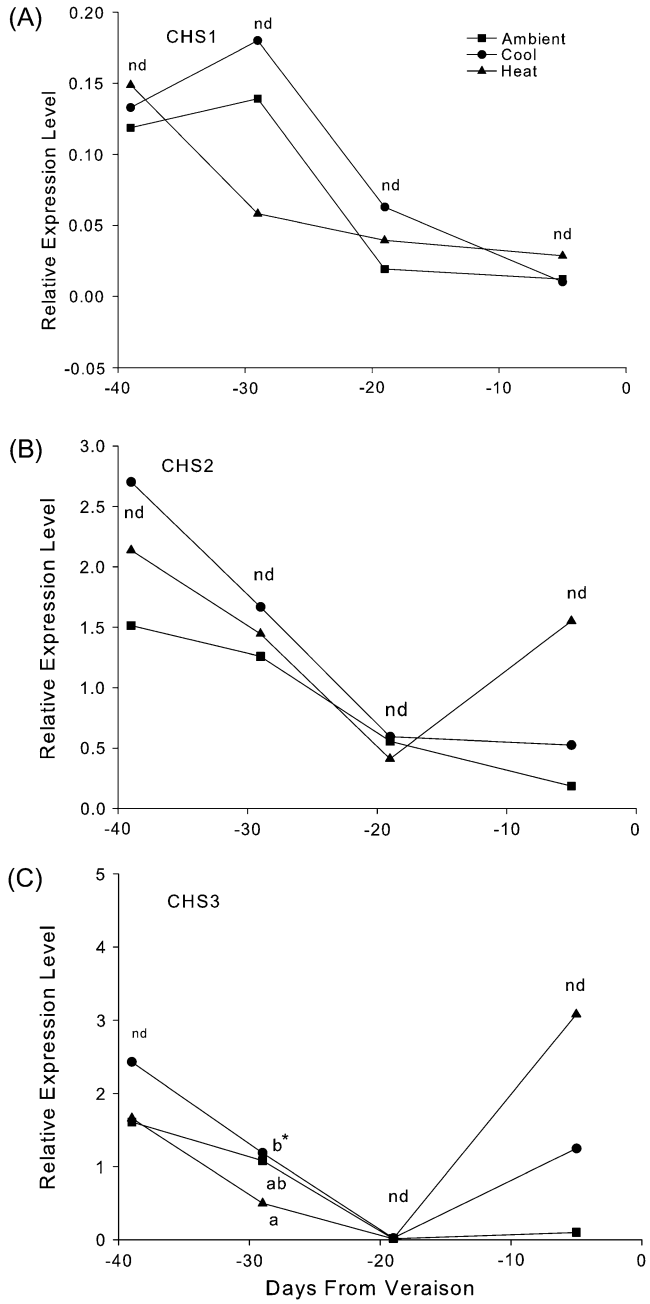


**Fig. 6.** Expression of *VvCHS1* (A), *VvCHS2* (B), and *VvCHS3* (C) in Merlot skins during 2008. Expression levels are relative to the expression of *VvUbiquitin*. Significant differences are denoted by letters (a, b, c) based on Fisher's LSD (*$P \leqslant 0.1$, **$P \leqslant 0.05$, ***$P \leqslant 0.01$); nd=not different.

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

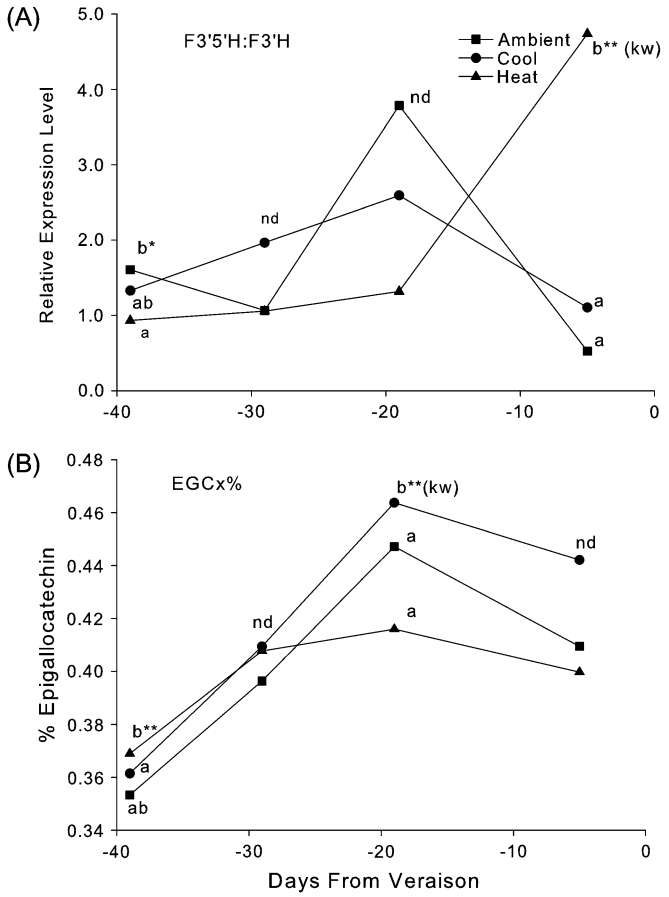


**Fig. 7.** Expression of *VvF3′5′H* to *VvF3′H* (A), and the proportion of EGCx relative to total PA content (B) in Merlot skins during 2008. Significant differences are denoted by letters (a, b, c) based on Fisher's LSD (*$P \leq 0.1$, **$P \leq 0.05$, ***$P \leq 0.01$); nd=not different, kw=Kruskaw–Wallis *P* value.

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 (Kobayshi *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.*, 2007*b*; 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.*, 2003*a*; 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.*, 2007*b*; 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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