AN ABSTRACT OF THE THESIS OF

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Title: Investigating the Effects of Temperature on Secondary Metabolism in *Vitis vinifera* L. cv. Merlot Berries.

Abstract Approved:

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James A. Kennedy

Grapes (e.g. *Vitis vinifera* L.) are one of the more important fruit crops from an economic standpoint with world market values over 550 million US dollars in 2007 and 2008, second to apples (United States Department of Agriculture). While a satisfactory level of ripeness is of primary concern, the quality of wine grapes is determined the contribution of secondary metabolites. Secondary metabolites provide the color, aroma, flavor, and tactile sensations associated with wine and are believed to be of biological value to humans. Anthocyanins provide the color to red grapes and wine while flavonols are thought to provide color stability to wine through co-pigmentation with anthocyanins. Proanthocyanidins (PAs) provide astringency or a tactile response in the mouth, which is a primary element of wine quality. For the

plant, secondary metabolites are involved in signaling, seed dissemination, and protection against biotic and abiotic stresses.

In this work, the temperature of field-grown grapes, *Vitis vinifera* L. cv. Merlot, was manipulated by cooling and heating berries relative to ambient temperatures during three growing seasons. A range of thermal time was investigated by delivering cold air during the day (Cool) or warm air at night (Heat). Additionally, the effect of reducing the diurnal temperature range (DTR) was investigated by daytime cooling *and* nighttime heating without a significant change in the accumulated thermal time compared to ambient berries. The impact on PAs was studied by imposing treatments from fruit set to véraison during active PA biosynthesis. Treatments imposed from véraison to harvest maturity (≥ 22.0°Brix) were intended to assess the impact on flavonol and anthocyanin accumulation and changes in PAs during ripening. In 2008, experiments focused on PA accumulation by examining metabolites and the expression of genes involved in PA biosynthesis at ten-day intervals up to véraison.

Treatment effects described here demonstrate a complex response of grape berries to shifts in temperatures. The data suggest periods of sensitivity with respect to secondary metabolism that may dictate the response to changes in temperatures. To the authors' knowledge, the data presented here is the first of it's kind; examining the effect of temperature on active PA biosynthesis in both the skin and seed of grape as well as the effect of altering the DTR of berries grown under field conditions. This research has implications in understanding fundamental plant responses to their environment and the impact of climate shifts and seasonal temperature variations on

grape berry composition.

Investigating the Effects of Temperature on Secondary Metabolism in *Vitis vinifera* L. cv. Merlot Berries.

by
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APPROVED:

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I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Seth D. Cohen, Author

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Dr. Julie M. Tarara assisted with editing of the text in "Daily temperature excursions have limited impact on grape skin proanthocyanidins and related gene expression before fruit ripening" and "Compressing the diurnal temperature range of *Vitis vinifera L. cv* Merlot hastens berry development and partitioning of flavonoid metabolites". Dr. Mark A. Matthews and Gregory A. Gambetta assisted in the discussion and text of "Daily temperature excursions have limited impact on grape skin proanthocyanidins and related gene expression before fruit ripening". Dr. James A. Kennedy assisted in the development of all documents included herein.

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Chapter 1

Flavonoid structure, biosynthesis, and function

The purpose of this thesis was to describe the effects temperature, as a fundamental element of climate, on the accumulation of various secondary metabolites in *Vitis vinifera* L. Merlot berries. Particular attention was paid to flavan-3-ols and polymers thereof, referred to as proanthocyanindins (PAs) or tannins. The novelty of this work is in the temperature manipulation of established vines growing under standard viticultural practices. Temperature manipulations were made in constant reference to ambient berries under the same exposure regimen to incident solar radiation. This resulted in a 'natural' diurnal temperature pattern while treatments simply modified maximum or minimum temperatures experienced by fruit. Data collected over three years (2006 to 2008) is presented with discussion explaining observations and interpretations. This includes general berry morphology and development patterns; metabolites (primarily flavonoids); and gene expression related to flavonoid metabolism during one year of experimentation. The goal of this research was to provide insight into the influence of temperature on biosynthesis and accumulation of PAs and other flavonoids in grape berries.

Introduction

The cultivation of grapes and production of wine occurs in nearly every country in the world and is integrated into the culture of many nations. In most cases, wine production represents an economic platform by providing employment in grape and wine production as well as related tourism. While the price of many agricultural crops is dictated by the market, the price of wine is often determined by its perceived

quality. Therefore, those producing grapes and wine strive to produce the highest quality product, often at the expense of quantity or economic potentials.

In general, grape growers have a target quality metric based on berry composition as assessed by laboratory and sensory analysis. It would follow that maximizing yield (per unit area) while maintaining the desired quality is the most efficient means for maximizing profit. Research in the field of viticulture and enology often focuses on finding balance between maintaining quality while maximizing output and / or decreasing input. Examples range from determining the water and nutritional requirements of grapes, the effect of defoliation and other canopy manipulations and general farming practices such as conventional versus non-conventional management.

Equivalent enological research includes the impact of oak on wine, various extraction techniques and times, and types of bottle closure on wine quality. In all cases, the production decisions represent real costs in terms of resources (natural or man-made), labor or time. It is imperative, therefore, to understand the ramifications of our decisions, and make them based on the best knowledge at hand.

Several have discussed the relationship of climate (temperature, sunlight and moisture) to wine quality, often by statistical modeling of climate data and vintage reports (Jackson and Lombard, 1993; Caprio and Quamme, 2002; Petrie and Sadras, 2008; Soar et al., 2008). The data indicate that temperatures during early berry development and late in ripening are important to wine quality and can vary by growing regions. Similarly, hours of sunlight during the growing season correlate positively and rainfall during ripening correlates negatively with quality. The

implications are that moderate temperatures (above 10C below 40C) and low water stress are conducive to fruit set and early berry development. Moderate to warm temperatures (between 10C to 40C) after fruit-set encourage rapid berry growth and synchronous development. In later stages of ripening, moderate temperatures and the absence of water (soil moisture and rain) are preferred for the development of flavor and aroma. In all cases, humidity or moisture and warm temperatures around fruit represent potential for biotic stress (fungal pressure etc.). It follows that the favorable conditions described for each region involve encouraging uniform berry development and reducing the threat of fungal infection. However, in warmer climates, maximum temperatures tend to be problematic due to hastened ripening, reduced flavor development and reduced acidity) while in cooler climates, the opposite is true (difficulty in ripening; increasing sugar and reducing acid). It is the culmination of events that determine the success of a vintage in any area. What is unclear is *if* and *how* each of these environmental elements influence berry composition, as they are closely related. This leads to questions regarding the compositional differences of berries during an exceptional vintage and that during a lesser vintage.

Experimental studies relating to grape production in the natural environment are difficult due to the multitude of environmental factors present at any given time. Because of this, many researchers have attempted to isolate one specific element of the environment and determine the effects as related to grape development, composition or some quality parameter. In this case, we attempt to describe the response of grape berries to differences in temperature during different stages of phonological development. The remainder of this thesis will describe the nature of

flavonoids in grapes, wine, and human health, their biosynthesis and function in plants, and a discussion of data collected during this research.

The first section should provide the reader with a chemical and biosynthetic background of flavonoids and their implications and relevance to wine quality and human health. It is important to establish the relevance and value of this research within the field of grape and wine research as well as the human health potentials associated with functional dietary ingredients. This section will also demonstrate interactions between plants and their environment; while the primary focus is on abiotic stress (e.g. water, temperature and solar radiation), these cumulatively dictate the presence of biotic stresses and it is therefore useful to acknowledge this factor. The following sections (three) will discuss the results from this research: 1) investigating the effect of temperature on skin PA accumulation during early berry development and 2) investigating the influence of modifying the diurnal temperature range of grape berries during early development and again during berry ripening.

Flavonoid Structure

Plant flavonoid compounds are the primary focus of this research. These are a diverse group of metabolites sharing a common chemical backbone and differing by conformation and chemical substitution patterns. Therefore, it is valuable to briefly discuss the nomenclature, diversity, and general differences between the compounds of interest.

Flavonoids (Figure 1.1) belong to a group of compounds commonly referred to as phenolics, characterized as a benzene ring bearing a hydroxyl group (-OH), and are produced via the phenylpropanoid pathway (Harborne, 1997; Ferrer et al., 2008).

Phenolic acids (ellagic acid, salicylic acid, coumaric acid) are produced early in the biosynthetic pathway and are precursors to a host of other downstream compounds. Lignin, which provides mechanical strength to cell walls in woody plants, is composed of phenolic acid derivatives such as coumaryl, sinapyl, and coniferyl alcohols (Monties, 1989; Ferrer et al., 2008). Stilbenes (e.g. resveratrol) are characterized by having two benzene rings linked by ethane or an C_2 bridge and are also produced upstream of flavonoids (Gorham, 1989).

The flavonoid (or pehnylpropanoid) backbone consists of a C6-C3-C6 (15 carbon) skeleton (Figure 1.1) with the 3 ring structures generally labeled as "A-C-B" respectively. The distribution and diversity of these compounds is quite large and involves the addition of various moieties (esters, methyl esters, glycosides, etc.). These chemical substitutions dictate their relative stability / reactivity, solubility (localization and extraction properties), and interaction with enzymes and transporters (protein / substrate specificity) (RiceEvans et al., 1996; Iwashina, 2000; Heim et al., 2002; Ferrer et al., 2008). Most structural differences mentioned within this text relate to the substitution patterns on the B-ring (C-1′ through C-6′) and C-ring, (C-3 and C-4). Further, the three classes of primary interest are the flavonols, flavan-3-ols, and anthocyanins.

Flavonoid biosynthesis

Biosynthesis of the flavonoids proceeds along a common pathway with branch-points leading to flavonols, flavan-3-ols, and anthocyanins in relative order of appearance (Figure 1.2). At the head of the pathway is the synthesis of L-arogenate from erythrose-4-phosphate (pentose phosphate pathway) and phosphoenolpyruvate

(glycolysis) via the shikimate metabolic pathway. The metabolism of L-arogenate leads to L-tyrosine and L-phenylalanine, the latter of which is the main building block for plant phenolics or phenylpropanoids. Phenylalanine proceeds through the pathway to yield cinnamic acids that can be further modified to hydroxycinnamoyl CoA's (e.g. p-coumaryl CoA). The flavonoids arise from condensation reactions between hydroxycinnamoyl CoA's and (3) malonyl-CoA's (Krebs cycle).

The first committed step in the flavonoid biosynthetic pathway is mediated by chalcone synthase (CHS) followed by chalcone isomerase (CHI), which closes the 3-ring structure at the C1 position (Ferrer et al., 2008; Boss and Davies, 2009). Three CHS isogenes (CHS1, CHS2, and CHS3) are found to be active in grapevine and are differentially expressed throughout berry development. The CHS's are part of a multigene family in *V. vinifera*; CHS1 and CHS2 are found to be similar (>90% amino acid similarity) while CHS3 is more divergent (<80% amino acid similarity) (Goto-Yamamoto et al., 2002; Castellarin et al., 2007). Further, CHS1 and CHS2 were found to be present in green berries (those lacking anthocyanins and red berries before véraison) while CHS3 was associated with the presence of anthocyanins in Cabernet Sauvignon. In Merlot berries, CHS1 expression did not exhibit a relationship to anthocyanin biosynthesis while CHS2 and CHS3 show considerable increases approaching véraison when anthocyanins appear in the skins of red grape sports (Ageorges et al., 2006; Castellarin et al., 2007). This, and work with CHS in other plant species, demonstrates that the isogenes may be differentially regulated both during development and between different tissues (Koes et al., 1989).

Homologous genes, while active in the same pathway, serve distinct functions due to either metabolite or tissue specificity or synchronization with vine phonological development. Shimada et al. identified multiple isogenes of both CHI and dihydroflavonol 4-reductase (DFR) in *Lotus japonicus* L. demonstrating expression differences based on tissue specificity and development (Shimada et al., 2003; Shimada et al., 2005). Substrate specificity is likely related to evolution and the conservation of genes contributing to the metabolite diversity found within the *Vitis vinifera* species (Kimura et al., 2001; Shimada et al., 2005). The evolution of flavonoids is proposed to be rather linear, following progression of the biosynthetic pathway, and driven by the plants response to their environment (biotic and abiotic interactions) (Cooper-Driver and Bhattacharya, 1998). Helen Stafford proposed a similar theory of the evolution of plant flavonoids, stemming from aquatic algae to the spread of land-based plants with different requirements for UV protection and other abiotic / biotic stress (Stafford, 1991). Therefore, the diversity of flavonoids and related genes is likely indicative of the dispersion and divergence of plants into new environments where advantageous traits, including secondary metabolites, have been selected over time (Matsuki, 1996). Examples of this diversity are evident among cultivars of *V. vinifera*, such as the lack of anthocyanins in the form of –acetyl and –coumaroyl-glucosides in Pinot noir compared to other cultivars (Mazza et al., 1999). Similarly, the distribution flavan-3-ols in grape seeds and skins is found to differ based on cultivar type and geographic location (Mattivi et al., 2009).

Flavonoid B-ring hydroxylation (C-3′ and C-5′) is dictated by two genes; flavonoid-3′-hydroxyalse (F3′H) and flavonoid-3′-5′-hydroxylase (F3′5′H) (Bogs et

al., 2006). The former yields di-hydroxylated flavonols, flavan-3-ols, and anthocyanins (e.g. quercetin, catechin, cyanidin, peonidin) whereas the latter results in the tri-hydroxylated counterparts (e.g. myricetin, gallocatechin, delphinidin, malvidin) differing in their relative stability. Castellarin et al. identified two F3′H genes, only one of which was expressed, and two functional F3′5′H genes in Merlot (Castellarin et al., 2006). The expression of the hydroxylase genes is typically coordinated with CHS however some tissue specificity exists. In grapes, the majority of flavonols and flavan-3-ols tend to be di-hydroxylated (e.g. quercetin and (+)-catechin / (-)-epicatechin considering all tissue) while anthocyanins tend towards tri-substitution (e.g. malvidin-3-O-glucoside).

Flavanone-3-hydroxylase (F3H) is a core requirement for flavonoid synthesis as it is responsible for the hydroxyl group at C-3. Generally, F3H expression is coordinated with that of CHS, CHI and other upstream genes (Boss et al., 1996; Pelletier and Shirley, 1996). As expected, in absence of F3H flavonoid biosynthesis is inhibited (Meldgaard, 1992).

Flavonol synthase (FLS) represents a branch-point in flavonoid biosynthesis and may divert substrate away from PA production as such (Downey et al., 2003; Czemmel et al., 2009). FLS diverts dihydroflavonols towards the production of flavonols, which are often glycosylated via a UDP:Glucosyl transferase (Iwashina, 2000; Almeida et al., 2007). In grape, at least two FLS genes are identified (VvFLS1 and VvFLS2), although only VvFLS1 activity appears to reflect changes in metabolite content (Downey et al., 2003; Czemmel et al., 2009). Additionally, substrate specificity (mono- and di-hydroxylated forms) is common in FLS as found

in petunia (Forkman et al., 1986). In grape, the predominant flavonols detected are quercetin derivatives with lower levels of kaempferol and myricetin (Spayd et al., 2002; Downey et al., 2003; Cohen et al., 2008). Conversely, flavan-3-ols and anthocyanins accumulate with a high proportion of tri-hydroxylation/substitution, which could be due to the specific activity of FLS or F3'5'H transcripts.

Dihydroflavonol-4-reductase (DFR) activity represents a committed step towards production of flavan-3-ols and anthocyanins. The products, leucocyanidin and leucodelphinidin (or chemically equivalent), may constitute the extension subunits found in grape PAs however the mechanism of polymerization is still unknown (Dixon et al., 2005). The products are transformed downstream into either flavan-3-ols or anthocyanins. Therefore, DFR activity is expressed throughout development in all tissues accumulating those compounds. Early in grape berry development (and other fruit) DFR activity is directed primarily towards production of flavan-3-ols and increases concomitantly with the incorporation of anthocyanins in epidermal tissues (Boss et al., 1996; Coombe and McCarthy, 2000; Takos et al., 2006; Almeida et al., 2007). Multiple DFRs have been identified in both Lotus japonicus and Medicago truncatula showing differences in tissue localization, substrate specificity, and activity in response to pH and temperature (Xie et al., 2004; Shimada et al., 2005). Further, Johnson *et al*. (2001). demonstrated that a single amino acid variation in DFR was responsible for the lack of mono-substituted anthocyanins in *Petunia* due to substrate specificity of the enzyme Due to the pivotal roll of DFR in flavonoid biosynthesis it would be valuable to identify and characterize DFR variants in *V. vinifera*.

The expression of leucoanthcyanidin reductase (LAR), leucoanthocyanidin dioxygenase (LDOX), and anthocyanidin reductase (ANR or BAN) are considered the final steps in production of flavan-3-ols and PAs. While LAR results in the 2,3-trans-flavan-3-ol (catechin (C)) the concerted steps of LDOX and ANR result in 2,3-cis-flavan-3-ols (epicatechin (EC) and epigallocatechin (EGC)) thought to be the starting or terminating units of PAs (Abrahams et al., 2003; Tanner et al., 2003; Xie et al., 2003; Bogs et al., 2005; Xie and Dixon, 2005; Terrier et al., 2009). In general, the distribution of flavan-3-ols in grape skins is limited to derivatives of the di-and tri-hydroxylated forms. In seeds, considerable quantities of flavan-3-ols accumulate with 3-*O*-gallate esterification (Kennedy et al., 2000). While the nature of galloyl transfer has been examined in oak and sumac (via a β-gluco-gallin intermediate), the mechanism remains speculative (Niemetz and Gross, 1998, 2005). It should also be noted that LDOX often displays side activity similar to FLS resulting in accumulation of flavonols (Turnbull et al., 2000; Turnbull et al., 2003; Stracke et al., 2009). This may represent the redundant function and relative promiscuity of some genes in the flavonoid pathway, possibly related to accumulation of beneficial metabolites when the primary regulators or structural genes may be inactive.

As mentioned, the undetermined step of PA biosynthesis is the production and incorporation (polymerization) of extension subunits. Linkages between flavan-3-ols are most often of the B-type, with a single link between the C4 of one unit and either C6 or C8 of the second unit, with the latter being the predominant form. A-type linkages are found less frequently, having two links between units; one between C4 and C8 and another between C2 and either C5 or C7 via an ether linkage (Abrahams

et al., 2003; Tanner et al., 2003; Xie et al., 2003; Bogs et al., 2005; Xie and Dixon, 2005; Terrier et al., 2009). In general, the majority of PAs are found in the B-type (C-4→C-8) with extension units in the 2,3-*cis* conformation (eg. (-)-epicatechin (EC) and (-)-epigallocatechin (EGC)) as found *V. vinifera* (Kennedy et al., 2001; Kennedy et al., 2002; Xie and Dixon, 2005). A question that arises is how extension sununits are produced; DFR results in 2,3-*trans* flavan-3,4-diols suggesting further modification to obtain the *cis*-epimer. Various mechanisms have been suggested including epimerization / oxidation (enzymatic and chemical), utilization of flavan-3,4-diols (via DFR), quinine methide intermediates, a novel epicatechin 3′-glucose, enzyme complexes (such as DFR and LDOX), and vacuolar transport prior to polymerization (Dixon et al., 2005; Xie and Dixon, 2005). Work by Turnbull et al. (2000, 2003) suggests that a mechanism between DFR and LDOX may be feasible, as LDOX activity can result in the production of a 2,3-*cis*-dihydroflavonol, which in turn may be utilized by a DFR enzyme to produce a 2,3-*cis*-flavan-3,4-diol (Turnbull et al., 2000; Turnbull et al., 2003; Stracke et al., 2009). This type of mechanism is postulated by Dixon et al. however there is no experimental data to support it (Dixon et al., 2005; Xie and Dixon, 2005). Work by Debeaujon et al. and Kitamura et al. collectively demonstrate vacuolar transfer mechanisms requisite for PA accumulation in the seed coat of *Arabidopsis*; there is likely a homologous mechanism in *V. vinifera* but it has not been elucidated to date (Debeaujon et al., 2001; Kitamura et al., 2004; Marinova et al., 2007). It is probable that combinations of proposed mechanisms are involved in condensation of flavan-3-ols, including transport and enzyme-coupling that would appear to be substrate specific.

Following PA biosynthesis is that of anthocyanins. The activity of LDOX results in the production of anthocyanin precursors (e.g. cyanidin) which are stabilized by the addition of glucose (or other sugar moieties) via UDP:glucose flavonoid transferase (UFGT) and can be further methylated (Methyltransferase), acylated (with acetate or coumarate) or further glycosylated (e.g. di-glucosides) (Boss et al., 1996). As mentioned above, PA accumulation in grape skins generally occurs prior to that of anthocyanins. This involves down regulation of PA directed genes and increases in those related to anthocyanin biosynthesis (Boss et al., 1996; Bogs et al., 2006). Recently, mechanisms of anthocyanin accumulation have been identified in *V. vinifera* involving a glutathione transferase (GST) and vacuolar trafficking via a MATE-type protein (Multidrug And Toxic Extrusion) (Ageorges et al., 2006; Gomez et al., 2009). Castellarin *et al*. (2007) subsequently demonstrated tight coordination of GST expression and anthocyanin accumulation during the development of grape berries. Upon searching for MATE-type genes within the grape genome, Gomez et al. found numerous candidates with phylogenetic similarity to *TT12* identified in Arabidopsis (Marinova et al., 2007). Further, the MATE genes that were characterized (AM1 and AM3) showed considerable selectivity for acylated anthocyanins. The implications from these studies are that the accumulation of flavonoids is dependent on a complex coordination of enzymes, many of which are independently regulated (based on localization and development) and exhibit considerable substrate specificity.

Beyond the specific structural genes related to flavonoid biosynthesis, many transcription factors (TF) have been identified that regulate groups of genes in a

coordinated fashion. Many of the TFs are of the R2R3-type MYB factors as described by Stracke et al. and are a closely related family of regulatory genes (Koes et al., 2005; Stracke et al., 2007). Flavonol biosynthesis, as directed by FLS, was recently found to be regulated by VvMYBF1, which also exhibited influence over CHI but little on down stream genes (Czemmel et al., 2009). Proanthocyanidin biosynthesis is regulated by numerous TFs such as MYB5a, MYB5b, MYBPA1, MYBPA2, MYBC and MYBD. The transcription factors MYB5a and MYB5b were found to regulate numerous flavonoid pathway genes leading to PA biosynthesis (except ANR by MYB5a) while having no effect on UFGT leading to anthocyanins (Deluc et al., 2006; Deluc et al., 2008). Both TFs were expressed in skin and seed before and after véraison, though MYB5a expression decreased during development. Transcription factors MYBPA1 and MYBPA2 were also found to regulate PA related genes without effecting UFGT (Bogs et al., 2007). The MYBPA1 TF is found to be active in both skin and seed before and after véraison (peaking at véraison), however MYBPA2 was more notably expressed in the skin tissue and showed little activity after véraison. Transcription factors MYBC and MYBD are also expressed in most berry tissues early in development; MYBC shows continued expression during berry ripening (Kobayashi et al., 2002; Castellarin et al., 2007). At véraison, MYBA1 (to some extent MYBA2) are found to be integral in the regulation of UFGT and other genes related to anthocyanin biosynthesis (Kobayashi et al., 2002; Jeong et al., 2004; Ageorges et al., 2006). Many MYB-type TFs have been identified involved in regulating grape flavonoid biosynthesis and additional genes continue to be

characterized. Regulation of many genes is under the control of more than one TF and the relationship between regulatory genes has not been determined.

Flavonoids and human health

The range of health benefits associated with plant phenolics is quite broad and has been demonstrated *in vitro* although *in vivo* studies have been conducted in humans and other animals. The most emphatic activity reported for flavonoids is their antioxidant capacity. This has been demonstrated for numerous flavonoids sourced from a wide variety of plants such as grapes, cranberries, tea, and rosemary (RiceEvans et al., 1996; Iwashina, 2000; Heim et al., 2002; Rice-Evans, 2004; Fernandez-Panchon et al., 2008; Ferrer et al., 2008). The health beneficial activities ranges from scavenging free radicals and preventing oxidation of lipids and inhibiting platelet aggregation to reducing oxidative damage to DNA (Frankel et al., 1993; Ahn et al., 2002; Fabiani et al., 2008; Hakim et al., 2008). Anti-cancer properties of anthocyanins, PAs and numerous other compounds have been attributed to the inhibition of cell proliferation, the modulation of apoptosis, and induction of enzymes related to cell detoxification (Santos-Buelga and Scalbert, 2000; D'Archivio et al., 2008; Wang and Stoner, 2008; Kaur et al., 2009). Further, PAs (more specifically A-type dimers) have been implicated in reducing the incidence of infections in the urinary tract by inhibiting adherence of causative bacteria (*Escherichia Coli*) to the epithelial cells (Prior and Gu, 2005). Aside from positive roles in human health, reviews have also covered negative aspects such as anti-nutritive effects (e.g. binding proteins), inhibition of vitamin and mineral availability through metal chelation,

possible carcinogenic properties (Santos-Buelga and Scalbert, 2000; Aron and Kennedy, 2008).

In grapes and wine, the "French paradox" first garnered attention when Renaud and de Lorgeril suggested diets inclusive of moderate red-wine were correlated reduced coronary heart disease (CHD) and increased life expectancy (Renaud and de Lorgeril, 1992). Subsequent work determined that phenolics extracted from red wine inhibited blood-lipid oxidation, which could contribute to a reduction in risk of CHD (Frankel et al., 1993; Frankel et al., 1995; Appeldoorn et al., 2005). Other studies have focused on specific compounds, such as resveratrol, as having exceptional antioxidant activity. Resveratrol (as with other flavonoids) may also possess some hormonal or estrogenic activity, and was most recently found to reduce inflammation and colitis in the gut as well as promoting beneficial flora (Kopp, 1998; Williams et al., 2004; Aron and Kennedy, 2008; Larrosa et al., 2009). As with other food items, the precise benefits of wine and grape consumption are uncertain but the biological activity of various purified compounds from grape is well documented and may contribute to human health (Frankel et al., 1995; Ahn et al., 2002; Gonthier et al., 2003; Appeldoorn et al., 2005).

While plant phenolics may exhibit beneficial *in vivo* activity, the compounds must first be absorbed and available to the body. Following ingestion, flavonoids travel through the small intestine and can be either excreted through the colon or further processed through the liver and travel to various tissues or through the circulatory system (Rice-Evans, 2004). It is suggested that most flavonoids entering the body are in the form of flavonoid-(β)-glycosides. These were first thought to be

resistant to hydrolysis by native microflora yet several enzymes with β-glycosidase activity were subsequently identified (Rice-Evans, 2001; Heim et al., 2002; Aron and Kennedy, 2008). Following degradation or hydrolysis, flavonoids can be conjugated with glucuronate, sulphate or methylated required for uptake and mobilization (Spencer et al., 2001; Rice-Evans, 2004).

Large PA polymers are not thought to be utilized intact; however hydrolysis and conjugation may occur (Santos-Buelga and Scalbert, 2000; Prior and Gu, 2005; Aron and Kennedy, 2008). In general, smaller PA oligomers (dimer / trimer) are thought to be absorbed into the intestine whereas the larger polymers are less permeable and are less susceptible to degradation in the intestine (Heim et al., 2002). Further, the B-ring catecholate configuration (e.g catechin) leads to higher relative antioxidant activity yet a higher rate of degradation and a reduction in availability (Rice-Evans, 2002). This emphasizes the point that the in vitro activity of compounds does not necessarily reflect in vivo activity, which must be independently demonstrated. Flavonoid derivatives have been recovered from waste, blood and organs showing various degrees of metabolism or modification and demonstrates some level of metabolism within the body (Gonthier et al., 2003; Spencer, 2003; Aura et al., 2005; Bieger et al., 2008).

Grape flavonoids and wine quality

Aside from proposed health benefits, grape derived flavonoids are critical to wine quality. The flavonoids of primary interest here are the flavonols, flavan-3-ols and anthocyanins found in grapes. These contribute primarily to the color and mouth-feel properties of wine. The flavonol content in grape berries is generally limited to

the skin, likely related to the function in UV protection of cells (Cheynier and Rigaud, 1986; Price et al., 1995). Further, the three main flavonols are derivatives of kaempferol, quercetin, and myricetin. These differ by the degree of B-ring hydroxylation, being mono-, di-, and tri-hydroxylated respectively. Compounds are typically found as glycosides (3-*O*-glucoside) or glucuronides however aglycones and other sugar-glycosides are found (e.g. 3-*O*-rhamnoside) in grapes and wine (Monagas et al., 2005). This glycosylation generally increases the solubility of flavonoids in water while the aglycones are less soluble. While flavonols may contribute to bitterness, an important role in wine quality is in stabilizing color (anthocyanins) and polymeric complexes through co-pigmentation (Drewnowski and Gomez-Carneros, 2000; Boulton, 2001).

Flavan-3-ols are found in the skin, seed and pulp of grapes (Verries et al., 2008). In all tissues, they exist in the form of monomer units as well as polymers (PAs). The predominant flavan-3-ols found in grape skin are (+)-catechin, (-)-epicatechin, and (-)-epigallocatehin; the former two are di-hydroxylated on the B-ring where the latter is tri-hydroxylated. (-)-epicatechin-3-*O*-gallate and (-)-gallocatetchin have also been found in the skin of some varieties, though tend to be in low proportion (Souquet et al., 1996; Hanlin and Downey, 2009; Mattivi et al., 2009). The polymer size or mean degree of polymerization (mDP) of skin PAs varies during development. While skin polymer sizes are in excess of eighty units, average sizes are around thirty units. In grape seed, PA mDP is typically much lower (under ten units) and flavan-3-ol monomers accumulate in substantial quantities in contrast to

skins. Further, (-)-epicatechin-3-*O*-gallate accumulates in considerable quantities while (-)-epigallocatechin is generally absent in the seed (Kennedy et al., 2000).

The sensory contributions of flavan-3-ols to wine include bitterness, astringency, and color. While the mechanisms involved in bitter recognition are not fully understood, molecular structure is thought to play a key role. Ligand receptors on the tongue have been identified and connected to the bitter response; however, many compounds eliciting bitter perception do not fit these models (Drewnowski and Gomez-Carneros, 2000; Rodgers et al., 2005). The astringent sensation elicited by PAs is due to their interaction with and precipitation of salivary proteins (proline rich proteins) in the mouth. This results in a reduction in lubrication on the surface of the tongue and increased apparent viscosity. The interaction between PAs and salivary proteins has been demonstrated and a positive correlation exists between concentration and protein precipitation (Naurato et al., 1999; de Freitas and Mateus, 2002; Gambuti et al., 2006). There does appear to be protein-specific binding threshold or saturation of PAs, which may be related to the differences in perceived astringency between subjects with high versus low salivary flow (Naurato et al., 1999; Peleg et al., 1999; Lesschaeve and Noble, 2005).

In general, monomeric and small oligomers of flavan-3-ols elicit bitterness, while larger polymers are thought to be more astringent (Drewnowski and Gomez-Carneros, 2000; Rodgers et al., 2005). Sensory research has supported this showing bitter intensity being inversely related to polymer size; in samples devoid of monomer flavan-3-ols (and small oligomers) bitterness was very low while astringency was quite intense (Peleg et al., 1999; Vidal et al., 2003; Lesschaeve and Noble, 2005).

The astringent quality of red wine is often described in terms such as “chalky”, “coarse”, “smooth”, “rough”, “aggressive”; terms that connect in-mouth sensations with familiar tactile responses (Gawel, 1998; Gawel et al., 2001; Vidal et al., 2003). The intensity of many of these attributes correlates positively to total phenolics, PAs, and polymeric pigments in wine; however this can be attenuated by the presence of polysaccharides and proteins (Gawel et al., 2001; de Freitas and Mateus, 2002; Vidal et al., 2004; Gawel et al., 2007). Work by Vidal et al. demonstrated a positive relationship between polymer size and perceived astringency as well as quality attributes associated with astringency (Vidal et al., 2004). Gallate esterification (e.g. (-)-epicatechin-3-*O*-gallate in seeds) tended to increase “rough” and “coarse” attributes of astringency while trihydroxylation (e.g. epigallocatechin found in skins) reduced the “coarse” and “dry” attributes and increased that of “fine grain”. In a similar manner, seed PAs are found to elicit astringency (protein binding) although bitterness was also found to be quite high (Arnold and Noble, 1978; Peleg et al., 1999; Gambuti et al., 2006). The suggestion, and conventional wisdom, is that skin PAs (high mDP, high trihydroxylation) provide astringency while seed PAs (low mDP, high gallate-esterification) may contribute more to bitterness.

Several studies have investigated the relevance of seed PAs to wine quality by adding and removing seeds during production (Kovac et al., 1995; Canals et al., 2008; Lee et al., 2008). Collectively, higher seed content results in small increases in the proportion of (-)-epicatechin-3-*O*-gallate and decreases in the proportion of (-)-epigallocatechin as expected. The differences in wine quality were not significant and in some instances seed additions resulted in a higher preference for wines. Based

on the given evidence, the relationship between wine quality and PAs may be driven by the composition (not total content) and resulting molecular interactions (proteins, polysaccharides etc.). The composition and extractability of these compounds changes during berry maturation as cell walls begin to degrade, evident in the softening of berries. Informal sensory evaluation of wines conducted in our lab (Dr. James Kennedy; Oregon State University) repeatedly show a preference (for mouth-feel and flavor) for wines made from more mature berries, until the overripe stage when preference declines. Evaluation of wines produced by Moreno et al. (unpublished sensory data) showed similar results, where aging berries up to four days after excision from the vine resulted in preferred wines (based on mouth-feel) (Moreno et al., 2008). Work by Cerpa-Calderon and Kennedy (2008) demonstrates that the rate and plateau of PA extracted is higher with increased maceration of berries during fermentation (crushing), a scenario that is expected in more mature berries when cellular integrity is compromised. Additional methods are employed to manage phenolic extraction and development during vinification and encourage attenuation or "softening" of PAs and color stability (generally through production of polymeric pigments); these include saignee (pre-fermentation), thermal treatment, use of cell-wall degrading enzymes, pre- and post-fermentation maceration, and mechanical disruption (punchdown / pumpover) (Sacchi et al., 2005).

The color of red wine is due to the presence of anthocyanins. Berry cells accumulating anthocyanins are isolated to the skin tissue of most *V. vinifera* cultivars used in wine production. Some cultivars accumulate anthocyanins within the pulp and these are used primarily for providing color to wine (Wang et al., 2003).

Additionally, anthocyanins in *V. vinifera* cultivars are differentiated by their B-ring substitution pattern (Figure 1.1), which can contain hydroxyl or methoxyl groups. The five predominant anthocyanins in winegrapes are cyanidin, delphinidin, peonidin, petunidin, and malvidin. These typically exist as 3-*O*-monoglucosides and may be acetylated or gallolylated through the sugar moiety (Lamuela-raventos and Waterhouse, 1994; Downey et al., 2004; Cortell et al., 2007). While anthocyanins exist as monomers in solution, they also form complexes (as in co-pigmentation) and polymers with flavan-3-ols and other phenolics over time (Es-Safi et al., 1999; Salas et al., 2003; Wrolstad et al., 2005). The polymerization of anthocyanins results in a shift from blue to red / orange and is generally responsible for the color stability of red wines (Cheynier et al., 2006). In addition, anthocyanins are found to have some impact on the mouth-feel properties of wine although it is suggested this may be due to the presence of additional compounds (Vidal et al., 2004).

Functions in plants

Phenolic compounds protect from microbial damage, Ultraviolet (UV) radiation, and predation among other environmental stresses during plant growth (Swain, 1975; Bennett and Wallsgrove, 1994; Robards and Antolovich, 1997; Parr and Bolwell, 2000). They are found in developing roots, vegetative tissues, leaves, and reproductive tissues. Aside from protection, they are involved in signaling within and between plants and other organisms and as such, aid in pollination, seed dispersion and reproduction. These compounds can be constitutive or inducible in nature; the former implying a basal, pre-existing level and while the ladder accumulate as part of a stress response (Levin, 1971).

Biotic Stress

Phytoalexin are antimicrobial compounds produced in response to pathogens (Hammerschmidt, 1999; Parr and Bolwell, 2000). Examples of pathogen induced phenolic accumulation are widespread though the interaction between pathogen / metabolite is not well understood. Kofalvi et al. (1995) showed a 1.5 fold increase of phenolics in wheat infected with wheat streak mosaic virus. Infected plants accumulated higher amounts of cinnamyl alcohols and activity of cinnamyl alcohol dehydrogenase (CAD) while the level of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) did not increase. This suggests an increase in lignin biosynthesis due to infection; however activity of oxidase enzymes and lignin content did not appear to change. Work with *A. thaliana* infected with *Xanthomonas campestris* provides additional explanation by identifying two cinnamoyl-CoA reductase genes (CCR1 and CCR2) related to lignin biosynthesis (Lauvergeat et al., 2001). It was found that CCR1 (involved in lignin biosynthesis) was not induced by infection while CCR2 (not involved in lignin biosynthesis) activity did increase. The conclusion is that that lignification is generally a delayed or secondary response to infection whereas accumulation of simple phenolics (e.g. cinnamyl alcohols via CCR2) is more likely a phytoalexin response and may otherwise strengthen the cell wall (Barber et al., 2000; de Ascensao and Dubery, 2003).

Punyasiri et al. (2005) studied the effect of *Exobasidium vexans* infection on tea plants (*Camellia sinensis* L) to determine the role of flavonoids in plant defense. (-)-epicatechin and (-)-epigallocatechin both decreased significantly upon infection while (-)-epicatechin-3-*O*-gallate increased. The increase in (-)-epicatechin-3-*O*-gallate

could result from (-)-epicatechin esterification with gallic acid; having higher antibacterial, antiviral and antioxidant capacity. Additionally, pathogen response and resistance was found to be positively related to the content of both PAs and anthocyanins, suggesting systematic induction of the flavonoid pathway.

In several plant / pathogen studies salicylic acid (SA; a phenolic compound) accumulation was coordinated with the pathogen response (Rasmussen et al., 1991; Lauvergeat et al., 2001). In strawberries (*Fragraria x ananassa* Duch.) inoculated with powdery mildew, SA levels generally followed the accumulation of phenolics in the leaves and fruit, however minimal differences in hormones (conjugated or free) or phenolic content were observed in infected plants (Hukkanen et al., 2007). While endogenous SA levels where relatively high, application of benzothiadiazole (BHT; analog of SA) to infected plants resulted in modest increases in phenolics. There was, however, a considerable reduction in the extent of infection in the leaves. As the authors suggest, most differences were evident in the cell-wall bound phenolic content, which is generally more difficult to accurately quantify due to limited extraction and hydrolysis. The results indicate some role of SA in pathogen response, likely targeted at cell-wall fortification, and efficacy of exogenous BHT in inhibiting infection.

Similar results were found upon application of BHT to grapes infected with grey mold (*Botrytis cinerea*); BHT reduced the incident of infection and increased the levels of PAs, stilbenes (resveratrol), and anthocyanins (Iriti et al., 2004; Iriti et al., 2005). In a previous survey of wines from a 12 year span years of high fungal pressure resulted in lower resveratrol levels in wine (Jeandet et al., 1995). The

rationale for this discovery is that the fungus produces an exo-enzyme (laccase-type) capable of oxidizing resveratrol in self-defense. This is supported by evidence of Landrault et al. that shows temporal increases in stilbenes in response to infection followed by a decline towards maturity (Landrault et al., 2002). Analysis of grapes exposed to *Botrytis cinerea* shows accumulation of resveratrol in areas surrounding fungal infection suggesting a localized effect of the antimicrobial compound (Jeandet et al., 1995). A similar localization of phenolics at the site of infection was seen in response to powdery mildew (*Uncinula necator*) suggesting a localized, cellular response, though it was not the primary mechanism for resistance (Ficke et al., 2004). Contrary to the response to *B. cinerea*, stilbene levels showed a positive relationship to the degree of powdery mildew infection in grapes (Romero-Perez et al., 2001). This discrepancy is proposed to be due to the inability of *U. necator* to degrade or oxidize the stilbene metabolites and further demonstrates the evolved relationship between plant phenolics and plant pathogens.

There are numerous additional examples of biotic interactions eliciting plant phenolic accumulation including interactions with nematodes and rhizobia associated with root tissues some (Feeney, 1970; Herms and Mattson, 1992; Matsuki, 1996; Hammerschmidt, 1999; Parr and Bolwell, 2000; Mishra et al., 2006).

Abiotic Stress

Water

Water, including rainfall, is an integral element of the environment / climate and a fundamental requirement for plant survival, allowing for transport of solutes

and maintenance of cellular integrity. In many cases, irrigation strategies are used to manage crop vigor and quality. The rate and timing of irrigation is a critical factor for the management of wine grapes (Winkler et al., 1974; Gladstones, 1992; Deloire et al., 2004). In most studies, reductions in irrigation imposed before and after véraison resulted in decreases in berry size (Hardie and Considine, 1976; Matthews and Anderson, 1988; Kennedy et al., 2002; Roby et al., 2004; Castellarin et al., 2007; Castellarin et al., 2007). Hardie and Considine (1976) showed that periodic water deficit (22 days) applied at anthesis hampered fruit set and berry size and that late season deficit (approximately 30 days before harvest) did not significantly reduce berry weight or volume. As discussed by Kennedy et al. (2002), the differences in berry size can influence the reported concentration of metabolites and it is therefore relevant to consider both parameters.

Roby et al. imposed irrigation treatments on Cabernet Sauvignon vines from véraison to harvest (Roby et al., 2004; Roby and Matthews, 2004). Berries of similar size were compared between treatments receiving twice (H) the volume of water from control (C) and withholding water (L) when mid-day leaf water potential was between -1.0 and -1.5 MPa. It was found that skin PA and anthocyanin concentrations tended to be higher in L and lowest in H berries. Seed PA concentration was generally unaffected by irrigation. Using a similar irrigation regimen, Kennedy et al. (2002) found skin PAs, flavonols, and anthocyanins to be highest following deficit irrigation on a *per gram* basis although minimal differences appeared *per berry*. Ginestar et al. (1998) had comparable results with Shiraz under post-véraison deficit; however values were significantly higher under deficit when

expressed on a mass basis and significantly lower *per berry*. A similar observation was made by Castellarin et al. (2007) where metabolite differences (PAs and anthocyanins) were only evident in anthocyanins when expressed on *per gram* basis. Sivilotti et al. (2005) also report higher total phenolic and anthocyanin content in Merlot berries under post-véraison water stress; however data is presented on a mass basis and berry mass was reduced due to treatments. The explanation provided is that water stress results in compositional changes including increased PA mDP; this was based on reduced extractability using ethanol compared to methanol and is not directly assessed. Kennedy et al. (2002) show compositional differences due to deficit irrigation (increased (-)-epigallocatechin extension subunits) however increases in mDP were not significant. The increase in proportion of (-)-epigallocatechin agrees with Castellarin et al. (2007) who observed an increase in the activity of F3′5′H relative to F3′H following both pre- and post-véraison deficits. Long-terms water stress of Merlot vines (~bloom through harvest) did show differences in anthocyanin content on a mass and *per berry* basis accompanied by up-regulation flavonoid biosynthetic genes (Castellarin et al., 2007). PA content was not reported in the latter study so it is unclear whether the up-regulation of the flavonoid pathway affected their biosynthesis in a similar manner to anthocyanins.

In work conducted by Ojeda et al. 2002 water deficits were imposed either before véraison and relieved or from véraison to harvest. In general, post-véraison stress resulted in the highest content of flavonoids on a mass and *per berry* basis. Strong pre-véraison deficit (30% evapotranspiration relative to control) resulted in greater reductions in all parameters than moderate (50%) pre-véraison or strong post-

véraison treatments. Further, while flavonol and anthocyanin contents were highest *per berry* from post-véraison deficit, flavan-3-ol and PA contents were not significantly higher relative to control. Data from Castellarin et al. (2007) shows that early season deficits impacted PA accumulation until véraison (during active biosynthesis), however at maturity all treatments came to similar levels. Anthocyanin content was maintained at higher levels in both deficit treatments until the sampling at maturity, when treatment differences disappeared. A similar trend was observed regarding the composition of anthocyanins; deficits resulted in higher proportions of tri- relative to di-hydroxylated anthocyanins (Castellarin et al., 2007). The authors also noted a considerable hastening in the inception of véraison due to early season deficits, which was also seen in induction of genes dedicated to anthocyanin biosynthesis. Similar observations have been made regarding inception of coloration; however data from Ojeda et al. do not show this and Matthews et al. clearly state the observation was not made, though it may be expected (Hardie and Considine, 1976; Matthews and Anderson, 1988; Ojeda et al., 2002; Castellarin et al., 2007). In the latter study skin phenolics and anthocyanin contents were found to be highest from early season deficit, followed by late season and lowest from continually irrigated vines (Hardie and Considine, 1976; Matthews and Anderson, 1988; Ojeda et al., 2002; Castellarin et al., 2007). The data presented here clearly show that water stress can have an impact on berry development and phenolic biosynthesis but is dependent upon timing (evident in metabolites and gene transcripts). The discrepancies between trials may be due in part to differences in cultivar response to stress or the degree or

exact timing of treatments. It can be concluded, however, that water stress is more likely to affect flavonoids produced after véraison.

Solar / UV radiation

One of the primary roles of plant flavonoids is protection from the deleterious effects of solar radiation, specifically that in the UV spectrum, such as photo-oxidative stress (Dixon and Paiva, 1995; Close and McArthur, 2002; Winkel-Shirley, 2002). Zucker (1965) first demonstrated that PAL, the first step toward flavonoid biosynthesis, was inducible by white light. Deikman and Hammer (1995) later demonstrated light-dependent induction of PAL, CHS, CHI, and DFR in *Arabidposis* as well as a temporal pattern of expression. They found that all genes were coordinately expressed and followed a circadian clock; levels were reduced several hours before the dark period then increased again before darkness. This demonstrates that flavonoid biosynthesis is not only light-sensitive but is active during the night and day.

In a mutant barley strain (*Hordeum vulgare* L.) deficient in PAs and total flavonoids (7% of wild-type), UV radiation resulted in an decline in plant status (leaf weight, rigidity, vigor), increased UV penetration into the leaf, and potential reduction in photosynthetic potential compared to the wild-type (Reuber et al., 1996). Further, the non-mutant strain showed induced accumulation of flavonoids in response to UV exposure where the mutant strain did not. Liu et al. also observed UV-B induced accumulation of barely flavonones (lutonarin and saponarin), which was correlated with an increase in PAL acitivity (Liu et al., 1995; 1995). These results are in accord with studies in canola (*Brassica napus* L.) and rye (*Secale*

cereale L.) where exposure to UV-B resulted in increases in flavonoid contents, chlorophyll and photosynthetic activity (Tevini and Teramura, 1989; Alenius et al., 1995).

Work with other mutant plant lines has also provided evidence for the UV protective roll of flavonoids and phenolics. Li et al. (1993) identified several *A. thaliana* mutants that are hypersensitive to UV-B radiation; the *transparent testa-4* (*tt4;* CHS mutation) showing moderate sensitivity and *tt5* (CHI mutation) showing even greater sensitivity. It was determined that wild-type plants showed UV-B induction of PAL, CHS and increases in sinnapate esters and, most notably, flavonol-type compounds (e.g. kaempferol). The moderate sensitivity of *tt4* was attributed to the accumulation of sinnapate esters under UV-B, although flavonols failed to accumulate. The *tt5* line showed considerable damage at even moderate UV-B levels, which was attributed to the lack of induction of UV absorbing compounds. In a contrasting fashion, Bieza and Lois (2001) identified an *Arabidopsis* line with increased tolerance for UV-B radiation (*uvt1*). In this case, the *uvt1* mutant exhibited higher basal levels of CHS, which resulted in greater induction of UV absorbance in the leaf, greatly reduced UV transmittance, and exhibited little tissue damage at high UV levels compared to wild-type. The protective roll of flavonoids on the photosystem II (PSII) was demonstrated by Booij-James et al. (2000) using similar *A. thaliana* mutant lines deficient in proteins related to flavonoids (CHS and CHI) and sinapate esters (ferulate-5-hydroxylase; F5H). They determined that the two proteins forming the core complex of the PSII system (D1 and D2) displayed slightly different UV sensitivity; however both sinapate esters and flavonoids provided protection from

UV-B radiation. The considerable turnover of D2 in the F5H mutant (still capable of flavonoid biosynthesis) compared to those competent of sinapate ester production prompted the authors to question the essential role of flavonoids over other phenolic compounds in UV protection.

Mellway et al. (2009) recently identified *MYB134*, a transcription factor in poplar (*Populus* spp) analogous to *tt4* found to regulate PA biosynthesis in *Arabidopsis* and similar to *VvMYB5a* and *VvMYBPA1* in grapes. In order to determine the regulatory role in PA flavonoid biosynthesis they subjected leaves to UV-B radiation as well as physical damage. Immediately following exposure to UV-B radiation (3 hours) flavonoid pathway genes up to FLS were up-regulated, coincident with accumulation of flavonol glycosides, however *MYB134* was not. Similarly, downstream genes directed towards PA biosynthesis were not immediately induced, though after 48 hours of light exposure *MYB134* and PA related genes (AND, DFR, LAR, and MATE (TT12 homolog from *A. thaliana*)) were strongly up-regulated. Wounding resulted in a similar response, though it was noticeable with 12 hours of treatment and had no effect on FLS activity. In addition to wounding, fungal infection resulted in up regulation of the *MYB134* and related genes while FLS was not affected. Over expressing *MYB134* resulted in up-regulation of all PA related genes and increased PA content in all tissues analyzed. It was noted the two additional TFs were co-induced with *MYB134* however levels were comparatively low in all cases. This elegantly demonstrated the differential regulation of flavonoids responding to the same stress, and the specific response of PA biosynthesis to wounding. Paolocci et al. (2005) found similar UV-induction of PAs in *Lotus*

corniculatus L. transgenic plants expressing the maize TF (*Sn*) related to anthocyanin biosynthesis. All plants showed an increase in PA containing cells following exposure to UV radiation, however over expression of the *Sn* gene resulted in further enhanced PA levels.

In research with *V. vinifera* UV radiation or exposure to sunlight has also been shown to influence flavonoid composition. Kolb et al. (2001) have demonstrated the protective effects of flavonoids (particularly flavonol glycosides) on the photosynthetic properties of grape leave. UV-B had a large effect on flavonol accumulation in the upper epidermis of the leaf, providing UV-B screening and reducing transmittance. Phenolic acids were induced slightly by visible light but were lower under UV exposure. Price et al. (1995) showed an increase in grape skin flavonols from sun-exposed berries compared to shaded. Anthocyanin content was not found to be different based on exposure. Similar results concerning grape skin flavonols has been reported elsewhere; sun exposure is positively related to flavonol content. As determined by Mellway et al. (2009), this is likely due to the specific regulatory mechanisms involved in flavonoid biosynthesis. Recently identified TFs in grape (*VvMYBF1*) and *Arabidopsis* (*MYB11, MYB12, MYB111*) are found to regulate FLS activity and the accumulation of flavonols only, explaining why flavonol biosynthesis may respond differently to UV than related, down stream flavonoids (Stracke et al., 2007; Czemmel et al., 2009).

Light stimulation of flavonoid biosynthesis in *V. vinifera* has been demonstrated in numerous studies. Potted vines (Cabernet Sauvignon and Pinto noir) were placed in a phytotron in the presence or absence of light during various stages of

development (Dokoozlian and Kliewer, 1996). In all cases, berries exposed to standard daylight accumulated highest amounts of skin anthocyanins and total phenolics. Berries shaded before véraison or at the lag phase of development showed inhibited coloration compared to control. Additionally, the phenolic contents of berries was similar regardless if shading occurred before or after véraison and lowest in berries shaded during the entire experiment. In two independent studies anthocyanin content tended to be higher in exposed berries during development, however not all differences were maintained by harvest (Crippen and Morrison, 1986; Bergqvist et al., 2001). Bergqvist et al. (2001) also found a positive correlation between sun exposure (measured as photosynthetically active radiation (PAR)) and total phenolic and anthocyanin content. However, south-facing berries showed at decline in phenolic content at highest levels of PAR, attributed to thermal damage associated with solar radiation (Crippen and Morrison, 1986; Bergqvist et al., 2001).

Smart et al. (1988) observed a linear decrease in both anthocyanins and total phenolics in skins under increasing layers of shade cloth. Supplementation with red light (660nm) resulted in increased contents in both untreated and heavily shaded berries. In this study treatments were imposed two weeks prior to véraison, which suggests PA biosynthesis would be minimally affected although post-biosynthetic modifications may take place. Spayd et al. (2002) used UV transmitting and absorbing cloth as well as natural shading techniques (leaf coverage) to modify the light environment around clusters. Reductions of UV transmission clearly reduced flavonol concentrations. Anthocyanins were not affected by blocking UV transmission alone; however concentrations did tend to be lower under natural shade.

There were additional effects of temperature on biosynthesis as well as partitioning of anthocyanins based on substitution patterns. This was investigated further by Tarara et al. (2008) who determined that shading alone had minimal impact on anthocyanin accumulation, though shading in combination with higher temperatures inhibited anthocyanins. It was determined that the effects of solar radiation alone were not as profound as the impact of temperature on anthocyanin content.

Jeong et al. (2004) studied the accumulation of anthocyanins under exposed or naturally shaded (20% incident light) conditions and followed the expression of core anthocyanin pathway genes (from CHS to UFGT) and the anthocyanin TF *VvMYBA1*. Exposed berries accumulated greater amounts of anthocyanins than berries that were shaded during the entire experiment, resulting in a 2-fold increase at harvest. Additionally, the expression of all genes assayed was up-regulated in exposed berries. Similar shading and exposure methods were initiated by Matus et al. (2009) at véraison. In addition, a leaf removal treatment (at véraison) and a delayed exposure treatment (tucking leaves 6 weeks after véraison) were used to investigate sink-source and timing effects. Exposure of grapes at véraison resulted in increases in the content of all anthocyanins monitored however leaf removal resulted in a slight delay in accumulation. Delayed exposure had minimal effect on anthocyanin accumulation; however flavonol content did increase concomitant with exposure. Flavonols were also highest in exposed fruit, though leaf removal inhibited flavonols compared to moving leaves to provide fruit exposure. Expression of related regulatory and structural genes was well correlated to anthocyanin accumulation in treatments other than leaf removal. While the TF *MYBA* was higher following removal of leaves,

other TFs and structural genes including a hexose transporter were reduced. This suggests a considerable disconnect between the expression of structural genes and metabolite accumulation following removal of leaves (carbon source). The expression of FLS and a related putative TF (*MYB12*) was well coordinated with the concentration and timing of flavonol accumulation.

In two different studies light was excluded from developing clusters by enclosing them in specifically designed plastic boxes (Downey et al., 2004; Cortell and Kennedy, 2006). In both studies, flavonol content was drastically reduced by shading. Anthocyanins were found to be slightly higher in exposed Shiraz berries at harvest though were not different in Pinot noir. In both studies, skin PA content reached maximal levels in exposed berries at véraison though by harvest there were no differences in Shiraz (Downey et al., 2004; Cortell and Kennedy, 2006). The increased skin PA content in Pinot noir berries was maintained at harvest, and in both studies, skin PA mDP was higher in exposed fruit at all sampling points (Downey et al., 2004; Cortell and Kennedy, 2006). Seed PA content showed minor and variable differences in both studies. Downey et al. (2004) found higher levels of flavan-3-ol monomers in exposed berries at véraison and harvest, while terminal and extension units were higher in exposed berries at both sampling points. There were no differences in flavan-3-ol monomers in Pinot noir though shading resulted in slight increases in extension and terminal units at harvest (Downey et al., 2004; Cortell and Kennedy, 2006). There were no differences in seed PA mDP reported at harvest. A commonality here, and in other studies, is an increase in the proportion of tri-hydroylated flavonoids (PAs and anthocyanins) in exposed fruit (Spayd et al., 2002;

Koyama and Goto-Yamamoto, 2008; Tarara et al., 2008). Further, results from Downey et al. (2004), show a general increase in the proportion of anthocyanins as acetyl-glucosides; Pinot noir does not accumulate acylated anthocyanins so there is no similarity in Cortell et al. (2006). From these studies it clear that flavonoid accumulation in grape berries does not necessarily require light. This is somewhat contrary to work by Diekman and Hammer (1995) but suggests flavonoid regulation in the berry may be linked to signals produced elsewhere within the plant that manage the circadian clock.

Fujita et al. (2007) also followed the accumulation of PAs in seed and skin under shade and exposed conditions, though monitored the expression of LAR1, LAR2, and ANR. Similar to the previous studies, PA levels (*per berry*) tended to be higher in exposed grape skins until harvest, when differences narrowed. Seed PAs showed minimal differences though were slightly higher in exposed berries around véraison and declined to similar levels by harvest. The expression of all three genes tended to be highest in exposed skins until véraison, after which levels declined; however the decline in LAR1 and LAR2 was delayed in the skins of shaded berries. In seeds, LAR1 and ANR were expressed earlier in development where LAR2 was delayed by twenty days. In general, relative expression levels were similar to those observed in skins. Expression levels are in reasonable agreement with metabolite data early in development; however PAs appear to accumulate after véraison when most gene expression has declined. From the work presented, it is clear that the exposure to UV or solar radiation can influence flavonoid accumulation from phenolic acids to anthocyanins.

Temperature

Within the *Lotus* species, several authors have reported variability with respect to PA accumulation and temperature responses. Angura et al. (1993) found that PA content increased in one species (*L.pedunculatis*) while decreasing in another (*L. corniculatus*) as day and night temperatures were elevated. Carter et al. (1999) also report a decrease in PA content in *L. corniculatus* as temperatures increased from 18 / 10°C to 25 / 15°C (day / night). In that case, PA levels were found to decrease in the leaves and slightly in the stems, though not in the roots; in all cases the differences were driven by changes in the amount of extractable versus bound PAs. Similar observations were made in birch seedlings (*Betula pendula* L.) upon increasing the ambient temperature by 2°C (Kuokkanen et al., 2001). Decreases were observed in leaf total phenolics, catechins, PAs, cinnamic acids, and flavonol glycosides, while flavonol aglycones increased. Similar decreases were observed in the stem, although only in the developing portion and not in the basal sections. Oak leaves (*Quercus robur* L.) showed an increase in PA content when grown at 3°C above ambient temperatures; however the increase was not significant until 45 days after initial expansion of the leaves (Dury et al., 1998).

In tomato and watermelon leaves, temperature related increases in phenolic content were coordinated with increased PAL activity and decreased oxidase activity (peroxidase and polyphenol oxidase) (Rivero et al., 2001). The sensitivity of the two plants was found to be quite different; watermelon exhibited a decrease in phenolics as temperatures increased from 15 to 35°C while tomato had highest levels at 35°C, followed by 15°C and lowest at 25°C. In this case the response to temperature, and

likely the composition/function of phenolics, differs between the two species. Caldwell et al. (2005) reported a decreasing trend in the isoflavones in soy (*Glycine max* L.) as temperatures increased from 18 to 28°C. They also observed temperature related partitioning between the predominant isoflavones (towards the methylated derivative) and a positive influence from both drought and carbon dioxide levels. High-temperature treatments (39 / 19°C) applied to rose buds (*Rosa hybridia* L.) were ineffective after only one day, but caused a reduction in anthocyanins when maintained for 3 days (Dela et al., 2003). The treatment was most pronounced during the middle stages of bud development and resulted in a shift towards the mono-hydroxylated anthocyanin (pelargonidin) (2:1) normally in equilibrium with cyanidin. While the decrease in anthocyanins was correlated with CHS and DFR activity, the level of F3′H protein was not found to be different. This suggests either preferential degradation of the di-hydroxylated anthocyanins or reduced enzyme activity due to temperature inhibition. Contrary to many of the previous examples, p-coumaroyl glucoside, flavonols and anthocyanins in strawberry fruit increased as day / night temperatures were elevated (Wang and Zheng, 2001). From these examples it is clear the effect of elevated temperatures varies by metabolite and plant species as well as tissue.

Opposing the general inhibitory effects of high temperature on anthocyanins, low temperature has been shown to enhance anthocyanin accumulation in several plant species. Christie et al. (1994) demonstrated that transient (24 hour) 10°C treatments of maize induced rapid up-regulation of anthocyanin biosynthetic genes and consequent anthocyanin content. Core flavonoid genes (PAL and CHS) remained

up regulated up to 48 hours after treatment while others, including UFGT, showed more rapid decline during the first 12 hours after treatment. In studies with apple and pear (*Malus domestica* B. and *Prunus persica* L. respectively), cold treatment was associated with increases in anthocyanin content (Leng et al., 2000). Plants grown outside showed peak accumulation of anthocyanins (and decreased leucoanthcyanidins) as temperature reached minimums in the winter months. Additionally, shoots subjected to sub-freezing temperature showed continual increases in anthocyanins until reaching lethal temperatures. A similar pattern of low temperature induction of anthocyanins and flavonols was observed in *Arabidopsis* when temperatures approached 10°C (Olsen et al., 2008). Metabolite levels in that study were tightly correlated with the expression of two PAL genes as well as the activity of PAL enzymes, suggesting some specific role of the two genes and possibly temperature induced protein modifications.

Previous research has demonstrated the effect of both high and low temperatures on anthocyanin accumulation in grapes. Early work by Kliewer et al. showed inhibition of anthocyanin development at higher temperatures (e.g. 35°C) while at moderate day temperatures (e.g. 25°C) anthocyanins accumulate more when the difference between day and night temperatures is reduced (Kliewer and Torres, 1972; Kliewer, 1977). This was also determined by Kobayashi et al. (1967) along with hastening of berry coloration pre-véraison with increased night temperatures; however those same temperatures resulted in lower color levels of berries when applied during maturation. An investigation of the timing of such treatments found temperature variations at 1 to 3 weeks after berry coloration had the largest effect on

anthocyanin accumulation (Yamane et al., 2006). Holding the temperature at 20°C resulted in a doubling in anthocyanin concentration while 30°C resulted in considerable (2-3 fold) reduction at harvest.

Research over the past ten years has confirmed the reduction of anthocyanins at higher day (above 30°C) and night temperatures (30°C vs 15°C) and temperature related partitioning of anthocyanins (Kliewer, 1977; Mori et al., 2005; Yamane et al., 2006; Tarara et al., 2008). The latter point was postulated by Kliewer (1977) as contributing to the variability observed between different grape cultivars(Kliewer, 1977). The partitioning of anthocyanins generally favors tri-hydroxylation and acylation in response to higher temperatures. This was clearly demonstrated by Tarara et al. (2008) and the chemical stability afforded by these modifcatoins was shown by Mori et al. (2007). Further, heat-mediated reduction of anthocyanins has been related to reductions in gene transcripts and enzyme activities; most notably that of UFGT (Mori et al., 2005; Yamane et al., 2006; Mori et al., 2007).

While considerable research has focused on the temperature influences on anthocyanins, few have examined flavonols or PAs directly. Mori et al. (2004) showed slight increase in the flavonol content of berries held at 25 or 30°C compared to a 30 / 15° cycle, which may be due to the reduction in anthocyanins under the same conditions (Mori et al., 2005). They found no difference in PAs between berries held at 30°C or those at 30 / 15°C; however berries held at 25°C exhibited high concentrations from véraison until harvest, when all treatments reached the same levels. As PAs primarily accumulate before véraison, this work may suggest that a 30°C day temperature results in more rapid degradation or sequestration of PAs until

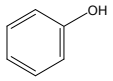
harvest. In two consecutive studies Spayd et al (2002) and Tarara et al. (2008) followed flavonol biosynthesis and determined there was little influence of temperature. Given the information provided by Mellway et al. (2009), PAs, flavonols and anthocyanins may share the same pathway; however their regulation and function in the plant is quite different.

The literature reviewed here shows considerable light and temperature effects on the biosynthesis and accumulation / degradation of various plant phenolics. Studies of grapes and sun exposure show considerable effects on flavonoid biosynthesis, though little has been done to uncouple the effects of light exposure and temperature. It is well documented that the surface temperature of exposed berries may exceed 10°C above ambient temperatures, expanding the actual temperature range of fruit and bringing in to question the complex nature of effects attributed to light exposure (Kobayashi et al., 1967; Kliewer, 1968; Smart and Sinclair, 1976; Kliewer, 1977; Bergqvist et al., 2001). Some of these relationships have been studied in regards to anthocyanins though very little information is available regarding PAs. Due to the importance on PAs in grape and wine quality as well as their roles in plant and animal health, it is valuable to improve our understanding of factors controlling their biosynthesis.

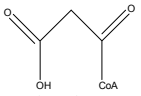
Research presented above has demonstrated different temperature responses based upon timing, duration and magnitude of treatments. Further, many studies have shown the effects of modifying both the day and night temperature (diurnal temperature range or thermoperiodicty) as opposed to uni-directional changes. In the

current research we have investigated the effects if manipulating day and night temperatures separately *and* in combination.

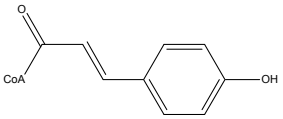
Figure 1.1 Structure of simple phenolic compounds and flavonoids within the flavonoid biosynthetic pathway.



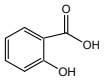
Phenol



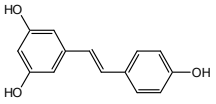
Malonyl- CoA
(intermediate)



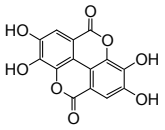
p-Coumaryl-CoA



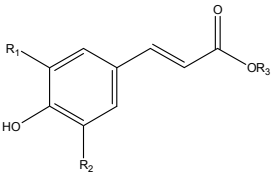
Salicylic acid

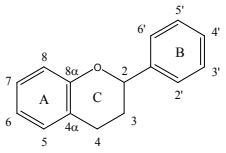


trans-Resveratrol

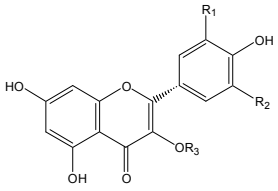


Ellagic acid



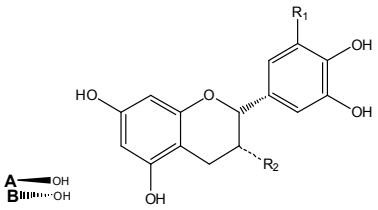


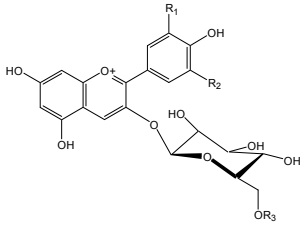
General Flavonoid
Backbone



Hydroxycinnamic acids	R1	R2	R3
p-Coumaric acid	H	H	H
Caffeic acid	OH	H	H
Ferulic acid	O-Me	H	H
Caftaric acid	OH	H	Tart

Flavonols	**R1**	**R2**	**R3**
Kaempferol	H	H	H
Quercetin	OH	H	H
Myricetin	OH	H	H

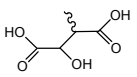


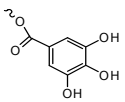


Flavan-3-ols	**R1**	**R2**
(+) Catechin	H	A
(-) Epicatechin	H	B
(-) Epicatecin-3-O-gallate	H	Gall
(-) Epigallocatechin	OH	B

Anthocyanins	**R1**	**R2**	**R3**
Cyanidin-3-O-glucoside	OH	H	H
Peonidin-3-O-glucoside	O-Me	H	H
Delphinidn-3-O-glucoside	OH	OH	H
Petunidin-3-O-glucoside	O-Me	OH	H
Malvidin-3-O-glucoside	O-Me	O-Me	H
[ ]-3-O-acetyl-glucoside	*as above*	*as above*	Ace
[ ]-3-O-coumaroyl-glucoside	*as above*	*as above*	Coum

Me = ~CH_3
Glc = ~Glucose
Ace = ~$COCH_3$

Tart = 

Gall = 

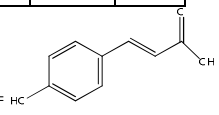
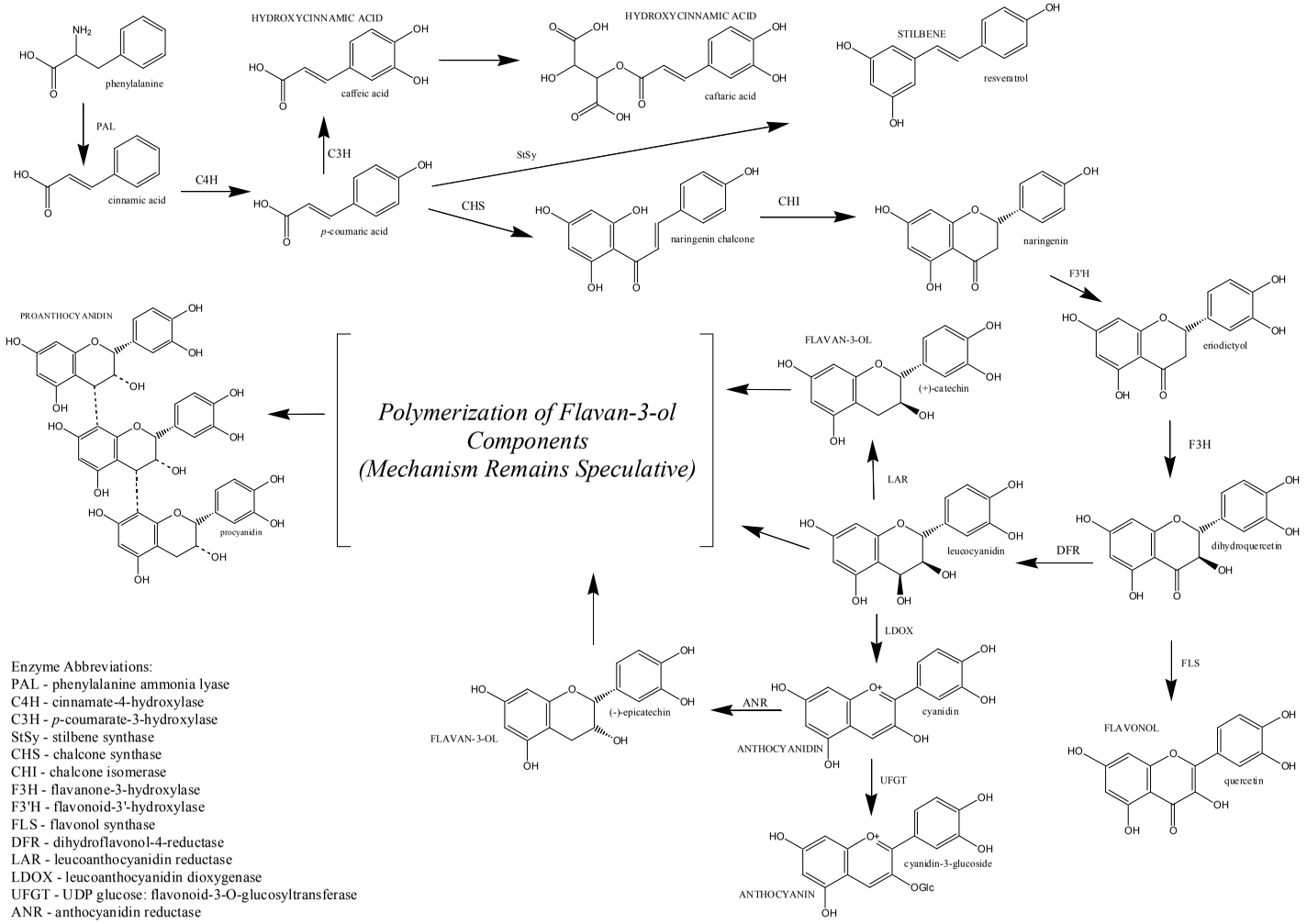
Coum = 

Figure 1.2 Generalized flavonoid biosynthetic pathway leading to flavonols, anthocyanins and proanthocyanidins.



Enzyme Abbreviations:
PAL - phenylalanine ammonia lyase
C4H - cinnamate-4-hydroxylase
C3H - *p*-coumarate-3-hydroxylase
StSy - stilbene synthase
CHS - chalcone synthase
CHI - chalcone isomerase
F3H - flavanone-3-hydroxylase
F3'H - flavonoid-3'-hydroxylase
FLS - flavonol synthase
DFR - dihydroflavonol-4-reductase
LAR - leucoanthocyanidin reductase
LDOX - leucoanthocyanidin dioxygenase
UFGT - UDP glucose: flavonoid-3-O-glucosyltransferase
ANR - anthocyanidin reductase

Chapter 2

Daily temperature excursions have limited impact on grape skin proanthocyanidins and related gene expression before fruit ripening.

Abstract

Temperatures during grape berry development and ripening are of critical importance with respect to berry composition and resulting wine quality. The impact of temperature on metabolism of sugars, organic acids and anthocyanins has been established, however little is known regarding the impact on proanthocyanidins (PAs) in grape skins. In this study, field grown grapes (cv. Merlot) were cooled during the day or heated at night by +/- 8 °C, from fruit set to véraison in 2006 the 2008, to determine the effect of temperature and thermal time on PA accumulation. Berry morphology, PA content and composition, and the expression levels of several genes involved in flavonoid biosynthesis were determined. The PA content *per berry* was highest in heated berries and lowest in cooled berries in 2006 and not different in 2007 or 2008. Cooling berries resulted in an increase in the proportion of (-)-epigallocatechin as an extension subunit, which was significant in 2006 and 2008 ($p < 0.05$) and less so in 2008 ($p = 0.076$). Heating and cooling berries altered the rate of berry development compared to ambient berries evident in berry morphology and the expression of several structural genes within the flavonoid pathway.

Introduction

Plant secondary metabolites have been the focus of an array of research, warranted by their diversity of structure, function and occurrence. They are involved in plant defense from biotic and abiotic stresses, aid in survival (e.g. seed dispersion), and have proposed bioactivity in humans and other foraging animals (Dixon and Paiva, 1995; Hammerschmidt, 1999; Rice-Evans, 2001; Winkel-Shirley, 2002; Xie and Dixon, 2005; Fernandez-Panchon et al., 2008). 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 cinnamic acids, lignin, and flavonoids, which include three distinct classes of compounds; flavonols, anthocyanins, and proanthocyanidins (PAs).

Production of each class is putatively mediated by a committed enzymatic step to fulfill a specific function within the plant. Flavonols are thought to protect from UV radiation whereas anthocyanins are thought to provide some protection from UV exposure, thermal stress, and aid in seed dispersion (signaling between plants and other organisms) (Dixon et al., 2002; Winkel-Shirley, 2002; Adams, 2006). PAs are polymers of flavan-3-ol subunits, produced in the cytosol, stored in vacuoles, and may be incorporated into cell walls (Aerts et al., 1999; Marles et al., 2003; Gagne et al., 2006). They are thought to be feeding deterrents and have antifungal properties (Aerts et al., 1999). All of these flavonoids have antioxidant properties (Dixon and Paiva, 1995; Robards and Antolovich, 1997; Rice-Evans, 2001).

Flavonoids have garnered particular interest in wine grapes, *Vitis vinifera* L., due to their contribution to perceived wine quality and their potential health benefits. While anthocyanins are responsible for the color of red wine, flavonols are thought to contribute to red wine color via co-pigmentation (Boulton, 2001; Adams, 2006). PAs are found in seed and skin tissue and are responsible for red wine astringency (Gawel et al., 2001; Vidal et al., 2004).

One of the more topical concerns relating to grapes and many other crops is the effect of climate shifts on yield and quality parameters. With regard to *V. Vinifera,* the relationship to climate is evident in the geographic distribution of cultivars suitable for wine production. For example, cultivars such as Cabernet Sauvignon and Pinot noir were subjected to selection based upon their performance in different climates (Vivier and Pretorius, 2002; Bessis, 2007). Amerine and Winkler (1944) adopted a model for classifying wine growing regions based upon accumulated thermal time and to determine the suitability of various grape cultivars for different climates. This model has been effective for choosing cultivars of *V. vinifera* for planting based on general ripening parameters; however it does not account for seasonal variations that influence fruit composition. Several authors have discussed the implication of these decisions in the face of climate change, and from this, have concluded that wine growing regions in the world are likely to be reassessed in the decades to come (Kenny and Shao, 1992; Jones et al., 2005; White et al., 2006). While there is debate regarding the role of humans in recent climate change there are clearly recorded periods of extreme climate events (temperature and precipitation) (Easterling et al., 2000; Chuine et al., 2004; Mann et al., 2009).

Therefore it is important to consider the ramifications that climatic factors, such as temperature, may have on wine grapes and other crops of socio-economic importance.

Research has been conducted specific to the effects of temperature on grape development and composition. Kliewer *et al*. reported the effects of varied temperatures on metabolism of sugars, acids, and anthocyanins using growth chambers and greenhouses (Kliewer, 1964; Buttrose et al., 1971; Kliewer and Torres, 1972; Lakso and Kliewer, 1975; Kliewer, 1977). Additional studies have focused on the effects of exposure to solar radiation and temperature on berry composition; however, few have been able to isolate temperature *per se* as a contributing factor (Crippen and Morrison, 1986; Bergqvist et al., 2001; Downey et al., 2004; Mori et al., 2005; Cortell and Kennedy, 2006; Yamane et al., 2006; Mori et al., 2007, 2007). In limited cases, experiments were conducted under field conditions but have not assessed 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 isolated cluster temperature on PA biosynthesis in the skin of grape berries. Biosynthesis occurs during the first phase of berry growth leading up to véraison (onset of fruit ripening), and therefore this period of development was the focus of study (Coombe and McCarthy, 2000; Robinson and Davies, 2000; Kennedy et al., 2002; Downey et al., 2003). We significantly modified the thermal time accumulated by berries growing in the vineyard. This was achieved by two approaches: 1) cooling berries during the day and 2) heating berries during the night. The effect of temperature treatments on

temporal development, PA accumulation and composition, and several key genes of the flavonoid pathway are discussed.

Methods and Materials

Field Procedure

The three-year study spanned 2006 to 2008 at the Irrigated Agriculture Research and Extension Center in Prosser, WA, USA (46.30º N, 119.75º W). Rows of own-rooted 'Merlot' (*Vitis vinifera* L.) vines (planted in 1999) were oriented of north-south, trained to a vertical shoot positioned bilateral cordon at 1.2m above ground, and spur-pruned. Experimental clusters were selected on the east aspect of the vine and 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 to 4 mm) and concluded at the onset of véraison (Coombe, 1995). Temperature treatment classifications used are outlined below:

(1) ambient, untreated control

(2) convective control (*blower*)

(3) nighttime heated (*heat*)

(4) daytime cooled (*cool*)

Temperature was control by a forced-air delivery system (Tarara et al., 2000) modified by replacing a single chilling unit with a pair of units that operated alternately. Target temperature differences were 8 °C above or below (*heat* and *cool* respectively) ambient cluster tempereature. No chilled air was delivered if berry

temperature was below 10 °C. The temperature and wind velocity around ambient clusters was 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 diam.; Type T [copper-constantan]) each encapsulated in a 4 to 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 ten-day intervals (n = 4) from the start of the experiment (48 days before véraison (DBV)) to its conclusion (5 DBV) to provide a time course study. The last sampling point coincides with the same relative end point of the 2006 and 2007 seasons. Véraison was defined as the point at which clusters from a concurrent experiment involving day-cooling *in combination with* night-heating achieved approximately 50 percent coloration (e.g. “Damp”) (Cohen et al., 2008). Treatments replicates (n = 4 clusters) were harvested at approximately 0900 hours, placed on ice and held under refrigeration. Berries were excised from the

rachis (peduncles intact), 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 previously described (Cohen *et al.*, 2008). Berry volumes (50 to 100 berries) were estimated based on H2O displacement prior to manual dissection and separation of skin and seeds. Berry coloration was determined by counting berries exhibiting approximately 80 to 100 percent red color. Skin extracts were prepared by macerating lyophilized skin tissue in an acetone / water solution (2:1) for 24 hours under nitrogen gas as previously described (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 (Lamuelaraventos 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). In 2006, aqueous extracts were filtered using a syringe filter (Acrodisc PTFE (13mm, 0.45µm) 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) and malvidin-3-*O*-glucoside (Extrasynthese, Genay, France) were used as quantitative standards for flavonols and anthocyanins, respectively.

Compositional analysis of PAs was carried out following acid-catalyzed cleavage in the presence of phloroglucinol (phloroglucinolysis) as previously described (Kennedy and Jones, 2001). Appropriate aliquots of aqueous extracts were lyophilized and dissolved in MeOH prior to reacting with phloroglucinol reagent as previously described (Kennedy and Taylor, 2003; Cortell et al., 2005). Samples were immediately analyzed following addition of aqueous sodium acetate. Quantification of PA subunits and estimation of mean degree of polymerization (mDP) was calculated using (+)-catechin (Sigma-Aldrich) as a quantitative standard.

The size distributions of intact PAs were analyzed by gel permeation chromatography (GPC) as previously described (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 (0.15M LiCl in DMF containing 1% and 5% (v/v) acetic acid and water, 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 (DAD) detector and an external column oven when required (Eppendorf CH-430; Westbury, N.Y., USA). All data were analyzed using Agilent Chemstation software (V A.08.03).

Gene Expression Profiling

Quantitative real-time PCR was carried out in an ABI PRISM 7700 sequence detector (Applied Biosystems, Carlsbad, CA, USA) as previously described (Castellarin et al., 2007). Grape skins were collected from 10-12 berries maintained

in liquid nitrogen during dissection. Extraction of RNA from approximately 0.3g tissue followed the protocol outlined by Iandolino *et al*. (Iandolino et al., 2004). Samples were DNase treated and first strand cDNA was synthesized as outlined by Castellarin *et al*. (Castellarin et al., 2007). Reaction mixtures (20µl) contained 10 µl Power SYBR Green Master Mix (Applied Biosystems), 5µl of 1:100 diluted cDNA, and 250 nM of each primer. Each sample was run in duplicate and means reported. Thermal cycling was at 95°C for 10 minutes 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 described (Bogs et al., 2005; Castellarin et al., 2007). Expression values are reported as means of biological treatment replicates (n = 4). Primer pairs were the same as those described by Castellarin *et al*. (Castellarin et al., 2007; Castellarin et al., 2007). Primers for CHS1, CHS2, CHS3, DFR and LDOX were retrieved from Goto-Yamamoto *et al*. (Goto-Yamamoto et al., 2002). F3H, BAN, F3'H (coded F3-1) and F3'5'H (coded F35-1) were designed by Castellarin *et al*. (Castellarin et al., 2007) based on published sequence information. FLS (FLS1), LAR (LAR2), and GST were retrieved from the literature (Downey et al., 2003; Bogs et al., 2005; Terrier et al., 2005). Myb5a was retrieved from Deluc *et al*., MybC and MybD were from Kobayashi *et al*. (Kobayashi et al., 2002; Deluc et al., 2006).

Statistical Analysis

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

$$DD = \frac{1}{n}\sum_{i=1}^{n}\left(\overline{T} - Tb\right)$$

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 and Fishers LSD was used to determine separation of means (α=0.05). Non-normal data sets were analyzed using Kruskal-Wallis ANOVA.

Results

Temperature and Solar Radiation Environment

Berry temperature data (Figure 2.1) show daily patterns and typical treatment separations. Treatments were imposed for approximately 45 days before véraison. During that period, *cool* resulted in significant reduction in the amount of time berries were exposed to temperatures above 30°C and the DD accumulation compared to other treatments (Table 2.1). In contrast, *heat* resulted in considerable increases in nighttime temperatures and DD accumulation. Total thermal time showed little annual variation within each treatment across years ($p<0.10$; Table 2.1). In each year ambient and *blower* berries accumulated equivalent DD, showing little net-effect of increased air velocity on cluster temperature.

Weekly thermal time summations (Table 2.2) illustrate differences relating to interannual variability. These are of particular interest in attempting to identify periods of temperature tolerance or sensitivity during berry development as investigated by Yamane *et al*. (Yamane et al., 2006) where treatments were imposed on entire plants in constant-temperature growth chambers. While each year exhibited periods of above average temperatures and resulting DD accumulation (three-year weekly average ≈ 94 GDD), that period fell at different stages of development in each of the three years.

Solar radiation in the fruiting zone is included in Table 2.2 as daily averages calculated at seven-day intervals of the respective season. Solar radiation was similar between 2006 and 2007 and tended to be higher in 2008 on average. Temperature differences between years did not necessarily reflect differences in solar radiation as 2006 was generally warmer than 2008, however radiation incident to the fruiting zone tended to be lower.

Veraison Outcomes

Berry Development

In general, berry mass was slightly elevated in *heat* and *cool* berries compared to ambient, though significant only in 2006 ($p = 0.051$, Table A.2). Dry weight of skin per berry was not found to be different nor follow an annual trend. Berry percent coloration was always greatest in *cool* and *heat* berries however this was significant only in 2007 and 2008 ($p < 0.05$).

PA Accumulation

Proanthocyanidin data at véraison show differences by treatment and the interannual variability encountered in ambient berries (Table 2.3). Overall, total PA content in ambient berries was lowest in 2007. Average PA polymer size did not differ significantly between years. The proportion of (-)-epicatechin-3-*O*-gallate (ECGt) as a terminal subunit was highest in 2006 but contributes the least to the total concentration of terminal units (1.2 to 7.3% across years). Similarly, the contribution of (-)-epicatechin as a terminal subunit (ECt) was also highest in 2006 (8.6 to 14.5% across years). Conversely, (+)-catechin (Ct) as a terminal subunit was lowest in 2006 (78.2 to 90% across years). Together, (-)-epigallocatechin (EGCx) and EC extension (ECx) subunits constituted over 95% of the total PA pool in Merlot berries in this experiment. Between years, the proportion of EGCx subunits was highest and that of ECx was lowest in 2007.

Total PA accumulation *per berry* was lower in *cool* than *heat* berries in 2006. During that season PA accumulation showed a positive correlation with thermal time ($r^2 = 34.7$; $p<0.05$), with *heat* berries having the highest content *per berry* at véraison. Differences in 2007 and 2008 were not significant and did not follow the same trend with respect to thermal time ($r^2 < 1.0$; $p > 0.1$). Again, there were no significant differences relating PA content *per berry* directly to thermal time. While *heat* tended to increase PAs compared to ambient, the treatment effect was not significant. The effect of *cool* was much more variable across years, and more difficult to make a conclusion.

The composition of skin PAs showed little effect by treatments on mDP. Terminal subunits were dominated by Ct in all years otherwise no differences were

observed between treatments. Extension subunits were primarily ECx and EGCx, each totaling between 40-50% of the total PA pool. Considering all treatments and years (two-factor ANOVA), the proportion of EGCx was highest in 2007 ($p < 0.001$) (mean = 51%) compared to 2006 and 2008 (mean = 44% both years) and was highest in *cool* berries ($p < 0.001$, data not shown). This is contrary to data relating sunlight exposure to increases in EGCx relative to ECx in both Pinot Noir and Shiraz berries; reflecting a difference between temperature and light effects on PA partitioning (Downey et al., 2004; Cortell and Kennedy, 2006).

Temporal Trends, 2008

Temporal changes in PA content and composition were investigated in 2008; only data from ambient, *cool*, and *heat* are included in the time-course analysis.

Berry Development

The progression of berry mass from fruit set to véraison followed expected trends (Figure 2.2). Ambient and *heat* berries followed a similar, two-stage, growth curve with little increase in mass between 29 and 19 DBV. *Heat* berries were highest in mass by 39 DBV and remained so through the experiment. *Cool* berries gained little mass by 39 DBV (berry mass at 48DBV ≈ 0.2 grams *per berry*), yet by 19 DBV values were equivalent to *heat* berries while ambient berries were lowest.

Flavonol Accumulation

Accumulation of flavonols was followed during development as a potentially competitive branch point in PA biosynthesis as they are actively produced early in berry development (Figure 2.3). 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; however this represents < 5% by mass of flavonoids compared to PAs. There was no significant treatment effect or significant differences in temporal accumulation.

PA Accumulation

Total PA contents are displayed in *nmol per berry* (Figure 2.4) relevant to the flux of metabolites through the biosynthetic pathway. Initial PA content of ambient berries at the start of the experiment (48 DBV) was 2863 ± 268.1 *nmol per berry* (0.85 ± 0.081 mg *per berry*). Similar to berry mass trends, PA content in *heat* and ambient berries were parallel during development, increasing until 19 DBV, and then decreasing. *Cool* berries exhibited a lag in PA accumulation after 10 days of treatment (39 DBV) followed by an increase by 19 DBV. This deviation from ambient treatments is concomitant with the progression of berry weights, suggesting a shift in overall rate of berry development.

PA mDP of berries at the start of the experiment (48 BDV) was approximately 26 units and that of *heat* berries showed little change over time (Figure 2.5). *Cool* berries exhibited the most dramatic changes in mDP between the initiation of the experiment and 19 DBV. Differences in mDP were driven by the increases in EGCx subunits between 39 and 19 DBV. In order to qualify changes in PA polymers during development we used GPC. In all cases PAs exhibited a nearly bi-modal distribution (Figure 2.6) between High (HMw ≈ 52mDP) and Medium (MMw ≈ 25mDP) molecular weight polymers with contributions from smaller oligomers (≈ 6 and ≈ 2 mDP). Subtle differences were noted during berry development, primarily shifts in proportion between HMw and MMw polymers as illustrated in the chromatograms.

Heat showed less variation than *cool* in relative distribution until 5 DBV, when there was a slight shift towards MMw polymer and accumulation of dimers (or anthocyanins, observed at 520nm). *Cool* showed an initial increase in proportion of HMw polymers followed by a slight shift towards MMw polymers and dimers (anthocyanins) by 5 DBV. Data from GPC confirms the observations based on PA phloroglucinolysis and provide a qualitative assessment of changes occurring during development. Further, total PA content determined by GPC (total area at 280nm) were in very good agreement with those determined by phloroglucinolysis (~ 95%, data not shown). The agreement between analytical methods suggests information provided following phloroglucinolysis accurately reflects PA composition during the experiment. While GPC provides broad knowledge based on absorbance (280nm) the accuracy of phloroglucinolysis is dependent upon quantification of known compounds and is less robust to oxidation or degradation in general.

In 2008, the contribution of ECGt was minimal and is not displayed. The pattern of accumulation of Ct and ECt was similar between *heat* and ambient berries (Figure 2.7A-B). The content of both terminal subunits was lowest in *cool* berries at 39 days pre-véraison. The discrepancies in Ct and ECt between treatments disappeared by 29 days pre-véraison, following a sharp increase in *cool* berries. All treatments showed maxima in terminal subunits by 19 DBV, though *cool* exhibited a higher rate of Ct accumulation. ECt subunits followed a similar accumulation pattern to Ct though *heat* exhibited the most pronounced increase at 19 DBV.

In general, the accumulation of extension subunits followed similar patterns in *heat* and ambient berries, with *cool* being most divergent. Accumulation of EGCx

appears to be responsible for the major differences observed in total PA accumulation (Figure 2.7C). *Cool* berries had considerably lower EGCx content initially followed by a dramatic increase by 19 DBV. ECx, Cx, and ECGx content exhibited the similar variation between treatments at the various time points (Figure 2.7D-F). All subunits reached an apparent maxima between 29 and 19 DBV. As with total PAs and EGCx content, *cool* berries showed a characteristic delay prior to a rapid increase in accumulation of ECx, Cx, and ECGx by 19 DBV.

Expression of Flavonoid Genes

To understand the biosynthesis and accumulation of PAs, we determined the expression of several key genes involved in their biosynthesis. As expected, nearly all genes assayed were expressed 39 DBV and declined to lower values 20 days later, similar to the temporal patterns previously reported (Boss et al., 1996). Genes associated with anthocyanin biosynthesis exhibit a second increase in expression approaching véraison, similar to other studies (Castellarin et al., 2007; Castellarin et al., 2007). Glutathione S-transferase (GST) is an exception showing minimal expression until véraison. This last gene, as discussed by Gomez *et al*. (2009), is involved in vacuolar transport of anthocyanins and serves as a biochemical indicator of véraison in this study.

Activity of three chalcone synthase isogenes (CHS1, CHS2, and CHS3) was followed during berry development representing the first committed step to general flavonoid biosynthesis (Goto-Yamamoto et al., 2002; Castellarin et al., 2007). The expression patterns of the three genes were similar, starting off higher and declining 4 to 5 fold by 19 DBV (Figures 2.8A-C). As with many other genes assayed,

expression levels of CHS1 and CHS3 were similar between treatments at 39 DBV yet *heat* exhibited a more rapid decrease over time. The pattern of CHS2 expression was similar in all treatments. These observations suggested a tight relationship between flavonoid accumulation and temporal development of berries. As reported elsewhere, analysis of ambient grapes (data not shown) harvested ten days after véraison showed that levels of CHS2 and CHS3 continued to increase after véraison; however, CHS1 did not due to the involvement of the former genes in anthocyanin biosynthesis (Castellarin et al., 2007).

Expression of downstream structural genes related to flavonoid partitioning showed similar patterns to those of the CHS genes. Core pathway genes involved in general flavonoid biosynthesis are shown in Figures 2.9A-F: Flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR), leucoanthcyanidin reductase (LAR2), leucoanthocyanidin dioxygenase (LDOX), anthocyanidin reductase (ANR), and glutathione transferase (GST). Genes involved in the hydroxylation of flavonoids and related metabolite proportions are shown in Figures 2.10A-C: flavonoid-3′-hydroxylase (F3'H), flavonoid-3′-5′-hydroxylase (F3'5'H), and flavanone-3-hydroxylase (F3H). Most differences were manifested at 29 DBV when *heat* showed a more pronounced decrease in expression of F3'H, LDOX, and ANR. Similar trends were observed for other pathway genes however no significant differences were determined (F3'5'H, F3H, DFR). Expression of LAR2 (putatively related to Ct biosynthesis) and FLS (flavonol synthesis) exhibit more divergent patterns compared to other genes. The expression of FLS was counter to down-stream 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 (GST) is tightly related to anthocyanin biosynthesis and was not detected until 5 DBV and was coordinated with berry coloration. Other genes associated with anthocyanin biosynthesis showed similar increases at 5 DBV including F3'H, F3'5'H, F3H, DFR, and LDOX. In most cases, increases were more pronounced in *heat* berries, which is likely due to the apparent acceleration of berry development. Expression levels of LAR2 and ANR, generally committed to flavan-3-ol biosynthesis, showed little indication of increased levels at this last time point, coinciding with the general decline in PA accumulation.

Before veraison, the ratio of F3'5'H to F3'H expression (Figure 2.11A) is in general agreement with the ratio of tri- to di-hydroxylated PA subunits (Figure 2.11B). *Heat* showed a lower ratio between F3'5'H and F3'H and a similar reduction in the proportion of tri-hydroxylated PAs. The expression of F3'H increased more in *cool* berries at 5 DBV and was contrary to the increase in F3'5'H in *heat* berries. While overall differences in expression were not significant, this may be indicative of temperature response related to partitioning of anthocyanins (Downey et al., 2004; Cohen et al., 2008; Tarara et al., 2008).

Three *Myb* genes (*Myb*5a, *Myb*C, and *Myb*D; Figures 2.12A-C) involved in regulating flavonoid biosynthesis early in berry development were expressed in skin tissues in Merlot (Kobayashi et al., 2002; Adams, 2006; Deluc et al., 2006; Castellarin et al., 2007). The expression profiles of *Myb*5a and *Myb*D showed differences at 39 DBV, otherwise they appear to decrease by 5 DBV. *Myb*C showed

a decline by 19 DBV yet was followed by an apparent increase approaching véraison in *heat*.

Discussion

The goal of this study was to determine the effect of temperature on PA accumulation in field-grown grape berries during the first stage of berry development. To do this we manipulated the temperature around the cluster by delivering cold air during the daytime or warm air at night. This system has been effective in similar studies and was successful based on DD values and daily temperature fluctuations achieved (Spayd et al., 2002; Cohen et al., 2008; Tarara et al., 2008). In 2006 we found a positive relationship between DD accumulation and PA content at véraison (Cohen *et al.*, 2008). Because of this, we expanded the investigation across multiple seasons and considered berry metabolism and the expression of key flavonoid biosynthetic genes during temporal development up to véraison.

Data from this experiment displayed considerable interannual variation and variable impact of treatment between seasons. These types of variability are present in other multi-year studies and are thought to reflect the complex response of the plant to the multiple factors that cumulatively define a particular season (Downey et al., 2004; Paolocci et al., 2005; Gagne et al., 2006; Pilati et al., 2007; Bavaresco et al., 2008). The accumulation of PAs in grape skins was found to increase with DD accumulation by véraison in the first year of this study. In subsequent years, *heat* resulted in negligible increases in PA content compared to ambient conditions. The effects of cooling had inconsistent effects on PA accumulation yet did result in

consistent shifts in PA partitioning towards a higher proportion of PAs in the tri-hydroxylated form.

While treatments had a variable influence on PA accumulation between years, the relative differences in berry development was similar each season. Investigation of annual temperature trends show a period of high temperature around 20 DBV in 2006. In 2008 berries exhibited an approximate peak in PA content at 20 DBV, which appeared to be related to the developmental stage of the berry. In general, *cool* berries were acclimated to lower temperatures (< 30°C, Table 2.1) while *heat* and ambient berries were subjected to elevated temperatures for longer periods of time (> 35°C). High temperatures 20 DBV in 2006 may have been inhibitory to *cool* berries while having less of an effect on *heat* or ambient berries due to the temperatures they were acclimated to beforehand. Similar trends were not observed in subsequent years when high temperature stress may have been less evident. Conversely, cooling may have reduced the effect of high temperature stress during week four, when ambient and *heat* berries accumulated PAs in response. Considering data across years and the knowledge of anthocyanin biosynthesis and temperature related degradation, the former explanation is more a plausible scenario (Mori et al., 2007, 2007). Treatment differences would reflect variation in gene expression or enzyme kinetics in this case. If chemical degradation was a factor (i.e. oxidation) one would expect higher temperatures to correlate with lower PA content, which was not evident in this study.

The relationship between thermal time and PA accumulation in 2008 is shown in Figure 2.13A. The graph illustrates the amount of PAs that accumulated between the time-points indicated, starting at the initiation of the experiment (48 DBV), as a

function of thermal time accumulation (DD) during the same period. Ambient and *heat* berries showed the same relationship between DD accumulation and PA content, consistent with elevated PA content in *heat* berries. *Cool* berries exhibit disconnect between DD accumulation and PAs compared to other treatments. Both the increase and subsequent decrease in PA content was greater in *cool* berries under considerably lower amounts of thermal time accumulation. This suggests PA accumulation was not directly related to accumulation of thermal time and more likely related to berry development.

There is a linear relationship ($r^2 = 0.75$; $p < 0.001$) between berry mass and PA content up to 19 DBV in 2008 (Figure 2.13B). A second-order relationship ($r^2 = 0.55$; < 0.001) reflects the decline in PA content approaching véraison (5 DBV), which could reflect oxidation or reduced extractability due to incorporation into cell wall material or polymerization (Kennedy et al., 2000; Downey et al., 2003; Geny et al., 2003; Adams, 2006; Gagne et al., 2006). A similar relationship was observed between berry mass and DD accumulation from the start of the experiment to 5 DBV (data not shown; $r^2 = 0.64$, $p < 0.001$, S.E. = 0.075gr.) confirms that temperature has a direct influence on development. The data demonstrate a connection between berry development and PA biosynthesis where changes in berry mass explain much of the differences in PA accumulation.

Previous work has shown that both day and night temperatures affect the rate of berry growth; moderate temperatures (~20°C) are generally associated with more rapid growth where both higher and lower temperatures may impede growth (Kobayashi et al., 1967; Hale and Buttrose, 1974). In this study, higher day and night

temperatures clearly accelerated development from the start of the experiment while lower day temperatures initially retarded development but recovered by 29 DBV; both *cool* and *heat* showed coloration sooner than ambient berries. The initial growth inhibition of *cool* berries likely reflects differences in the rate of cell division and / or enlargement and may be related to the activity of hormones such as the auxin, indole-3-acetic acid (IAA). In *Arabidopsis thaliana* L., low temperatures (20 °C vs 29 °C) were found to inhibit auxin-mediated cell elongation (Gray et al., 1998). In grapes, IAA is found to coordinate with early berry enlargement and to be antagonistic to coloration at véraison, which is hastened by abscisic acid (ABA) (Coombe and Hale, 1973; Shiozaki et al., 1995; Davies et al., 1997; Costantini et al., 2007). Regardless of the mechanisms relating to the differences in developmental rate, the inhibition observed in *cool* berries was overcome before véraison as was the coordinated lag in PA accumulation. This recovery in development and metabolism could be due temperature acclimation or a compensatory response to the temperature treatments imposed (Usadel et al., 2008).

The expression of PA related genes and accumulation of skin PAs during berry development is followed in numerous studies in grapes and other fruits (del Rio and Kennedy, 2006; Takos et al., 2006; Castellarin et al., 2007; Akagi et al., 2009; Carbone et al., 2009; Gagne et al., 2009; Hanlin and Downey, 2009). Both gene expression and metabolite accumulation are dynamic processes, showing considerable fluctuations over relatively short periods of time. Spikes in gene expression and PA accumulation observed in this study and in literature (around the time of fruit coloration) illustrate that PA biosynthesis is likely to occur during brief

periods of time coordinated with particular developmental stages of the berry. Therefore, environmental factors that influence the rate of berry development and the duration of a particular developmental stage may impact metabolite accumulation. Further, environmental factors that elicit a stress response in the plant may have a different impact based upon timing and coordination with accumulation of specific metabolites. Considering the treatment effects in this study, differences in PA biosynthesis are likely the result of shifts in temporal development (indirect effect) and direct temperature effects (e.g. shift in subunit proportions). In general, expression of genes related to PA biosynthesis was in reasonable agreement with the pattern of PA accumulation in 2008, considering *heat* versus *cool* berries.

In the context of temperature effects and grape flavonoid metabolites, much of the direct research has focused on anthocyanins. Numerous researchers have cumulatively shown that sun exposure, in coordination with moderate temperatures, encourages anthocyanin accumulation while high temperatures are generally inhibitory due to differences in gene expression and chemical degradation of metabolites (Buttrose et al., 1971; Haselgrove et al., 2000; Downey et al., 2004; Mori et al., 2005; Yamane et al., 2006; Cortell et al., 2007; Mori et al., 2007; Tarara et al., 2008; Matus et al., 2009). In general, higher temperatures result in increased proportions of tri-hydroxylated anthocyanins, opposing the observation here regarding PA partitioning. Low temperature induction (15.0°C to 5.0°C) of flavonoids and related structural genes has also been observed in *Zea mays* L. and *Arabidopsis thaliana* L. in a light dependent manner (Christie et al., 1994; Leyva et al., 1995; Janas et al., 2000; Olsen et al., 2008). While the mechanism linking

anthocyanins to light and temperature is still under research, it is clear that temperature effects accumulation and composition in a predictable manner. In this experiment, the effect of temperature (day and night) on PA biosynthesis was less evident before véraison and limited to metabolite partitioning. Studies in forage crops have shown relationships between temperature and PA accumulation; however, discrepancies exist that could be attributed to additional environmental stresses or different sensitivities between plant species (Anuraga et al., 1993; Lees et al., 1994; Dury et al., 1998; Carter et al., 1999). To date, there is limited evidence of an absolute response of PA biosynthesis to variations in temperature.

Flavonoids play a role in the photoprotection of plants (Close and McArthur, 2002; Winkel-Shirley, 2002). In *Arabidopsis* UV-B radiation induced expression of CHS and a general increase in UV absorbing metabolites (Bieza and Lois, 2001). Similarly, UV-B radiation was shown to induce accumulation of flavonoids in various other plants and have deleterious effects on those lacking sufficient quantities (Li et al., 1993; Reuber et al., 1996; Burchard et al., 2000; Kolb et al., 2001; Paolocci et al., 2005; Mellway et al., 2009). Mellway et al. report that FLS and upstream genes show a rapid response to light exposure where genes directed towards PA biosynthesis show a considerable delay in induction (48 hours) indicating differential regulation between classes of compounds. Exposure of grape berries to UV radiation has resulted in significant increases in skin PAs at véraison and increases in flavonols before and after véraison (Downey et al., 2004; Cortell and Kennedy, 2006; Fujita et al., 2007; Tarara et al., 2008). In those experiments reporting PA content, exposure resulted in a higher proportion of PAs in the tri-hydroxylated form and higher PA

mDP in general, counter to the effect of temperature observed here. Flavonol content was not found to respond to temperature; as the contribution of tri-hydroxylated forms was not reported a direct comparison cannot be made here. Compared to the effects of UV radiation, berry temperature appears to impose little influence on overall PA biosynthesis or accumulation. While the light environment was maintained across treatments each year annual differences are more difficult to control. Differences in light environment may contribute to the interannual variability as apparent in ambient berries; lower PA content in 2007 may relate to the reduced solar radiation impinging on the fruiting zone compared to 2008 and lower temperatures later in development (Tables 2.2 and 2.4).

Conclusion

Seasonal variations in air temperatures (barring permanent damage from excessive heat or freezing temperatures) have limited effect on PA biosynthesis in grape berries. Light environment and transient temperature stress would likely have a greater impact on PA accumulation and composition. The general photo-protective role of flavonoids has been discussed by others and deserves considerable attention based on these experiments (Close and McArthur, 2002). In this study, temperature treatments effected berry development by hastening the inception of véraison confounding a direct effect of temperature on biosynthesis alone. Increases in thermal time accumulation led to limited increases in PA content while cooling berries resulted in a consistent increase in the proportion of tri-hydroxylated PAs across all years. *Cool* berries exhibited a compressed period of berry development inclusive of a delayed, rapid rate of PA accumulation and similar rate of PA decline

towards véraison, both concomitant with the higher EGCx proportions. This decrease is coordinated with a period of increased oxidation and changes in cell-wall structure, suggestive that PAs may become oxidized and / or incorporated into the cell wall at this point (Huang et al., 2005; Pilati et al., 2007). EGC would be more chemically reactive to reactive oxygen species than the di-hydroxylated counterparts (C / EC) thus a more rapid decrease due to oxidation would be expected. This may have relevance with respect to PA extractability in a wine system but requires further investigation (Cortell and Kennedy, 2006; Gagne et al., 2006; Ortega-Regules et al., 2006). It is expected that long range temperature changes of this magnitude may impact the rate of berry development but have minimal influence on PA composition. There is evidence of a robust compensatory response to temperature allowing for normal development and accumulation of certain secondary metabolites. Further work should address the relationship between variable light environment, temperature and PA biosynthesis as previous work has focused on presence or absence of light. The effect of transient temperature treatments and extended exposure to high temperature (>35 °C) should also be investigated as relevant climate variables. Further, it would be of interest to examine changes in enzyme kinetics and transcriptional modifications that may take place under such conditions as additional insight into mechanistic rationale.

Table 2.1. Accumulated thermal time, number of hours berry temperatures exceeded designated thresholds and treatment duration by véraison in 2006 through 2008.

	2006			2007			2008		
Treatment	Thermal Time[a]	> 35° C[b]	> 30° C	Thermal Time	> 35° C	> 30° C	Thermal Time	> 35° C	> 30° C
Ambient	605b[d]	58b	257b	595b	37b	236b	590b	38b	238b
Blower	597b	47b	236b	597b	37b	235b	589b	29b	226b
Cool	488a	1.4a	57a	494a	0.2a	48a	471a	0.0a	20a
Heat	683c	45b	257b	684c	28b	231b	665c	28b	215b
p-value[e]	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.0034*	*< 0.001*	*< 0.001*	*0.0085*	*< 0.001*
Duration[c]	*179-223 (45)*			*184-227 (44)*			*186-231 (46)*		

[a]Expressed as degree-days (°C) above base 10 °C.
[b]Hours above designated temperatures.
[c]Expressed as doy_{begin} to doy_{end} (# days).
[d]Values with same letters within week are not different; LSD, α=0.05.
[e]ANOVA p-value for thermal time data in italics unless otherwise noted.

Table 2.2. Accumulated thermal time by week for ambient clusters and average daily irradiance incident in the fruiting zone.

Year		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2006	Thermal Time[a]	99.8b[c]	90.9a	88.5	123.8c	84.0a	94.0b
	Solar Radiation[b]	NA	8.15±3.24	8.39±1.32	9.79±2.13	9.20±1.92	8.63±1.62
2007	ThermalTime	106.8c	119.5c	89.1	97.8b	92.3b	72.8a
	Solar Radiation	11.01±0.31	8.25±1.10	6.12±1.29	8.69±1.47	8.91±1.61	8.90±1.87
2008	Thermal Time	82.2a	96.2b	85.6	84.2a	89.0b	92.7b
	Solar Radiation	12.69±2.58	13.63±3.17	10.42±2.49	10.95±2.23	9.12±2.59	8.75±2.81
	p-value[d]	*<0.001*	*0.0073*[e]	*0.0749*	*<0.001*	*0.0053*	*0.0200*[d]

[a]Expressed as degree-days (°C) above base 10 °C.
[b]Expressed as MJ/m^2± St. Dev (n=3)
[c]Values with same letters within week are not different; LSD, α=0.05.
[d]ANOVA p-value for thermal time data in italics unless otherwise noted.
[e]Kruskal-Wallis p-value.

Table 2.3. Proanthocyanidin content, mDP, and composition in Merlot skins at véraison in 2006 through 2008.

		PA Content			Terminal Subunits[a]			Extension Subunits			
	Treatment	mg/berry	mg/g berry	mDP	C%	EC%	ECG%	EGC%	EC%	C%	ECG%
2006	Ambient	[b]1.16ab,y*	2.33b,y**	28.0	78.2x***	14.5y**	7.3y***	43.0a,x**	53.6b,y**	1.5	1.9xy*
	Blower	1.14ab	2.20b	27.3	81.1	11.6	7.3	43.8a	53.0b	1.5	1.7
	Cool	0.97a	1.62a	25.8	82.1	11.6	6.3	48.5b	48.3a	1.6	1.6
	Heat	1.46b	2.45b	28.2	86.0	7.7	6.3	41.2a	55.8b	1.4	1.6
	p-value	*0.0384*	*0.0272*	*ns*	*ns*	*ns*	*ns*	*0.012*	*0.005*	*ns*	*ns*
2007	Ambient	0.96,x	1.41,x	25.2	90.0y	8.8x	1.2x	49.2a,y	47.4b,x	1.3	2.2y
	Blower	1.18	1.98	29.5	88.1	10.2	1.7	52.4ab	44.5ab	1.1	1.9
	Cool	1.20	1.59	26.4	89.2	9.4	1.3	53.7b	43.4a	1.1	1.8
	Heat	1.11	1.65	27.4	90.1	8.2	1.7	49.2a	47.3b	1.2	2.2
	p-value	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.035*	*.03*	*ns*	*ns*
2008	Ambient	1.41,y	2.24y	27.7	89.3y	8.6x	2.1x	42.5ab,x	54.4ab,y	1.4	1.6x
	Blower	1.49	2.41	28.5	88.2	9.9	1.9	44.8ab	52.4ab	1.4	1.5
	Cool	1.43	2.08	27.3	90.2	7.9	1.8	45.9b	51.3a	1.3	1.5
	Heat	1.53	2.24	26.2	90.5	7.9	1.6	41.6a	55.3b	1.4	1.7
	p-value	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0756*	*0.0811*	*ns*	*ns*

[a] Mole percentage; terminal and extension subunits considered separately. C = catechin, EC = epicatechin, ECG = epicatechin-gallate, EGC = epigallocatechin.
[b] Values with same letters within grouping are not different. Letters x and y separate ambient across years (* p <0.1, ** p< 0.05, *** p<0.01); LSD, α=0.05.
[c] ANOVA p-value in italics unless otherwise noted (ns = not significantly different (p>0.05)).
[d] Kruskal-Wallis p-value.

Figure 2.1. Exemplary diurnal variation in berry temperature (°C) over four days (Day of Year 199-202, 2008) showing lower-than-ambient daytime temperature in *cool* berries and higher-than-ambient nighttime temperature in *heat* berries. In any 24-h period, the mean difference between ambient and *blower* berries was < 1.0 °C.

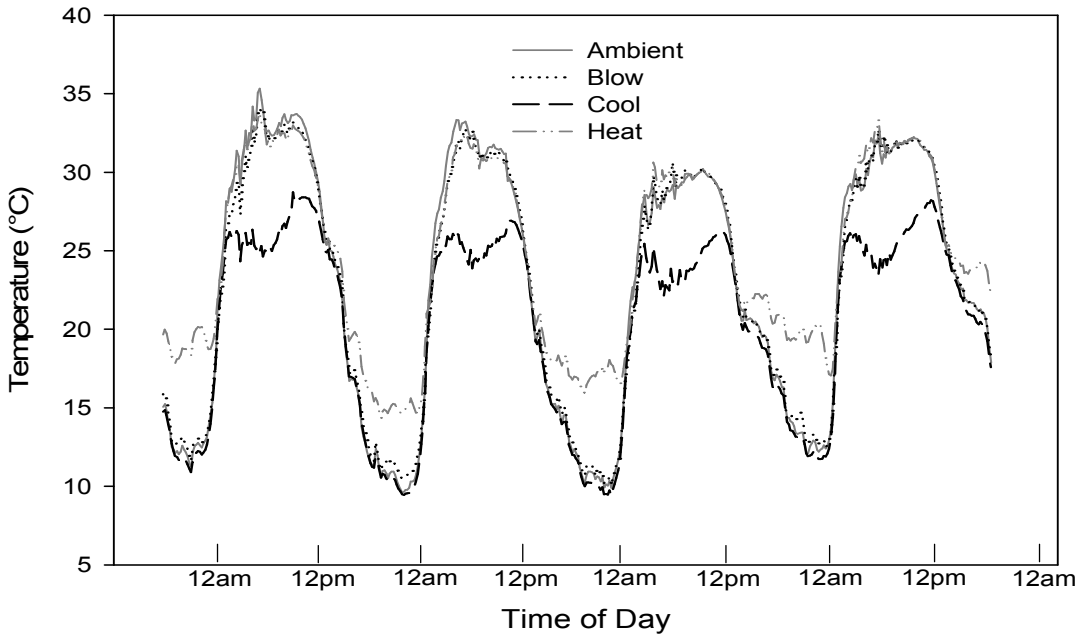


Figure 2.2. Pre-véraison berry mass (2008). Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

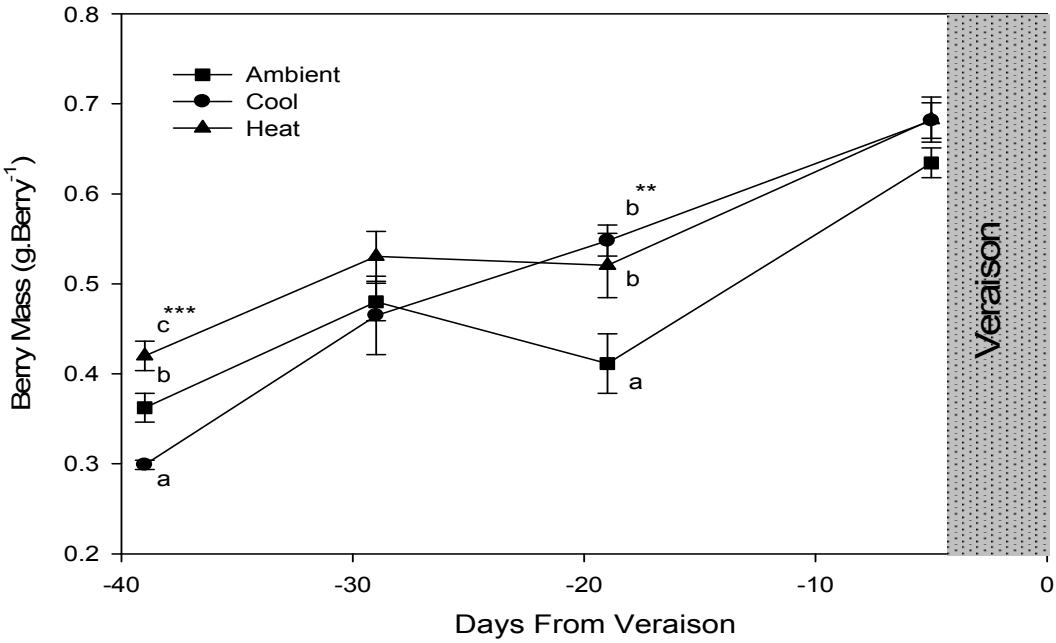


Figure 2.3. Pre-véraison flavonol concentrations (quercetin derivatives) in Merlot skins (2008). Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* $P \leq 0.1$, ** $P \leq 0.05$, *** $P \leq 0.01$), error bars represent SEM.

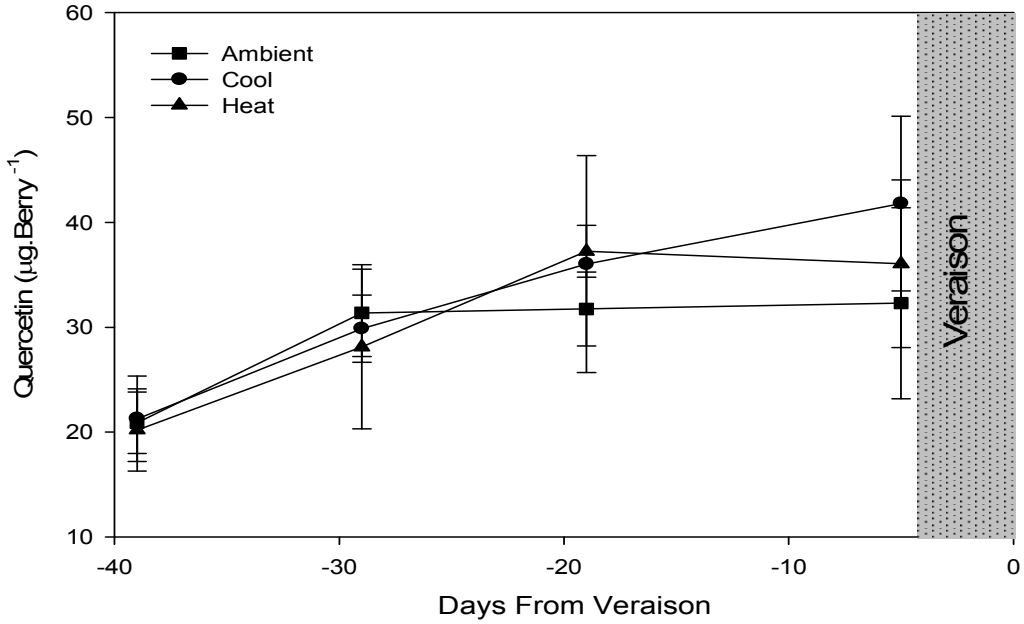


Figure 2.4. Pre-véraison PA content in Merlot skins (2008) in *nmol per berry*. Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P ≤ 0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

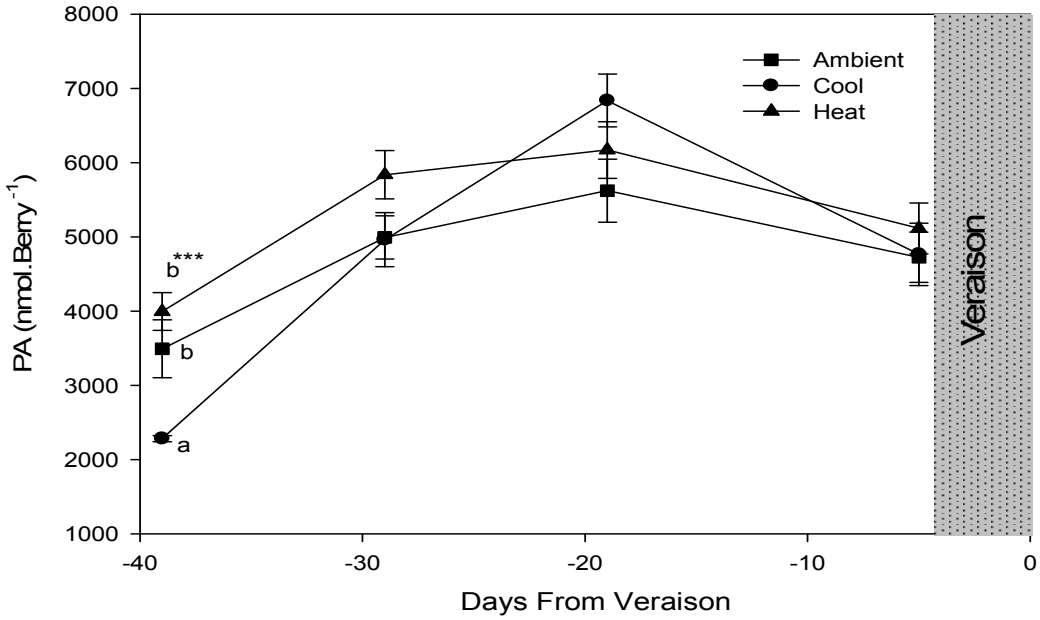


Figure 2.5. Pre-véraison PA mDP in Merlot skin (2008) as determined by phloroglucinolysis, error bars represent SEM.

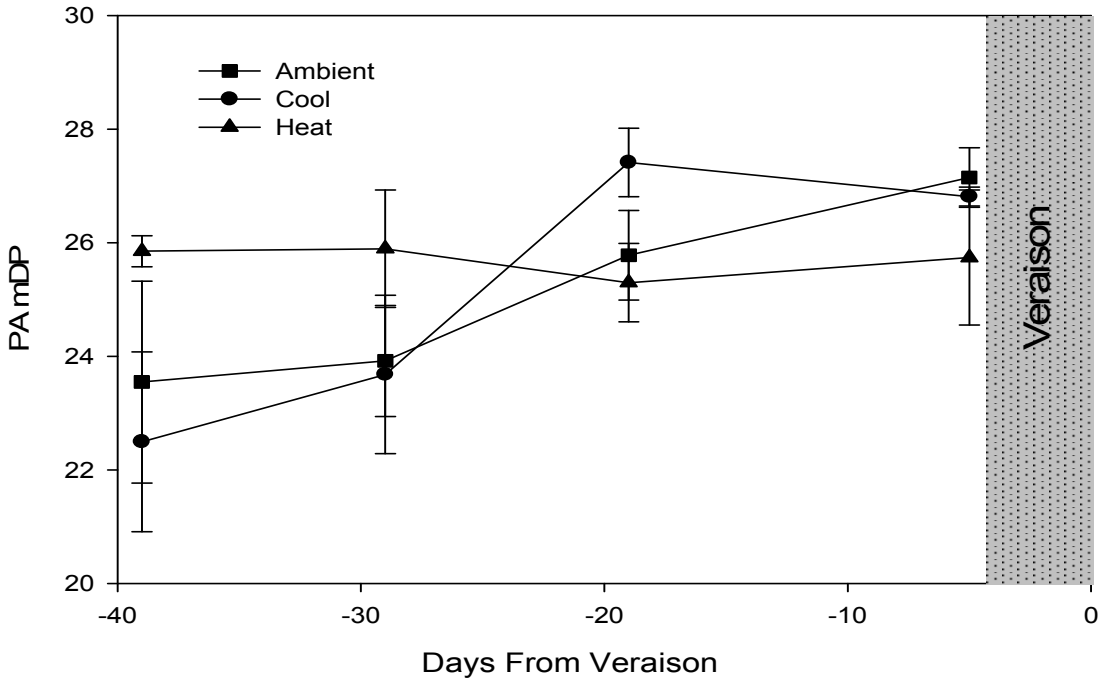


Figure 2.6. Size distributions of PA polymers in pre-véraison Merlot skins (2008) determined by gel permeation chromatography, for (A) *heat* and (B) *cool*. Temporal differences in the size distribution of polymers are shown for *heat* and *cool* between 39 and 5 days before véraison (DBV); relative mDP is shown above the X-axis based on estimated molecular mass, 50% mass elution. Values are averaged (n = 4) at each time point and normalized on the Y-axis (10.5min) to account for differences in absolute concentration.

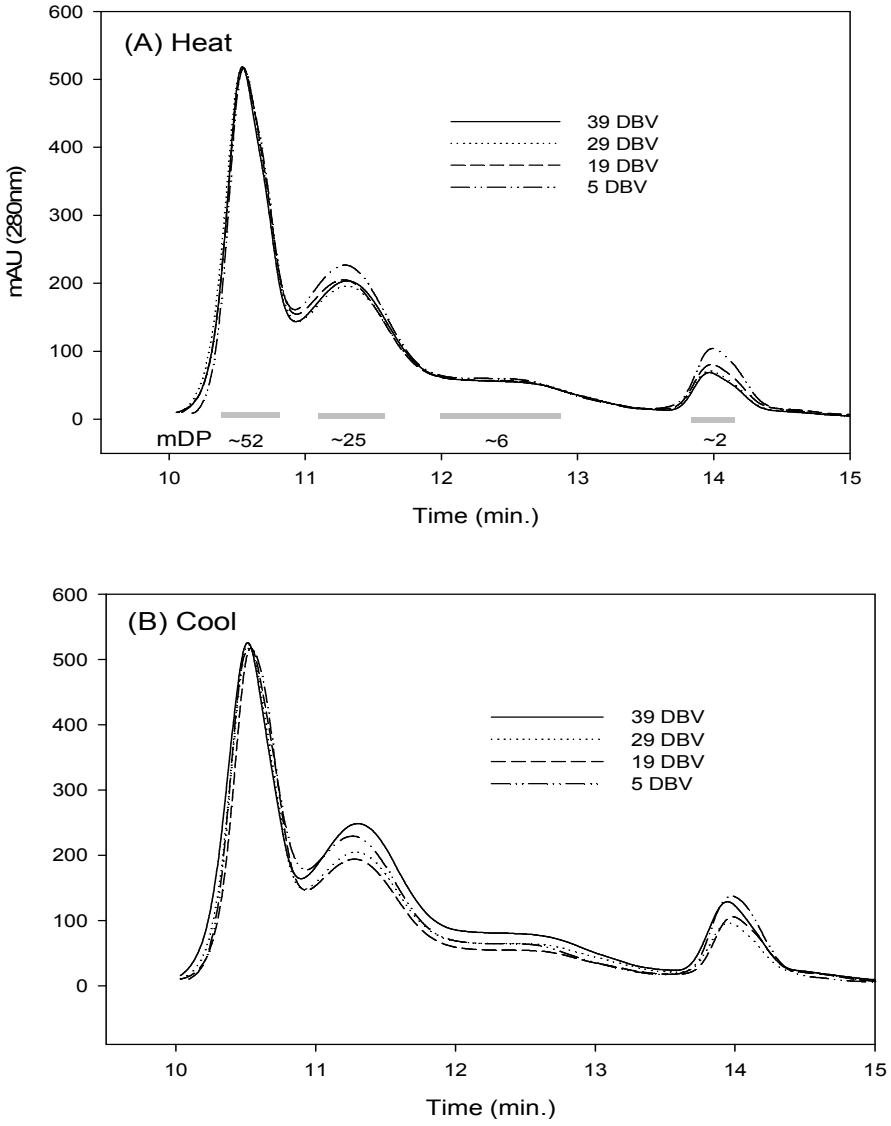


Figure 2.7. Concentrations of PA subunits in pre-véraison Merlot skins (2008). Panels A-F show terminal (A-B) and extension subunits (C-F) in *nmol per berry*. Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

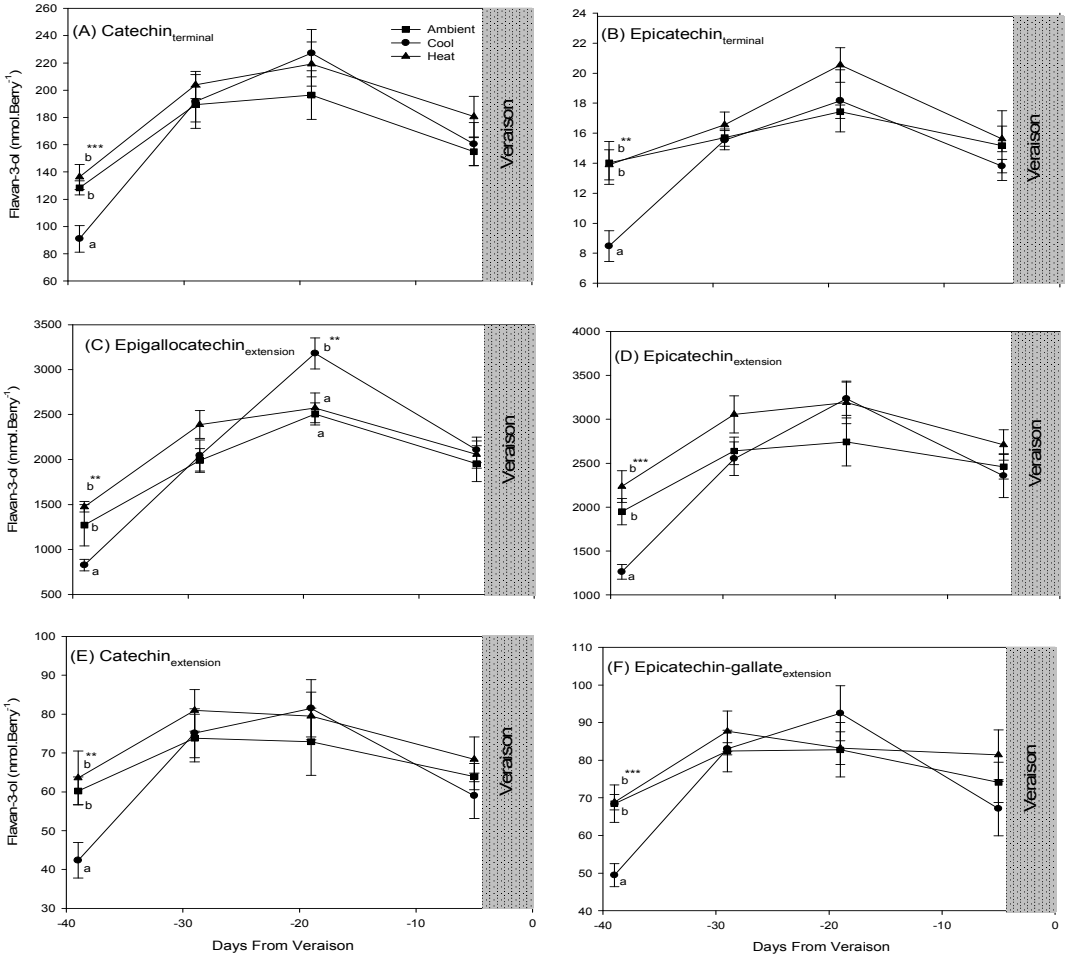


Figure 2.8. Expression of (A) CHS1, (B) CHS2, and (C) CHS3 in pre-véraison Merlot skins during 2008. Expression levels are relative to the expression of *VvUbiquitin.* Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

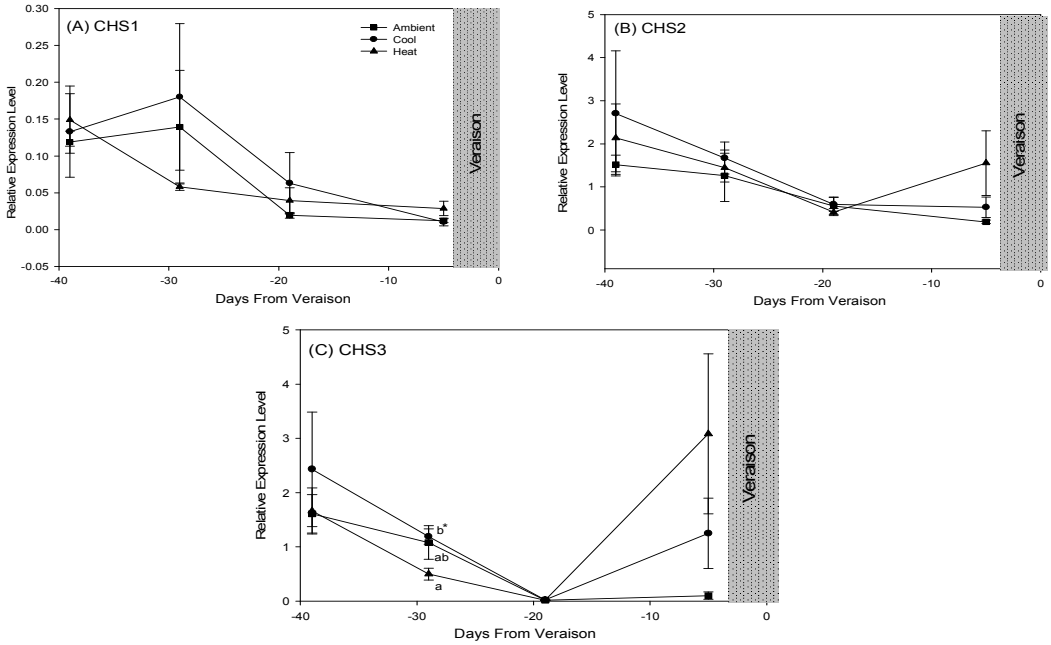


Figure 2.9 Expression of (A) FLS, (B) DFR, (C) LAR, (D) LDOX, (E) ANR, and (F) GST in pre-véraison Merlot skins during 2008. Expression levels are relative to the expression of *VvUbiquitin.* Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

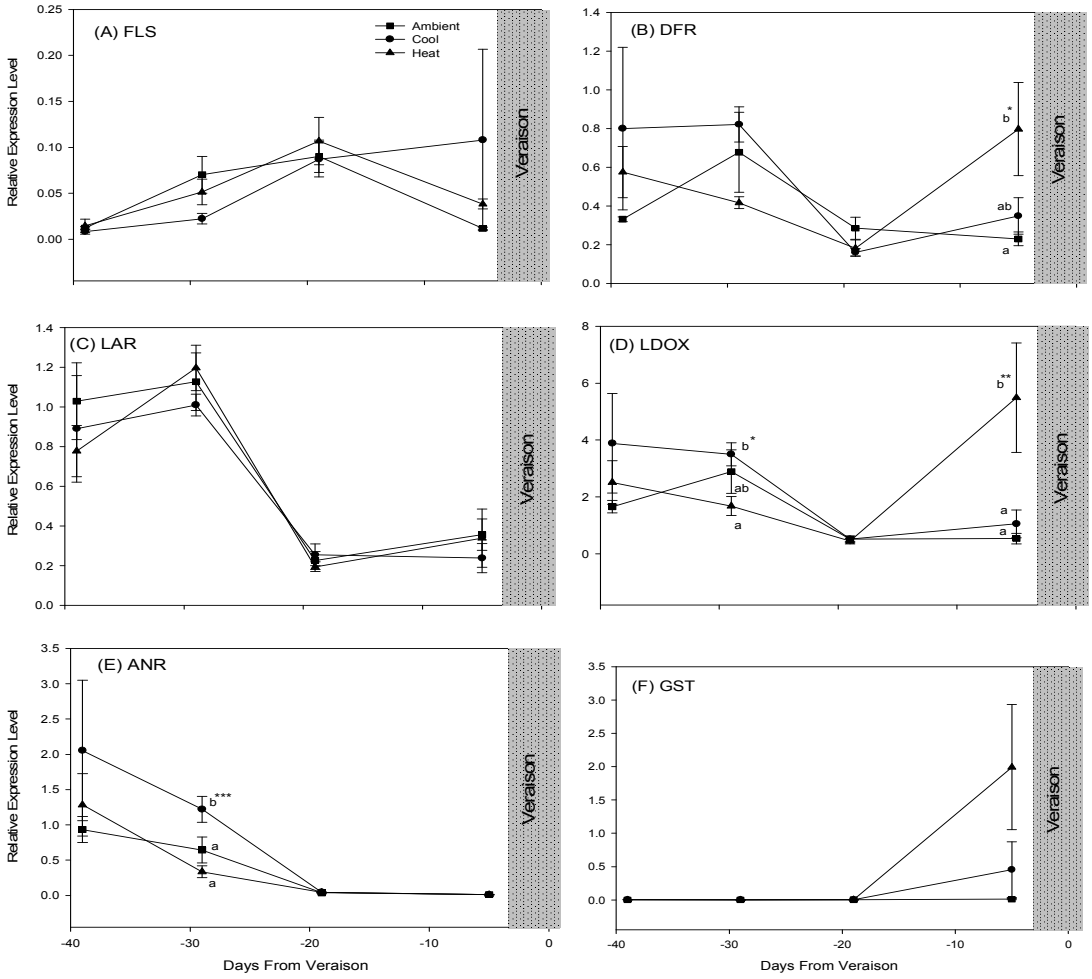


Figure 2.10. Expression of (A) F3′H, (B) F3′5′H, and expression of (C) F3H in pre-véraison Merlot skins (2008). Expression levels are relative to the expression of *VvUbiquitin.* Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

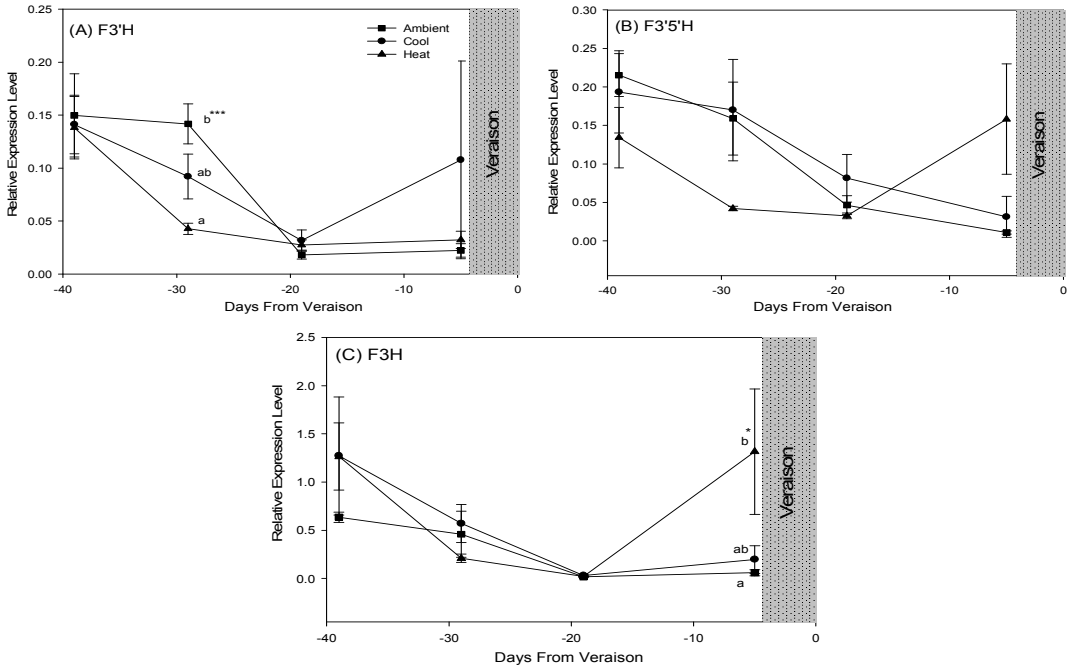


Figure 2.11. (A) Expression of F3′5′H to F3′H, and (B) the proportion of EGCx (of total PA pool) in pre-véraison Merlot skins (2008), error bars represent SEM.

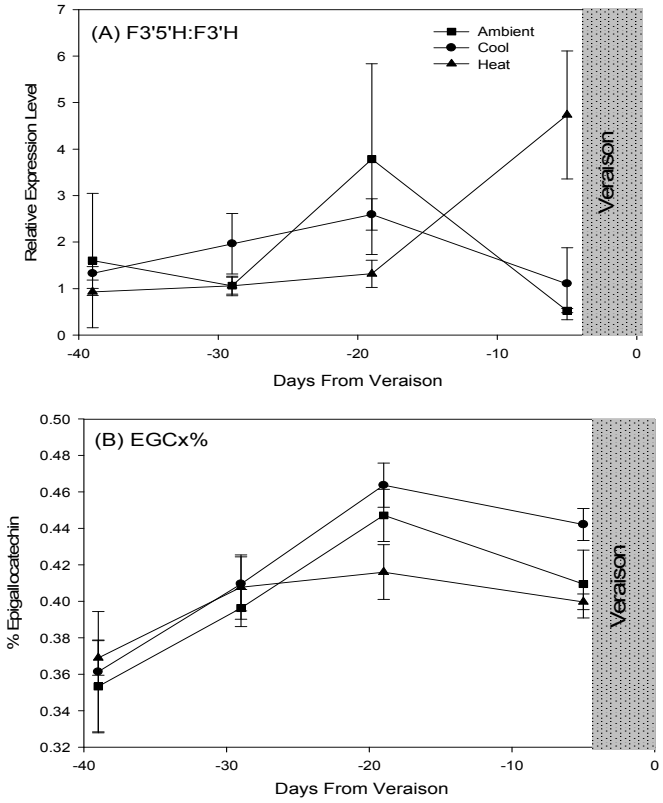


Figure 2.12. Expression of (A) Myb5a, (B) MybC, and (D) MybD regulatory genes involved in pre-véraison flavonoid biosynthesis in pre-véraison Merlot skins during 2008. Significant differences are denoted by letters (a,b,c) based on Fisher's LSD (* P≤0.1, ** P ≤ 0.05, *** P ≤ 0.01), error bars represent SEM.

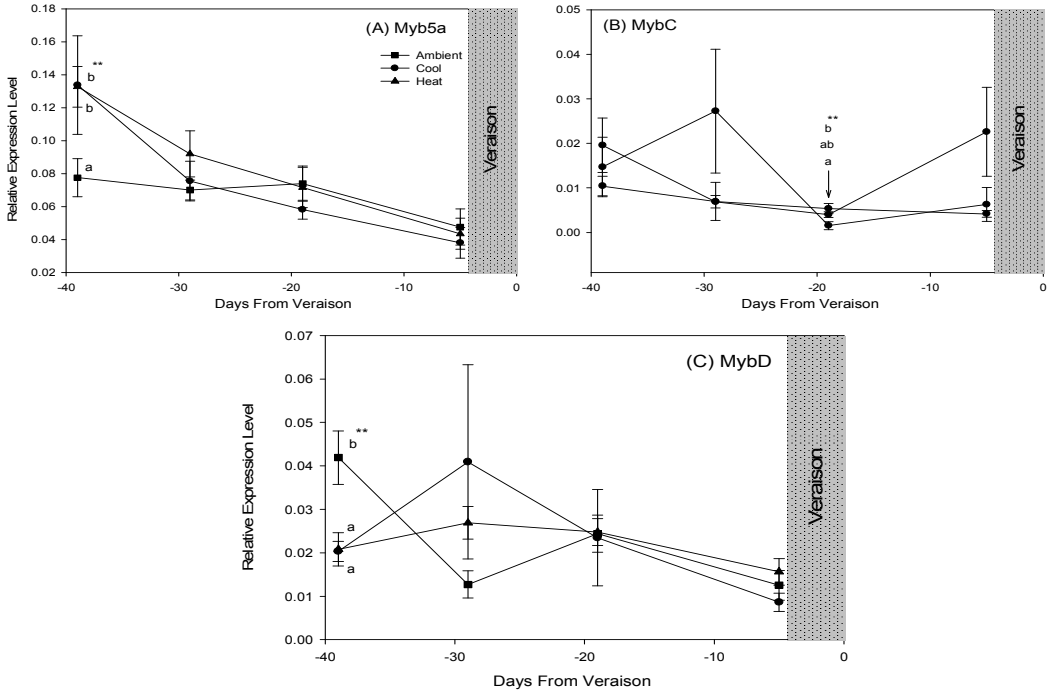
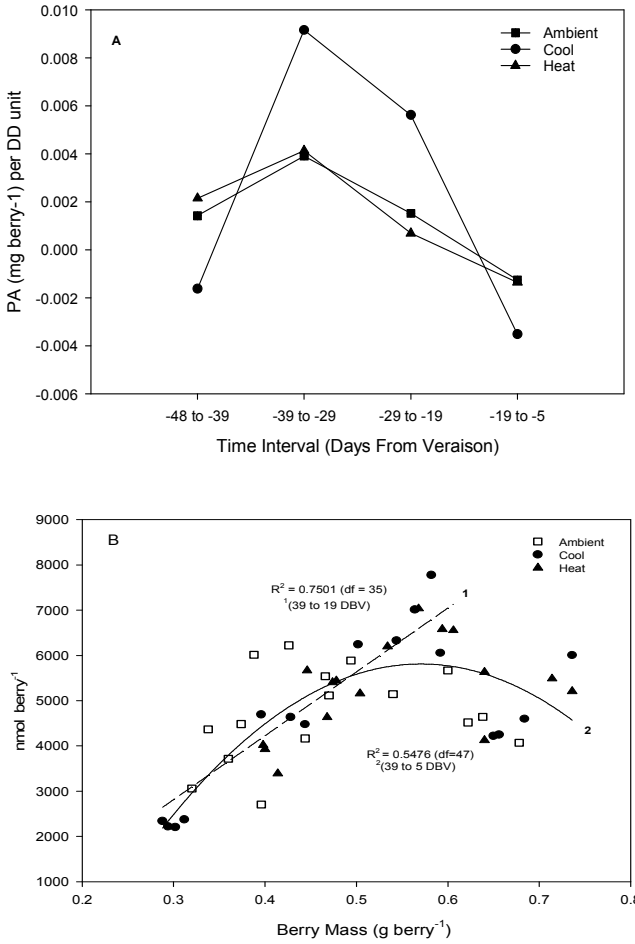


Figure 2.13. Panel A) PA concentration (mg *per berry*) over time in pre-véraison Merlot skins (2008) as a function of accumulated thermal time (DD). Panel B) Relationship between skin PA concentrations (nmol *per berry*) and berry mass in Merlot berries (2008).



Chapter 3

Compressing the diurnal temperature range of *Vitis vinifera L. cv* Merlot hastens berry development and partitioning of flavonoid metabolites.

Abstract

Temperatures during the day and night are known to influence grape berry metabolism and resulting composition. In this study, we have reduced the diurnal temperature range (DTR) of *Vitis vinifera* L. *cv* Merlot berries by cooling berries during the day and heating at night (±8°C). Before véraison, there were minor differences in proanthocyanidin (PA) composition in the skins and seeds due to temperature treatments. Reducing the DTR significantly hastened berry development and the inception of véraison. Treatments imposed after véraison had minimal impact of skin and seed PAs; however temperature-related partitioning of anthocyanins and flavonols was observed. Reducing the DTR of grape berries had a consistent effect on berry development and morphology and partitioning of flavonoid metabolites; however total flavonoid content was not significantly altered.

Introduction

The successful cultivation of wine grapes (*Vitis Vinifera* L.) involves growing cultivars selected for suitability of climate. While all elements of climate should be considered, annual temperature profiles have found specific utility. This is illustrated by the adoption of the degree-day classification system as described by Amerine and Winkler (1944). The basis for this system is the general temperature requirement for fruit to reach acceptable maturity, defined by sugar concentration and flavor development. Higher temperatures result in higher sugar concentrations and lower concentrations of organic acids, where the opposite is true at lower temperatures (Winkler et al., 1974; Coombe, 1987). While a primary goal of wine grape production is to achieve appropriate sugar and acid concentrations for the desired style of wine, secondary metabolites are responsible for the attributes most consider paramount to defining wine character and quality. Attributes such as color, aroma, flavor, and mouth-feel (astringency or tactile response) are the result of grape secondary metabolites. While the response of primary metabolites to climate is fairly well understood, that of secondary metabolites is not entirely clear. Further, the concentration of sugars and acids can be manipulated easily during wine processing whereas the concentrations of secondary metabolites are more difficult to manipulate after fruit harvest.

Geographic areas can be further categorized by annual climate variation. Cool, temperate, and Mediterranean climates in France have been differentiated based on average minimum and maximum temperatures and the difference between the two

(Carbonneau, 1985). Gladstones describes this aspect as continentality, which accounts for temperature differences between the warmest and coldest months in classifying a region (Gladstones, 1992). Such differences in temperature profiles can be attributed to variations in latitude, topography, and the proximity to coastal waters. In general, large bodies of water can provide some thermal insulation resulting in more gradual warming and cooling. This is evident in annual as well as daily temperature fluctuations or the diurnal temperature range (DTR).

The influence of day and night temperatures has been investigated Synthesis of organic acids occurs early in berry development and is most active at moderate temperatures (20-25°C) (Kliewer, 1964, 1965, 1966). During berry ripening, organic acid concentrations correspond negatively with temperatures due to considerable reductions in anabolism coupled with degradation and increased respiration ,particularly malic acid, at higher temperature ranges (Kliewer, 1964; Hardy, 1968; Kliewer, 1968; Buttrose et al., 1971). Sugar accumulation is low early in development and increases during maturation, during maturatin, lower night temperatures (10°C) and higher day / night combinations (35/25°C) were slightly inhibitory. This situation represents general differences associated with cool or temperate climates as compared to more arid climates. In regions (or seasons) characterized by higher temperatures (i.e. > 35°C), ripening occurs more rapidly and results in berries of higher sugar content and lower acidity. Alternatively, regions and seasons with lower temperatures achieving sufficiently high sugar content and reduction in acidity (specifically malic acid) can be difficult.

Development of secondary metabolites occurs at distinct phases of berry development. Proanthocyanidins (PAs), or flavan-3-ol polymers, accumulate in grape skins and seeds early during cell division and cell enlargement during stage I prior to lag phase in berry growth (Coombe and McCarthy, 2000; Robinson and Davies, 2000; Harbertson et al., 2002; Downey et al., 2003; del Rio and Kennedy, 2006). During this time flavonols begin to accumulate in the skin and continue through berry maturation or ripening. The transition between berry development and ripening, termed véraison, is characterized by accumulation of skin anthocyanins (in red or black grapes) and reduced PA accumulation (Pirie and Mullins, 1980; Hrazdina et al., 1984). While PAs are responsible for the mouth-feel properties of red wine, anthocyanins are the source of color. Flavonols are thought to provide some stability to both PA and anthocyanin-based polymers (Boulton, 2001). Due to the timing of accumulation of the various metabolites it is important to consider the impact of temperature during the various phases of grape development. For cultivation of wine grapes, coordinating the development of berry metabolites responsible for aroma, flavor, and mouthfeel with appropriate levels of sugars and acids is of foremost importance.

Previous research has demonstrated the effect of both high and low temperatures on anthocyanin accumulation. Kliewer et al. showed inhibition of anthocyanin development at higher temperatures (e.g. 35°C) while at moderate day temperatures (e.g. 25°C) anthocyanin accumulation is inversely related to DTR (Kliewer and Torres, 1972; Kliewer, 1977). A hastening of berry coloration was associated with increased pre-véraison night temperatures however those same

temperatures were inhibitive to anthocyanin accumulation when applied during maturation (Kobayashi et al., 1967). Subsequent work has confirmed an observed reduction in anthocyanin accumulation at higher day (above 30°C) and night temperatures (30°C vs 15°C) and temperature related partitioning of anthocyanins (Kliewer, 1977; Mori et al., 2005; Yamane et al., 2006; Tarara et al., 2008). Further, heat-mediated reduction of anthocyanins was related to reductions in gene transcripts and enzyme activities as well as degradation of existing anthocyanins (Mori et al., 2005; Yamane et al., 2006; Mori et al., 2007). Alternatively, anthocyanin accumulation and related gene expression was found to increase in maize (*Zea mays* L.) following exposure to low temperatures of 10°C (Christie et al., 1994).

Research regarding the direct effects of temperature on flavan-3-ols or PAs has been limited in grape as most research has focused on the impact of exposure to solar radiation and other environmental factors. Exposure to solar radiation (and U.V. light) resulted in increased PA accumulation in several cultivars of *V. vinifera* (Downey et al., 2004; Cortell et al., 2005; Fujita et al., 2007; Koyama and Goto-Yamamoto, 2008). However, little work has been done to uncouple the effects of light exposure and temperature resulting in uncertainty as to the exact mechanism behind current observations. It is well documented that the surface temperature of exposed berries may exceed 5-10°C above ambient temperatures, expanding the actual temperature range of fruit and bringing in to question the complex nature of effects attributed to light exposure (Kobayashi et al., 1967; Kliewer, 1968; Smart and Sinclair, 1976; Kliewer, 1977; Bergqvist et al., 2001).

The objective of this research was to determine the effects of reducing the diurnal temperature range on flavonoid accumulation in cv. Merlot berries grown under field conditions. Compression of DTRs was intended to simulate considerable differences that may exist between growing regions and site variation. While similar research with *V. vinifera* has occurred, it has focused primarily on the accumulation of sugars, organic acids, and anthocyanins and has been limited to growth chambers in most instances. Data presented here demonstrates the effects of reduced DTRs on grape flavonoids including skin anthocyanins and flavonols as well as PAs from both skins and seeds. Experiments were imposed on clusters in an established vineyard from the time of fruit set to véraison and again from véraison to full maturity to coincide with the active period of accumulation of the different classes of metabolites.

Methods and Materials

Field Procedure

The three-year study spanned from 2006 to 2008 at the Irrigated Agriculture Research and Extension Center in Prosser, WA, USA (46.30º N, 119.75º W). Own-rooted cv. Merlot vines (planted in 1999) were oriented north-south, trained to a bilateral cordon at 1.2m above ground, and spur-pruned. Experimental clusters were oriented on the east aspect and exposed to solar radiation by tucking shoots and leaves under a catch wire 1.5 m above ground. Treatments were applied to individual clusters, and each cluster was treated as a replicate (n = 4). Three treatment regimens were compared during the three years of study; seasons were divided into two phases based upon berry development. For Phase I experiments, studies were initiated at

stage 27-28 (berry diameter 2 to 4 mm) of a modified E-L classification system and concluded at the onset of véraison (Coombe, 1995). For Phase II experiments, studies were initiated at véraison and concluded at harvest or physiological ripeness (≥ 22Brix°). Both Phase I and Phase II experiments were conducted during the 2006 and 2007 seasons, while 2008 was limited to Phase I. Treatments included: (1) ambient (2) convective control (*blow*), and (3) diurnal dampened (*damp*). *Damp* treatments were achieved by delivery of cooled air during the day and heated air during the night. All *in situ* temperature control was accomplished by a forced-air delivery system modified by replacing the single chilling unit with a pair of units that operated alternately (Tarara et al., 2000). Target temperature differences were 8 °C below or above ambient clusters (day and night respectively); no chilled air was delivered if berry temperature was below 10 °C. The temperature and wind velocity around ambient clusters was not manipulated. Convective control (*blow*) refers to ambient air delivered to a cluster 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 diam.; Type T [copper-constantan]) each encapsulated in a 4 to 6 mm diameter bead of silicone before véraison. After véraison thermocouples were inserted directly below the skin surface and sealed with water-based glue. 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). Harvested berries were excised

from the rachis (peduncles attached to berries), counted, weighed, frozen in liquid nitrogen, and stored at -80°C prior to analyses.

Equipment and Protocols

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 (DAD) detector and an external column oven when required (Eppendorf CH-430, Westbury, N.Y., USA). All data were analyzed using Agilent Chemstation software (V A.08.03).

Berry volumes (50 or 100 berries) were estimated based on H_2O displacement prior to manual dissection and separation of skin and seeds. For soluble solids analysis, 15-20 frozen berries were crushed and incubated at 75°C for 1 hour and centrifuged (3500 *g*) before determination on a portable digital refractometer (WM-7; Atago, Tokyo, Japan).

Analyses of grape skin phenolics were carried out as previously described (Cohen et al., 2008). Skin extracts were prepared by macerating lyophilized skin tissue in an acetone:water solution (2:1) at 20 °C for 24 hours under nitrogen gas as previously described (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 (Lamuelaraventos and Waterhouse, 1994). In 2006, aqueous extracts were filtered using a syringe filter (Acrodisc PTFE (13mm, 0.45µm) Pall Corporation, East Hills, NY, USA). In 2007 and 2008 samples were centrifuged at

16,000g for 15 minutes to remove solids. Quercetin (Sigma Aldrich; St. Louis, MO, USA) and malvidin-3-*O*-glucoside (Extrasynthèse, Genay, France) were used as quantitative standards for flavonols and anthocyanins, respectively.

Compositional analysis of PAs was carried out following acid-catalyzed cleavage in the presence of phloroglucinol (phloroglucinolysis) according to the procedure by Kennedy and Jones (2001). Appropriate aliquots of aqueous extracts were lyophilized and dissolved in MeOH prior to reacting with phloroglucinol reagent as previously described (Kennedy and Taylor, 2003; Cortell et al., 2005). Samples were immediately analyzed following addition of aqueous sodium acetate. Quantification of PA subunits was calculated as previously described using a (+)-catechin (Sigma Aldrich, St. Louis, MO, USA) as quantitative standard (Kennedy and Jones, 2001). Proportions of flavan-3-ol subunits are presented as mole percentages to illustrate differences in metabolite partitioning, regardless of molecular mass.

Berry temperature data were summarized over time and by treatment in SAS (ver 9.1, SAS Institute, Cary, NC, USA) using the MEANS procedure. Thermal time or "heat accumulation" was computed using a trapezoidal method of integration of temperature over time reported as degree days (DD) with a 10°C base temperature (Tobin et al., 2001). Statistical analyses of metabolite and temperature data were performed using Statgraphics Centurion statistical software (Statpoint Tech. Inc., Warrenton, VA). Differences within years were determined using one-way ANOVA, two-factor ANOVAs (treatment and year) were used to determine treatment and year effects across all years of experimentation. Non-parametric data sets were analyzed using Kruskal-Wallis ANOVA as indicated in individual tables.

Results

Temperature Data

Temperature profiles for treatments over three experimental seasons are shown in Figures 3.1A-C. Due to the nature of treatments, annual daily average temperatures were similar for all treatments. Graphical representations of daily averages (ambient) and daily minimum and maximum temperatures for each treatment illustrate compression of DTR achieved with treatments. The difference in seasonal temperature oscillations between years is also illustrated, contributing to seasonal variability. Clearly, *damp* reduced maximum temperatures while increasing minimum temperatures relative to ambient. This resulted in nearly eliminating temperatures excess of 35°C or below 10°C (Figure 3.1).

Phase I

In 2006 DD (Table 3.1) values were slightly lower in *damp* berries during phase I of the study, in subsequent years there were no differences between treatments. Ambient and *blow* clusters were equivalent in the time spent at various temperatures above 25°C while *damp* clusters were consistently lower in all years. This demonstrates that temperatures of *damp* clusters were maintained near 20-25°C for a greater proportion of time during the experiment. In all instances and across all three years, *damp* clusters were subjected to significantly lower DTR than ambient and *blow* clusters, which were not different (Table 3.1; $p < 0.001$).

While the 2008 season exhibited a higher DTR on average ($P < 0.001$, not shown) the variability of average temperatures across the season is less apparent. Before and after adjusting thermal time summations for the duration of the

experiment, 2008 shows the lowest DD values ($p < 0.05$). There were periods of above average temperatures (e.g. ≥ 35°C) each year however the timing of periods was different. This is noteworthy as exposure to potentially stress-inducing temperatures would have occurred during different stages of berry development each year.

Phase II

Temperatures during berry maturation in both 2006 and 2007 resulted in no annual DD differences between treatments (Table 3.1). Similar to treatments during Phase I, hours spent at temperatures above 25°C were lowest in *damp* clusters in nearly all cases, where *blow* and ambient clusters were similar. Further, the DTR of *damp* was always lowest ($p < 0.01$) while ambient and *blow* were similar at nearly all times. 2006 appeared to be a warmer season in general, exhibited by higher temperatures and greater accumulation of thermal time ($p < 0.01$). The DTR in 2006 was also higher by week and by seasonal average ($p < 0.01$) than 2007.

In general, there were minimal differences in the temperature or metabolite profiles for ambient and *blow* berries. Hence, illustrations of data are limited to comparisons between ambient and *damp* berries and references to *blow* are made when relevant.

Berry morphology and composition

Phase I

Treatment effects on berry morphology and soluble solids at véraison are illustrated in Figure 3.2. *Damp* resulted in increased berry mass in all years ($p < 0.05$). Berry volumes were proportional to berry weights, following the same trends

($r^2 \approx 0.97$, data not shown). This, in combination with higher rate of coloration and higher soluble solids content, suggests considerable hastening of berry development compared to ambient. Mass of skin *per berry* was found to be highest in *damp* berries in 2006 and 2007 ($p < 0.05$) but was not significant in 2008. Data from a concurrent experiment showed that the rate of berry development of *damp* berries was greater than equivalent levels of daytime cooling or night time heating alone (Cohen et al., 2008). The three seasons resulted in significant differences in the number of seeds *per berry*, with the highest in 2008, followed by 2006 and then 2008 ($p < 0.001$, data no shown). However, there were no interannual differences in seed fresh weight or treatment effects on either seed number *per berry* or seed fresh weight.

Phase II

Berry morphology and soluble solids contents (°Brix) at the end of Phase II are shown in Figure 3.3. There were no differences in berry weights due to treatment or between years. Skin dry weights were similar in 2006 but tended to be lower in *damp* in 2007. Similarly, there were no differences in soluble solids in 2006 but levels were higher in *damp* berries in 2007. Soluble solids content of ambient berries was higher in 2007 than 2006 indicating a higher level of berry ripening, which may contribute to general differences between the two years. Consistent with Phase I experiments, there were no differences in number of seeds *per berry* or seed fresh weight due to treatments. Again, number of seeds *per berry* was higher in 2006 than 2007.

Flavonoids – Phase I

Seed PAs

The concentration of seed PAs *per berry* was not significantly affected by treatments, however *per seed* concentration was highest in ambient berries in 2008 (data not shown, $p < 0.01$). Overall concentrations of PA's *per berry* were lowest in 2007; however values were highest on a *per seed* basis, possibly related to the reduced seed numbers *per berry* ($p < 0.01$). Average polymer size (mDP) of seed PA's tended to be lower in ambient berries, which was significant in 2007 (Figure 3.4) and when considering three years of data together ($p < 0.05$).

At véraison, the seeds contained considerable quantities of flavan-3-ol monomers, which are also shown in Figure 3.4. Total concentrations of monomers followed similar patterns to total seed PA concentrations. *Damp* berries had higher concentrations in 2006, otherwise no differences were found between treatments. Further, overall concentrations of monomers *per berry* were not different between years whereas *per seed* concentrations were highest in 2007 ($p < 0.001$).

Differences in seed flavan-3-ol monomer composition were observed across the three years of this study. In 2006, the proportion of catechin (Cm) was highest in *damp* berries. Proportions of epicatechin (ECm) were not different in 2006 but were highest in *damp* berries in 2007 and 2008 and when considering all years together ($p < 0.001$). The proportion of epicatetchin-3-*O*-gallate (ECGm) was consistently lowest in *damp* berries in all years of the study. Overall proportions were highest in 2006 and lowest in 2007 ($p < 0.001$). Regression analysis (backward selection) of ECGm with DD and DTR as independent variables resulted in DD having no

significance ($p < 0.1$) in resulting models. Average DTR explained 83.2%, 51.9%, and 97.2% of variability in proportions of ECGm in 2006, 2007, and 2008 respectively ($p < 0.001$). Considering three years data together resulted in the following model:

%ECGm = 0.0113961 + 0.00634433*avg_dtr (adj. r^2 = 50.7%, std. Error = 0.0264, $p < 0.001$)

The data indicate that higher DTR's result in increases in gallate-esterification of seed flavan-3-ol monomers.

As with total PA's and flavan-3-ol monomers, accumulation of total PA polymers *per berry* was not influenced by treatment or year during this experiment. Relative differences in PA polymers due to treatment and year were similar to that observed with total PA concentrations.

Catechin extension subunits (Cx) were proportionately highest in *damp* berries in 2006 and across all years and highest overall in 2008 ($p < 0.001$). The proportion of epicatechin as an extension subunit (ECx) did not differ due to treatment although they were highest in 2007 ($p < 0.001$). Contrary to ECGm, the proportion of epicatechin-3-*O*-gallate extension subunits (ECGx) was lowest in ambient berries in 2008 but followed the same pattern with respect to year (2006 > 2008 > 2007; $p < 0.001$).

The distribution of terminal subunits showed little influence of treatment. In 2008 the proportion of epicatechin as a terminal subunit (ECt) was highest and that of epicatechin-3-*O*-gallate (ECGt) was lowest in *blow* berries, which does not suggest an effect of temperature. Overall, the proportion of ECGt was highest in 2006 followed by 2007 then 2008 ($p < 0.001$).

Skin PAs

The concentration and composition of skin PA's at véraison are shown in Figure 3.5 and are similar to distributions reported by Souquet et al. in Merlot (1996). Treatments had little effect on total skin PA content *per berry*. *Damp* resulted in the lowest PA content by berry mass in 2006 and across all years (not shown, $p < 0.01$) PA mDP tended to be lowest in *damp* berries, which was significant in 2006 and across all years of data ($p < 0.01$).

The concentration of flavan-3-ol monomers and terrminal subunits in berry skins showed no significant trend. No treatment effect was observed for Ct, ECt or ECGt during the study. The proportion of Ct was lowest in 2006, when that of epicatechin (ECt) and epicatechin-3-*O*-gallate (ECGt) terminal subunits were highest ($p < 0.01$).

Concentrations of skin PA extension subunits were not found to be different due to treatment. Additionally, there were no differences in PA extension subunit composition. In general, concentrations were highest in 2008 ($p <0.001$) as with total PA concentration. While day-cooling alone results in higher relative proportions of epigallocatechin (EGCx), *damp* treatments did not show the same differences (Cohen et al., 2008). Proportions of EGCx and ECGx were highest in 2007 when proportions of ECx were lowest ($p < 0.001$). Considering all observations regarding PA accumulation, there is considerable variability between years with respect to treatment. This may be related to differences in developmental stage when berries were harvested; however the relative differences were consistent across years. Therefore, interannual discrepencies may represent the influence of treatments

relative to annual patterns (i.e. treatment effect during periods of high or low temperature relative to developmental stage).

Skin Anthocyanins and Flavonols

Color development is illustrated in Figure 3.2 as the percent of berries exhibiting red coloration. *Damp* berries accumulated anthocyanins earlier than other treatments as determined by visual inspection. Analysis of anthocyanins simply confirms this with concentrations being undetectable or very low in ambient and *blow* berries and at or above 10 μg *per berry* in *damp* treatments (data not shown). Briefly, compositional diversity of anthocyanins tends to be lowest early in development (lacking acyl-derivatives) though develops quite rapidly by véraison. Due to low concentrations at this point, anthocyanin accumulation was considered only as an indicator of berry maturation in this study.

Flavonol concentrations (Figure 3.6) showed no effect of treatment on a *per berry* basis. However, the trend towards lower concentrations in *ambient* was significant when considered across three years of data ($p < 0.05$). Data presented on a mass basis shows the effect of berry size, where *blow* berries tended to be highest in concentration ($p < 0.01$; across three years). Total concentrations were considerably lower in 2006 ($p < 0.001$). Similar to anthocyanin accumulation, chemical diversity of flavonols was more limited early in development, similar to anthocyanins. Initially, quercetin derivatives (Di-OH) were dominant thus the proportion of Di-OH flavonols is higher in ambient and *blow* berries. *Damp* berries tend to accumulate slightly higher amounts of mono- and tri-hydroxylated flavonols by véraison, shifting the proportion of Di-OH downward.

Flavonoids – Phase II

Seed PA's

Accumulation of seed PA's at harvest displayed no significant treatment effect on *per berry* or *per seed* basis. *Per seed* PA concentrations were higher in 2007 than 2006 ($p < 0.001$), a difference possibly related to the reduction in seeds *per berry* in 2007 (data not shown). The concentration of seed flavan-3-ol monomers was also highest in 2007 on both *per berry* and *per seed* basis ($p < 0.01$) while no treatment effect was observed (Figure 3.7). Differences in seed PA mDP were minimal within each year though were higher in 2006 overall ($p < 0.001$).

Treatments had no effect on the proportion of Cm in seeds. There were no annual effects of treatment on ECm proportions, however concentrations were slightly higher in *damp* berries considering both years of data together ($p = 0.0501$). The proportion of ECGm in *damp* was lower than that in ambient berries in 2006 and slightly lower across both years ($p = 0.0719$). Overall, ECGm proportions were higher in 2006 than 2007 ($p < 0.001$).

Consistent with total PAs and flavan-3-ol monomers, there were no differences in total polymeric PAs between treatments (Figure 3.7), however concentrations were higher overall in 2007 ($p< 0.001$). The proportions of Cx and ECx were higher in 2007 while that of ECGx was lower ($p < 0.01$). The distribution of terminal subunits showed no effect of treatment though interannual differences were consistent with those for monomer and extension subunits. As observed previously, the increase in proportion of epicatechin as a gallate-ester is related to

higher temperatures in general, not necessarily related to DTR alone (Cohen et al., 2008).

Skin PAs

Treatments during maturation had little effect on accumulation of PAs in berry skins (Figure 3.8). Ambient had slightly higher PA's *per berry* in 2007 than other treatments ($p = 0.053$). Overall concentrations were higher in 2006 *per berry* and by mass ($p < 0.05$). There were no differences in PA mDP between treatments or years.

Overall, treatments had minimal effect on the distribution PA terminal subunits (Figure 3.8). In general, the proportion of Ct was lower in 2007 than 2006 ($p < 0.01$), likely due to the higher contribution of ECGt to the total pool that year. Interestingly, the concentrations of ECGt were lower at harvest in 2006 than at véraison, a shift not observed in 2007. This may represent a loss of ECG due to increased degradation or incorporation into polymers or cell wall material. This aspect is difficult to discern from this experiment but appears to be most pronounced in ambient berries.

The distribution of extension subunits was not affected by treatment in 2006. Total extension subunits were highest in ambient berries in 2007. In that year the proportion of Cx was higher and that of ECGx was lower in *damp* than ambient berries, however these differences were minor and represent less the five percent of total extension units. In general, there was little effect of temperature treatments on skin PAs during berry maturation.

Skin Anthocyanins and Flavonols

The flavonol concentration of berries at harvest followed the same relative trend as for skin PA's. Treatment differences were not significant in 2006 whereas *per berry* concentrations in *damp* were marginally lower in 2007 and considering both years of data together ($p = 0.0606$). Overall values were higher in 2006 than 2007 ($p < 0.05$). The proportion of Di-OH flavonols was highest in *damp* berries in 2007 and across both years of data ($p < 0.01$) but not in 2006 alone. The rate of Di-OH was highest in 2007 ($P < 0.001$), similar to that of anthocyanins yet opposing the trend in skin PA's.

Trends in total anthocyanins were opposing between 2006 and 2007. The increase in anthocyanins due to *damp* in 2006 was not significant however the decrease in 2007 was, following the same trend observed with seed and skin PA's and flavonols. Accounting for berry weights eliminates all differences in anthocyanins. In all circumstances the proportion of Di-OH anthocyanins was highest in *damp* berries. The overall proportion of Di-OH anthocyanins was considerably higher in 2007; differences were manifested in increases in cyanidin- and peonidin-glucosides concomitant with decreases in malvidin-3-*O*-glucoside and derivatives. This shift in hydroxylation is in accord with work by Tarara et al. (2008) where cooling sunlit berries resulted in the same observations. Data from a concurrent study also suggests these differences are predominantly the result of cooler daytime temperatures, where warmer night time temperatures alone did not produce the same result (Cohen et al., 2008). The proportion of anthocyanins in the form of acetyl glucosides was also found to be lowest in *damp* berries in both years and lowest in 2007 ($p < 0.001$). Similarly, coumaroyl-glucosides were also lowest in *damp* and highest in 2006

overall ($p < 0.001$). In general, *damp* resulted in lower proportions of anthocyanins in the Tri-OH, acetyl- , and couamoryl-glucoside forms. This is in general agreement with experiments with Merlot and Shiraz berries in response to reduced daytime temperature and in years with lower average temperatures (Downey et al., 2004; Tarara et al., 2008).

Discussion

The diurnal temperature range of field grown grape berries was compressed by delivery of cold air during the day and warm air at night. To the authors' knowledge, this is the first data of its kind collected outside of the growth chamber (Kobayashi et al., 1967; Kobayashi et al., 1968; Kliewer and Torres, 1972; Kliewer, 1977). As a result of this experiment we have altered the duration of time berries are exposed to 'optimum' temperatures for growth via compressing the DTR. Various experiments with grapes have estimated this temperature range to be approximately 20-25°C, with respect to photosynthesis and primary metabolism (Kobayashi et al., 1967; Kobayashi et al., 1968; Kriedemann, 1968; Buttrose and Hale, 1973; Gladstones, 1992; Jackson and Lombard, 1993). High (≥35°C) and low (≤10°C) temperatures have been shown to alter berry development and accumulation of sugars, acids and anthocyanins in gapes; however, responses can be cultivar dependent and vary with temporal development (Kobayashi et al., 1967; Buttrose et al., 1971; Kliewer and Torres, 1972; Buttrose and Hale, 1973; Haselgrove et al., 2000; Bergqvist et al., 2001; Yamane et al., 2006; Mori et al., 2007; Tarara et al., 2008). From many of the aforementioned studies it is thought that the range of temperatures experienced during a 24-hour day can be as important as absolute high

or low temperatures. In general, treatments altered developmental rate of berries and had an impact on the partitioning of various flavonoids within their biosynthetic pathway. There were no consistent effects of reduced DTR on total accumulation of skin or seed flavonoids during the study.

The effect of DTR is quite evident with respect to general berry development. As discussed by several authors, the interplay between light and temperature during a 24-hour day is a complex aspect of plant development (Heggie and Halliday, 2005; Jones, 2009). Based upon the photoperiod, plants adjust metabolism to protect against forms of abiotic stress, which includes high and low temperatures. Many of these changes involve shifts in regulatory hormones and genes, altering the rate of development in some cases. Due to the temporal pattern of metabolite accumulation in grapes, it is not surprising that shifts in development may effect metabolite accumulation in some manner. Reductions in DTR hastened development as evident in increased berry mass, volume, coloration, skin weight *per berry*, and soluble solids content at véraison. In concurrent experiments both day-cooling and night-heating alone showed similar results, however the magnitude of difference was considerably less (Cohen et al., 2008). This result is in accord with expectations considering existence of optimum metabolic temperatures as mentioned above, however hastening of development has typically been associated with higher overall temperatures (Kobayashi et al., 1967; Kobayashi et al., 1968; Winkler et al., 1974; Gladstones, 1992; Jackson and Lombard, 1993).

Treatments applied during berry maturation (véraison to maturity) had very little effect on berry morphology in general. The data have obvious implications with

regard to manipulating rate of berry development in the field; while it is unclear what the magnitude of DTR compression is required to achieve an affect, DTR compression of one-half the magnitude described in this study showed similar results concerning morphology at véraison ("damp" versus "double-damp"; (Cohen et al., 2008).

Due to the nature of seeded wine grapes, it is difficult to manage the differential extraction of PA's from skins and seeds from a production standpoint. When attempted, reducing seed PA's during wine production did not yield an absolute qualitative benefit; instead, seed additions have resulted in improvements in wine color and potential aroma character (Kovac et al., 1995; Canals et al., 2008; Lee et al., 2008). Although research with purified PA's suggests those originating from seed will affect astringency and may be 'coarse' or bitter, the relevance to wine production and quality remains uncertain (Peleg et al., 1999; Vidal et al., 2003; Gambuti et al., 2006).

In this study, temperatures were recorded just beneath the berry exocarp, which is expected to approximate seed temperatures. Therefore, the effects of temperature on seed PA's are better isolated; the direct influence of UV radiationare eliminated wherein the skin it is not. Further, this is one of the first studies to investigate the discrete effect of temperature on PA accumulation in grape seeds. While there were minimal treatment differences in total PAs at véraison there was an increase in ECGm in response to higher DTR's. From previous research, this response is likely driven by higher day temperatures (Cohen et al., 2008). A similar observation was reported in tea (*Camelia sinensis* L.) with increased levels of gallate

esterificatin of flavan-3-ols associated with leaves harvests during warmer months (Yao et al., 2005). In work by Cortell and Kennedy (2006), exposure to sunlight resulted in a decrease in ECGx and ECGt in Pinot noir seeds, though ECG was not reported as a monomeric subunit. In the current study, the proportion ECG as a monomer was always lowest in *damp* berries; however the proportion as extension and terminal subunits was not. Thus, the biosynthetic mechanism regulating monomeric and polymeric flavan-3-ols in grape seeds would appear to be somewhat independent.

Between véraison and harvest there is an overall reduction in seed PA's likely related to oxidative polymerization, visualized by browning of the seed coat (Marbach and Mayer, 1974; Devic et al., 1999; Kennedy et al., 2002; Debeaujon et al., 2003; Downey et al., 2003; Pourcel et al., 2005; Pourcel et al., 2007). Differences were most evident as reductions in the proportion of ECG as monomeric and terminal subunits between samples collected at véraison and harvest in 2006 and 2007. This may reflect changes due to seed maturation and development of the seed coat and differences in rate of polymerization attributed to flavanol galloylation in model solutions (Cheynier and Ricardo-da-Silva, 1991).

The treatment effect on skin PAs at veraison found to be minor across the years of study and was only significant in 2006 when accounting for berry mass. In that year PA concentration exhibited a moderate increase with heat accumulation (r^2 = 0.34, p = 0.007, std. error = 0.2); however this was not the case the following seasons (Cohen et al., 2008). As reported in Cohen et al. (2008), berries that were cooled during the day *only* had higher proportions of EGCx, counter to observations where

exposure to solar radiation resulted in increases in EGC (Downey et al., 2004; Cortell and Kennedy, 2006). In this study *damp* berries had the same proportion of EGCx as ambient berries, suggesting the higher nighttime temperatures act antagonistically to the lower daytime temperatures with regard to flavan-3-ol partitioning and composition. It is interesting to note that the relative differences in total skin PAs between ambient and *damp* berries across three years is opposite that that for seed PAs. While differences were not significant in each case, this represents differences in tissue specific PA regulation based on temperature and factors such as UV radiation that may be limited to the skin. As an example, aril juice from pomegranates (*Punica granatum* L.) grown in Mediterranean climates were found to have higher contents of sugars, acids, total phenolics and anthocyanins than those grown in an arid, desert, environment (Schwartz et al., 2009). However, while anthocyanins in the skin were lower, total phenolic and PA contents in the skin were found to be higher in those fruits grown in the desert.

It is generally accepted that minimal PA accumulation occurs after véraison, therefore, any changes are likely the result of berry maturation and cell wall modifications leading to differences in extractability (Adams, 2006). There were no differences in total PA's or composition due to treatment in 2006; however there was a slight increase in ambient berries compared to *damp* in 2007. Again, this could be due to higher temperatures or differences in maturity as berries were more advanced in 2007 and in *damp* versus ambient berries that year. It has been shown that both anthocyanins and PAs interact with grape cell wall material and may influence their extractability from the skin (Oretgea-Regules, 2006, Gagne et al., 2006). Cell walls

are subject to loosening and degradation during ripening and differences in their degradation may be related to variability in PA concentrations observations across vintages (Huang et al., 2005; Gagne et al., 2006). Therefore, the influence of the DTR on berry ripening may influence PA concentrations by means of altering extractability and not necessarily PA biosynthesis alone.

Accumulation of total skin flavonols at véraison did not appear to be affected by temperature treatments in any single year on a *per berry* basis. Previous work has shown the positive influence of light exposure on flavonol accumulation with minimal influence of temperature where relevant (Price et al., 1995; Spayd et al., 2002; Downey et al., 2004; Tarara et al., 2008). However, when temperature was considered, myricetin concentrations (Tri-OH) were not reported over the entire course of study, so direct comparisons with regard to rates of flavonol hydroxylation cannot be made. *Damp* berries tended to have lower proportions of Di-OH flavonols at véraison, which is likely explained by the higher contribution of mono- and tri-hydroxylated forms brought on by hastened maturity. In contrast, *damp* berries tended to have higher proportions of Di-OH flavonols at harvest, which is directly attributed to lower day temepratures.

Anthocyanin accumulation occurred earliest in *damp* in all three years of the study indicative of advanced maturity. During berry maturation, however, anthocyanin biosynthesis is active and shifts in metabolism were observed. Concentrations were lower in *damp* berries in 2007 on a mass basis, similar to the pattern observed for flavonols and skin PAs. In both years, the proportion of Di-OH anthocyanins was considerably highest in *damp* berries and the proportion of

anthocyanins in the form of acetate- and coumarate-glucosides was lowest. Similar shifts in proportions are observed in relation to reduced daytime temperatures in data associated with this study (Cohen et al., 2008) and work by Tara et al., (2008). Although there is insufficient data to substantiate the rationale behind interannual treatment discrepancies, the drastic difference in acyaltion and hydroxylation are indicators that the effects of reduced DTR are likely dependent on the upper range of temperatures.

In general, there were no differences in the concentration of skin flavonoids at harvest 2006 while *damp* resulted in lower concentrations of all classes in 2007 (PAs, flavonols and anthocyanins). Time spent at elevated temperatures and accumulation of thermal time was consistently lower in 2007 (all cases $p < 0.001$) than 2006 during phase II, which may illustrate a positive relationship between temperature and flavonoids during that period. In 2006, higher temperatures may have been inhibitory to berries such that *damp* berries were not as severely affected.

The data presented here confirms that the regulation of grape flavonoids differs between the stages of berry development and berry ripening. Lower daytime temperatures and, in this case, reduced DTRs shift partitioning of both anthocyanins and flavonols in skins towards those possessing di-hydroxylation of the flavonoid B-ring. Before véraison, during active skin PA biosynthesis, an opposing shift was observed in 2006 upon reducing daytime temperatures (Cohen et al., 2008). This may reflect differences associate with various MYB-type regulatory genes expressed throughout berry development and may also reflect differences in the nature of metabolites (Kobayashi et al., 2002; Deluc et al., 2006; Takos et al., 2006; Bogs et al.,

2007; Walker et al., 2007; Deluc et al., 2008; Czemmel et al., 2009; Terrier et al., 2009). Unlike anthocyanins, PAs are subject to polymerization and compositional differences may be the result of subunit condensation and associated differences in extractability impacting downstream analysis. From this and similar studies it is clear that differences in metabolites can be attributed to variability in biosynthesis however post-biosynthetic modifications are likely of equal importance.

The goal of this study is to provide a better understanding of factors thought to differentiate wine growing regions, sites or seasons. It is suggested that temperate climates, cool years in warm climates or warm years in cool climates produce superior quality wine grapes (Winkler et al., 1974; Gladstones, 1992; Jackson and Lombard, 1993). Often, canopy management is used to modulate vine microclimate thereby achieving optimum growing conditions for a specific site (Smart, 1985). Here, we have greatly reduced the incident of high and low temperatures; however few differences were observed with respect to flavonoid accumulation. At the same time, there was a consistent effect on the rate of berry development. While partitioning of seed flavan-3-ols, anthocyanins, and flavonols is under some influence of DTR, there is no such affect with regards to skin flavan-3-ols or PAs. The data presented here is suggestive that typical vintage (quality) variability is more likely due to the timing of events such as high or low temperatures, U.V. radiation and water availability than a seasonal summary. Reducing the incident of high and low temperature spikes will affect rates of development and may translate to quality differences in grapes at harvest. In moderate years, for example, a higher percentage of fruit may develop in better synchronization, reducing the tendency for variability at

harvest. Additional work concerning the effect of transient temperature treatments would be necessary to determine the existence of sensitive developmental stages that correspond to the various metabolites and contribute to vintage variability and efficacy of treatments or cultivation techniques. Temperature manipulations in this study were imposed for long periods of time such that an acclimation response could not be ruled out. Further, the impact of DTR and overall berry maturation on development of aroma active compounds is still of importance from a quality perspective and likely responsive to shifts in the rate of berry maturation.

Table 3.1. Thermal time summaries (DD), average DTR (Tmax - Tmin), and time (hours) spent above temperatures (± SD, n = 4) indicated during each phase of experiment in 2006 through 2008. Duration of experiment is shown in parentheses (days). Letters denote differences between values within each year (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 3.1 continued.

				Number of hours above indicated temperature			
	Treatment	**DD Sum[a]**	**DTR Avg**	**Hr > 40°C**	**Hr > 35°C**	**Hr > 30°C**	**Hr > 25°C**
2006	Ambient	605.2 ± 7.6y**	19.6 ± 0.7y***	2.7 ± 2.7	58.1 ± 11.2y***	256.7 ± 22.1y***	501.0 ± 15.3y***
Phase I	Blow	596.6 ± 9.3y	18.9 ± 0.4y	1.1 ± 1.2	46.6 ± 10.8y	235.5 ± 16.4y	481.1 ± 21.5y
(45)	Damp	580.5 ± 4.5x	10.2 ± 0.1x	0.0 ± 0.0	1.9 ± 1.0x	65.1 ± 3.7x	356.4 ± 12.1x
2007	Ambient	595.4 ± 17.1	18.9 ± 1.3y***	0.6 ± 1.2	36.9 ± 20.3y*	236.1 ± 44.8y***	504.6 ± 29.9y***
Phase I	Blow	597.5 ± 7.2	18.5 ± 0.7y	0.0 ± 0.0	37.2 ± 7.1y	235.3 ± 23.9y	503.4 ± 11.9y
(44)	Damp	592.3 ± 13.0	10.6 ± 0.5x	0.0 ± 0.0	6.1 ± 7.2x	76.9 ± 22.8x	382.5 ± 14.3x
2008	Ambient	589.9 ± 18.9	21.1 ± 1.4y***	0.5 ± 0.8	38.3 ± 22.4y*	237.6 ± 43.3y***	502.6 ± 18.0y***
Phase I	Blow	589.5 ± 15.0	10.3 ± 0.5y	0.0 ± 0.0	29.1 ± 7.6y	226.4 ± 26.7y	495.0 ± 21.8y
(46)	Damp	566.4 ± 9.3	11.8 ± 0.7x	0.9 ± 1.8	3.5 ± 6.9x	30.2 ± 15.8x	317.3 ± 27.8x
2006	Ambient	431.7 ± 11.0	24.1 ± 1.9y***	4.0 ± 5.0	43.4 ± 18.5y*	180.9 ± 21.9y***	310.3 ± 12.4y***
Phase II	Blow	417.6 ± 13.9	22.3 ± 1.8y	0.8 ± 0.5	26.0 ± 13.2xy	156.7 ± 23.2y	296.2 ± 16.4y
(43)	Damp	419.2 ± 11.5	14.4 ± 0.2x	0.3 ± 0.5	11.9 ± 1.8x	57.4 ± 3.9x	205.4 ± 6.4x
2007	Ambient	391.0 ± 5.0	21.8 ± 1.3y***	0.9 ± 1.8	14.6 ± 14.9	108.5 ± 15.5y***	276.2 ± 7.1z***
Phase II	Blow	380.4 ± 10.4	20.4 ± 1.9y	0.6 ± 1.1	9.4 ± 10.4	81.0 ± 24.8y	257.5 ± 15.7y
(41)	Damp	374.3 ± 3.2	12.7 ± 0.7x	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 1.7x	131.7 ± 8.6x

Figure 3.1 A-C. Average, maximum, and minimum daily temperature profiles (°C) in 2006, 2007, and 2008 (n = 4). Daily averages are shown for ambient, other treatments had equivalent values. Departures from daily maximum and minimum temperatures are shown for *damp* compared to those values for ambient and *blow* illustrating considerable compression of the diurnal temperature range encountered. Shaded regions demarcate the period of véraison (vertical bar) and potential optimum temperatures (horizontal; 20-25°C) flanked by temperature extremes (35 and 10°C) found to inhibit metabolism.

Figure 3.1 Continued

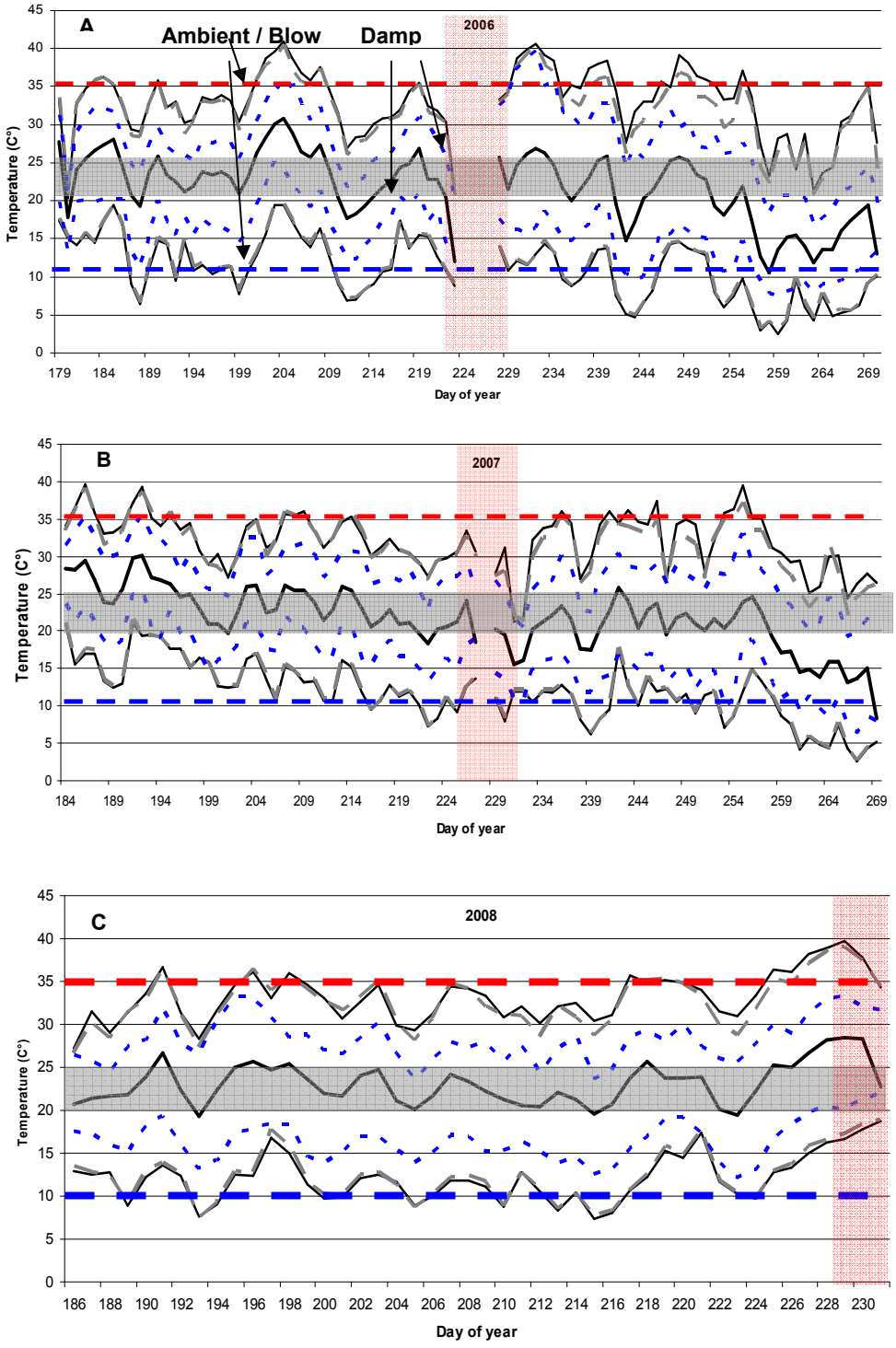
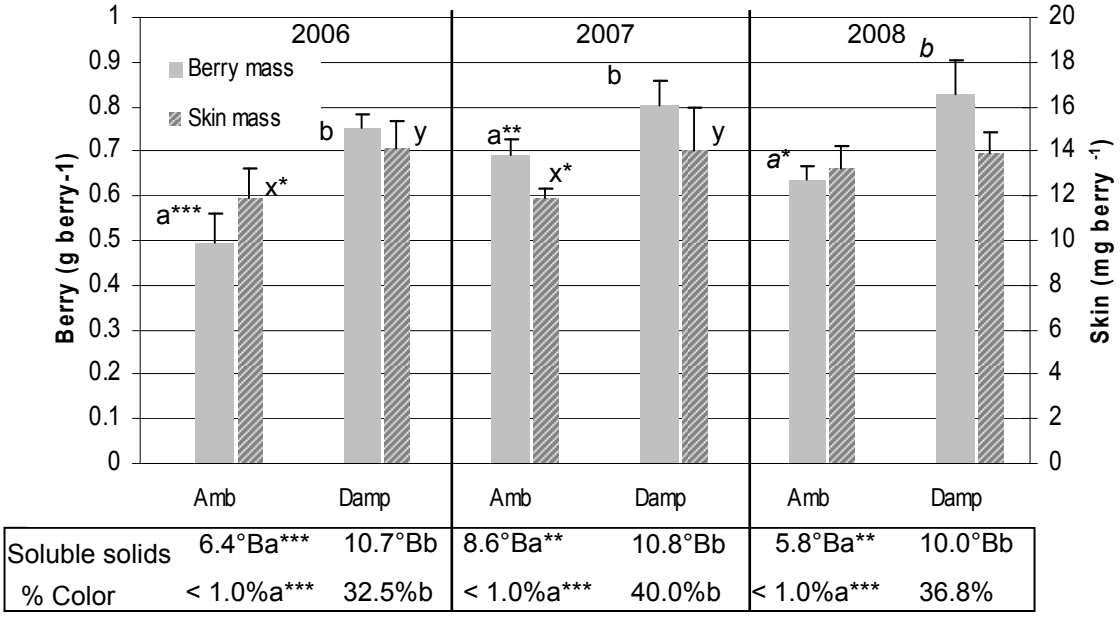


Figure 3.2. Merlot berry mass, skin mass *per berry*, total soluble solids (°Brix), and percent of berries showing red color at véraison in 2006 to 2008. Error bars indicate SD (n=4), letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



	2006		2007		2008	
Soluble solids	6.4°Ba***	10.7°Bb	8.6°Ba**	10.8°Bb	5.8°Ba**	10.0°Bb
% Color	< 1.0%a***	32.5%b	< 1.0%a***	40.0%b	< 1.0%a***	36.8%

Figure 3.3 Merlot berry mass, skin mass *per berry*, and total soluble solids (°Brix) at harvest in 2006 and 2007. Error bars indicate SD (n=4), letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

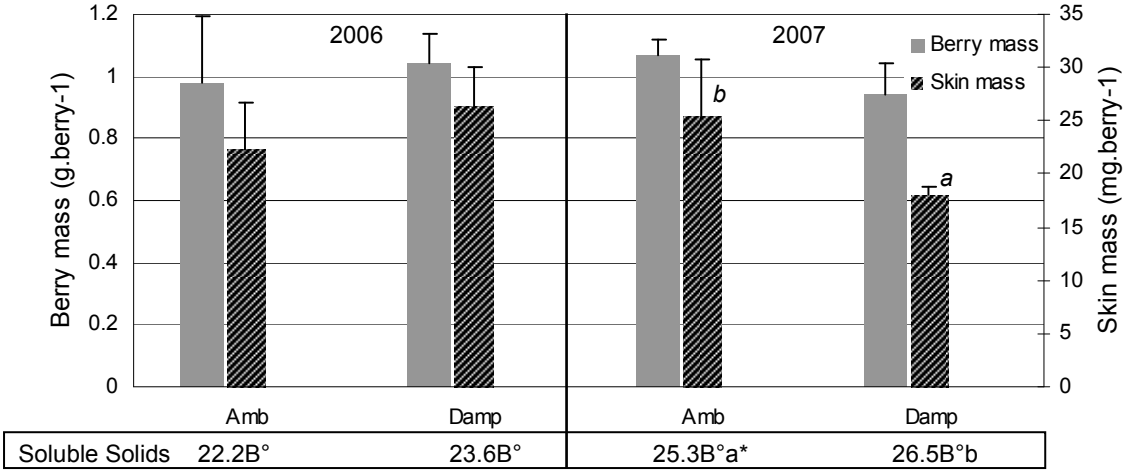
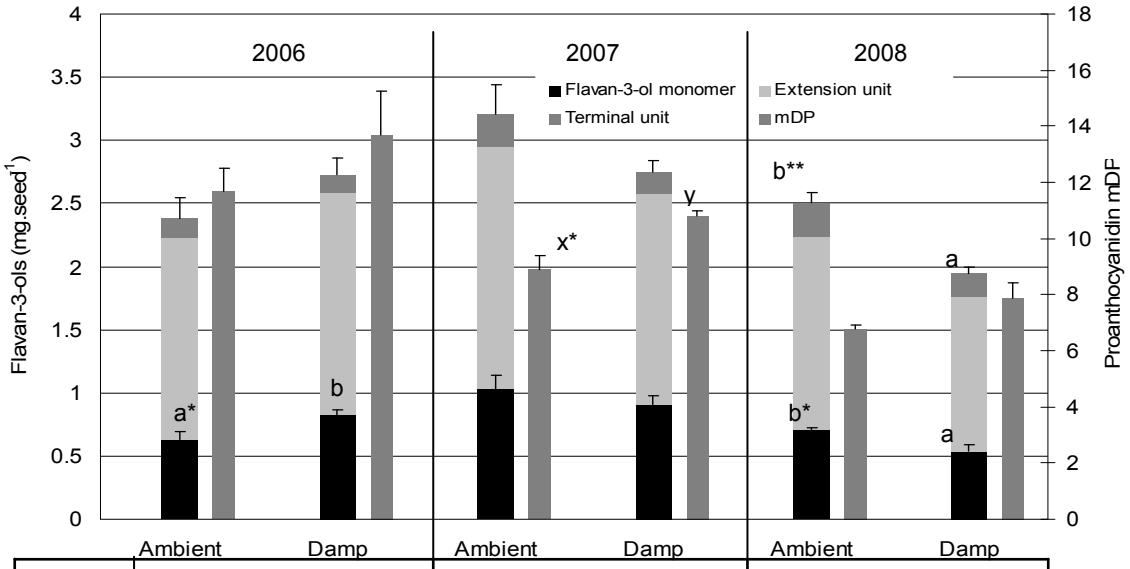
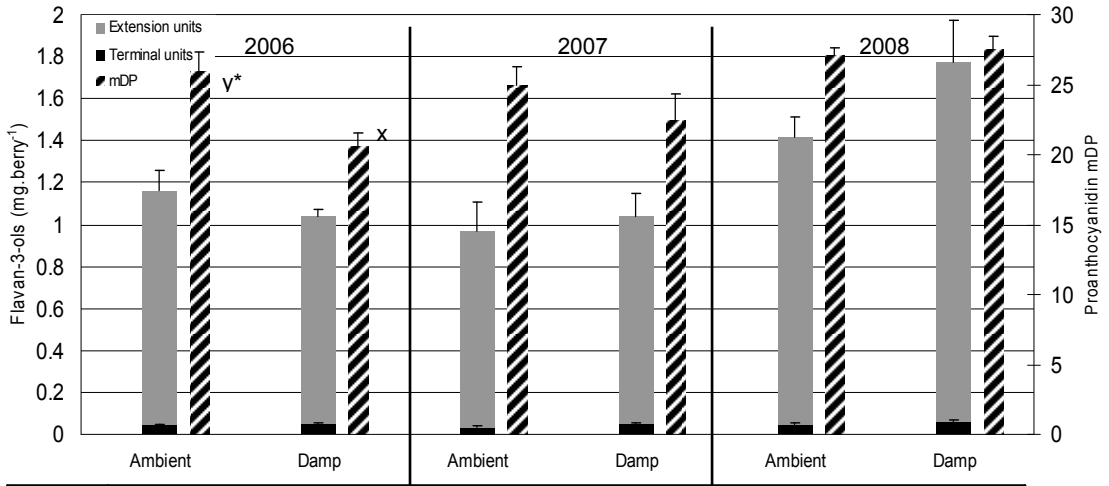


Figure 3.4. Flavan-3-ol content (*per seed)*, composition, and PA mDP in Merlot seeds at véraison in 2006 through 2008. Error bars represent SEM of total flavan-3-ol monomers, polymers, and PA mDP (n = 4). Mole percentages are shown for each subunit as a portion of total monomer, extension and terminal units (*m*, *x*, *t* respectively). Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



%ECG*t*	77.7	87.7	58.5	65.7	42.5a*	41.8a
%EC*t*	3.4	4.0	19.2	17.8	17.2b**	16.0b
%C*t*	18.9	8.3	22.4	16.5	40.2	42.2
%ECG*x*	21.5	20.6	13.4	13.3	14.6a*	15.9b
%EC*x*	70.1	68.4	77.2	76.7	73.3	71.6
%C*x*	6.9a***	9.1b	8.0	8.7	9.6	10.5
%EGC*x*	1.5	1.9	1.3	1.3	2.6	2.0
%C*m*	55.1a**	59.8b	58.0	56.0	53.7	51.7
%EC*m*	27.9	30.1	32.8a**	36.9b	31.4a**	41.3b
%ECG*m*	17.0b***	10.1a	9.2b**	7.1a	14.9b**	7.0a

Figure 3.5. Flavan-3-ol content (*per berry*), composition, and PA mDP in Merlot skins at véraison in 2006 through 2008. Error bars represent SEM of total flavan-3-ol extension and terminal units and PA mDP (n = 4). Mole percentages are shown for each subunit as a portion of total extension and terminal units (*x* and *t* respectively). Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



%EGC*x*	43.0	44.3	49.2	49.9	42.5	44.7
%C*x*	1.5	1.5	1.3	1.2	1.4	1.3
%EC*x*	53.6	52.6	47.4	46.8	54.4	52.4
%ECG*x*	1.9	1.6	2.2	2.1	1.6	1.6
%C*t*	78.2	84.6	90.0	91.4	89.3	90.7
%EC*t*	14.5	10.1	8.8	7.1	8.6	7.5
%ECG*t*	7.3	5.3	1.2	1.5	2.1	1.8

Figure 3.6. Flavonol content (*per berry*) and composition in Merlot skins at véraison in 2006 through 2008 in quercetin equivalence. Bars show the distribution of mono-, di-, and dri-hydroxylated flavonols (Mono-OH, Di-OH, Tri-OH respectively), error bars represent SEM of total flavonol content. Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

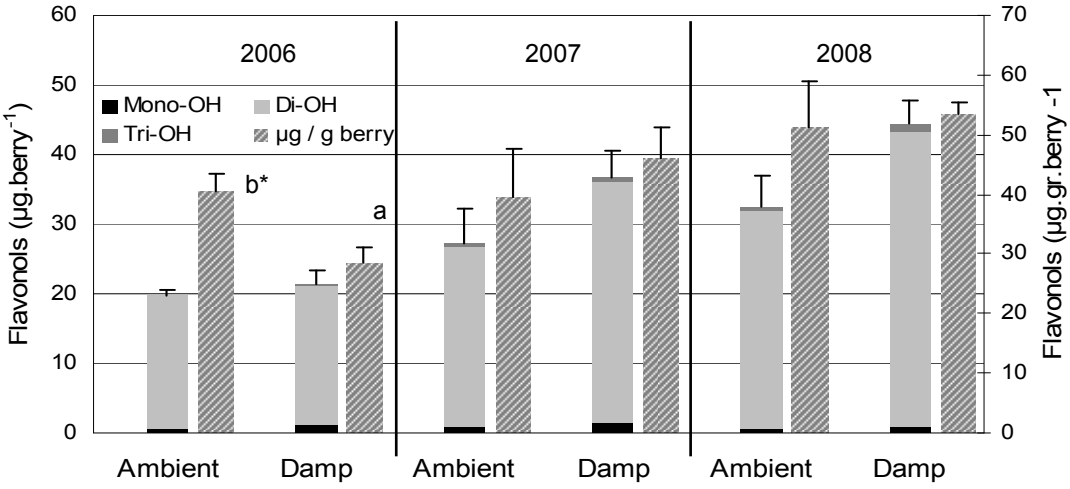
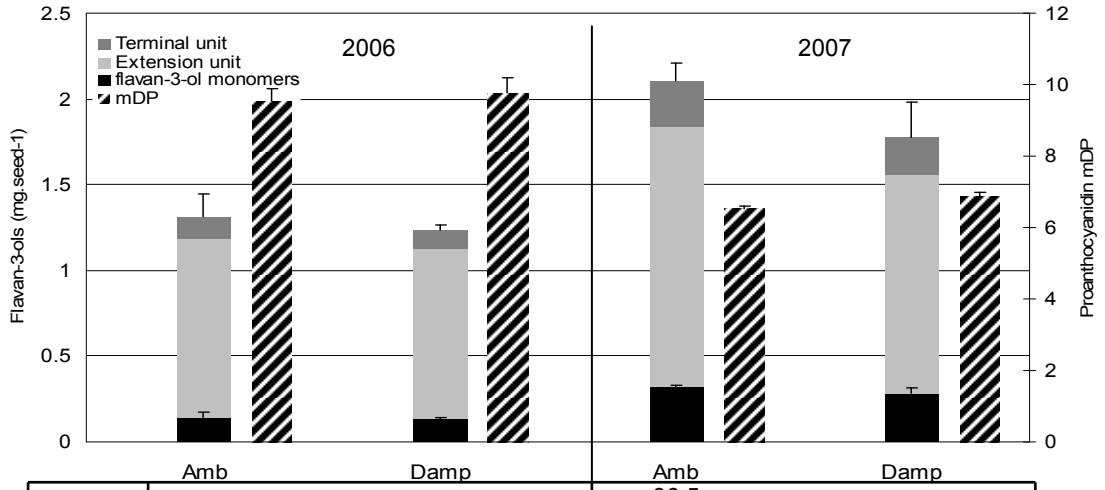
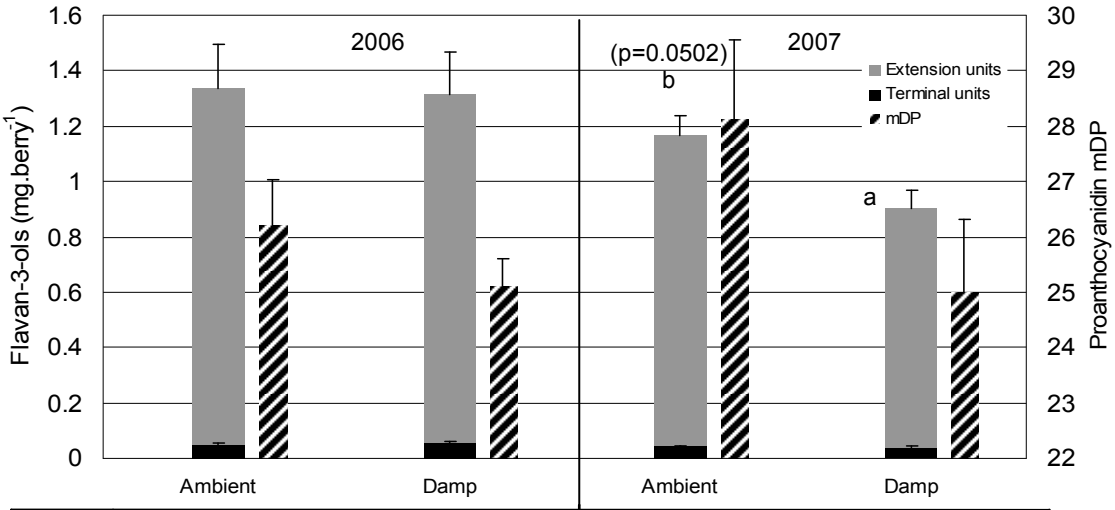


Figure 3.7. Flavan-3-ol content (*per seed*), composition, and PA mDP in Merlot seeds at harvest in 2006 and 2007. Error bars represent SEM of total flavan-3-ol monomers, polymers, and PA mDP (n = 4). Mole percentages are shown for each subunit as a portion of total monomer, extension and terminal units (*m*, *x*, *t* respectively). Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



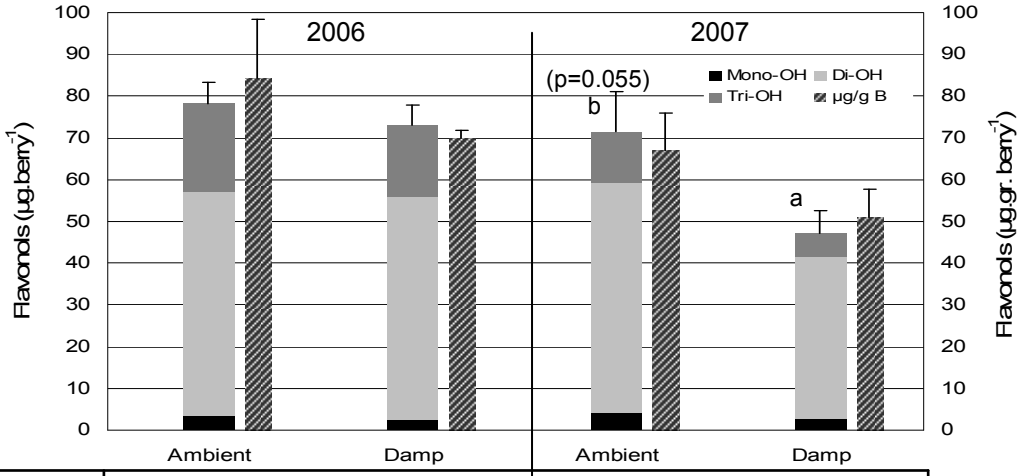
%C*t*	26.5	25.1	36.5	37.1
%EC*t*	17.8	18.1	25.9	24.7
%ECG*t*	55.7	56.8	37.5	38.3
%EGC	0.2*a**	2.9*b*	1.6	1.5
%C*x*	8.0	7.6	8.8	9.1
%EC*x*	69.6	68.0	76.0	75.6
%ECG	22.3	21.5	13.5	13.8
%C*m*	52.5	52.7	55.7	51.6
%EC*m*	37.8	40.2	38.1	41.8
%ECG*m*	9.7b*	7.1a	6.2	6.6

Figure 3.8. Flavan-3-ol content (*per berry*), composition, and PA mDP in Merlot skins at harvest in 2006 and 2007. Error bars represent SEM of total flavan-3-ol extension and terminal units and PA mDP (n = 4). Mole percentages are shown for each subunit as a portion of total extension and terminal units (*x* and *t* respectively). Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



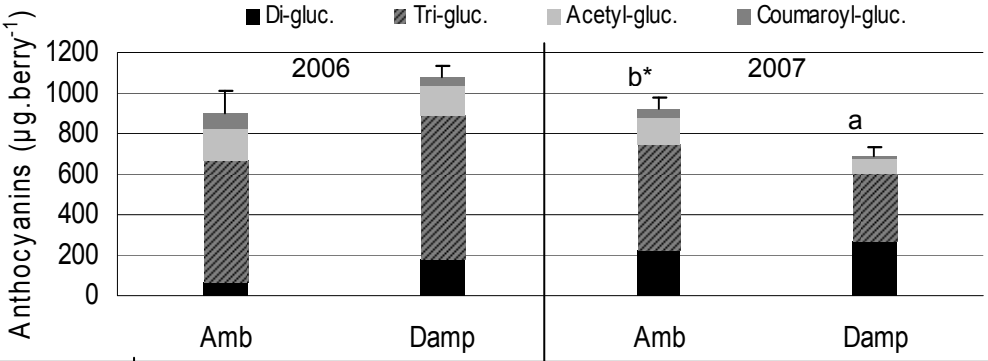
%EGC	42.8	44.6	49.0	45.6
%C*x*	1.4	1.4	1.4a*	1.7b
%EC*x*	54.4	52.8	47.6	49.9
%ECG	1.4	1.2	2.0a*	2.7b
%C*t*	89.0	84.6	76.5	77.8
%EC*t*	11.0	13.0	16.6	15.5
%ECG*t*	0.0	2.4	6.8	6.7

Figure 3.9. Flavonol content (*per berry*) and composition in Merlot skins at harvest in 2006 and 2007 in quercetin equivalence. Bars show the distribution of mono-, di-, and dri-hydroxylated flavonols (Mono-OH, Di-OH, Tri-OH respectively), error bars represent SEM of total flavonol content. Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



Mono-OH	4.6	3.7	5.7	5.3
Di-OH	67.5	72.6	77.1a*	82.3b
Tri-OH	27.8	23.8	17.3b*	12.3a

Figure 3.10. Anthocyanin content (*per berry*) and composition in Merlot skins at harvest in 2006 and 2007 in malvidin-3-glucoside equivalence. Bars and table show the distribution between di- and tri-hydroxylated, acetyl- and coumaroyl-glucoside forms of anthocyanins (Di-gluc., Tri-gluc., Acetyl-gluc., Coumaroyl-gluc respectively). Error bars represent SEM of total anthocyanin content. Letters denote differences between values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



%Di-OH	11.6a**	21.3b	30.1a***	44.1b
%Acetyl-gluc.	17.4b***	13.0a	14.6b***	10.5a
%Coumaroyl-gluc.	9.8b*	4.3a	4.7c***	2.1a

Chapter 4

General Conclusions

The objective of this research was to determine the specific effect of temperature on grape flavonoid accumulation. As discussed, there has been a considerable amount of research regarding the effects of solar radiation and temperature on anthocyanins whereas research on PA biosynthesis is more limited. Our goal was to contribute to this knowledge base in a practical manner as related to the production of wine grapes.

Previous research has shown a positive relationship between moderate temperatures and anthocyanin accumulation and the inhibitory effects of high temperatures. It has also been shown that the magnitude of diurnal temperature range can impact the accumulation of anthocyanins. In order to determine the relevance of these finding to PA biosynthesis we designed experiments to alter the day and night temperatures of field-grown grape berries. We were able to do this while maintaining a similar light environment around treated berries to isolate temperature as a variable.

Based on previous observations, we developed the hypothesis that PA accumulation would increase with accumulation of thermal units (DD). To test this we modified the total thermal time accumulation of berries by reducing daytime temperatures or increasing nighttime temperatures, shifting the mean daily temperature down and up respectively.

Our second hypothesis was that compressing the diurnal temperature range would increase PA accumulation by providing a more ‘optimal’ thermal environment. This experiment effectively reduced the variation between high and low temperatures

normally experienced without significantly altering the thermal accumulation. If PAs accumulate in response to thermal stress, one would expect the PA content of these berries to be reduced. However, if accumulation was limited by inhibitory high or low temperatures, berries grown under at reduced DTR should accumulate more PAs.. By conducting these two experiments we aimed to survey the effect of a range of temperatures and determine if a connection exists between thermal accumulation and PAs.

After the first season of experimentation (2006) we found a positive relationship between thermal time (DD) and skin PAs before véraison. Treatments after véraison showed minimal effect. This was somewhat expected as PAs are found to accumulate before véraison. For the second season (2007) we replicated the experiment and were able to impose an additional *full-season* study. The same experimental design was used; however treatments were maintained from initiation of Phase I to the end of Phase II (full maturity). Our objective was to determine if differences observed at véraison would translate to the end of berry ripening. Table 4.1 shows a summary of the data collected in 2007 at the end of Phase I, Phase II, and the full-season experiment.

The accumulation of PAs at véraison in 2007 showed no treatment effect (Chapter 2). The percent of (-)-epigallocatechin as an extension unit was slightly higher in cool berries when compared against ambient or heat only ($p < 0.05$). There were no differences in PAs during the Phase II or full-season experiment. As no differences were observed at véraison, and no effect has been observed in treatments

after véraison, it is not surprising that the full-season experiment showed no differences.

Anthocyanins and flavonols are not displayed for Phase I as they have been shown only to indicate rate of maturation. Total anthocyanins were lowest in damp and cool berries in the Phase II experiment but were not different in full-season. The reduction in anthocyanins under at lower daytime temperatures during Phase II is unexpected, however treatments applied after véraison may have briefly shunted berry development and the initial accumulation of anthocyanins, as seen with PAs in 2008. Yamane et al. (2006) reported considerable reductions due to heating and increases due to cooling when treatments were applied at véraison (Yamane et al., 2006). However, their treatments were transient (2 weeks) and in our study treatments were maintained through ripening. Heat was only added during the night thus the daily maximum temperature was not different from ambient. Further, heat and ambient berries accumulated more tri-substituted and acylated anthocyanins accounting for much of the difference between treatments.

Kitamura et al. (2005) reported the composition of anthocyanins in the hybrid variety Aki Queen to be similar to that of Merlot; however the proportion of tri-substituted anthocyanins is considerably less (≈25-30%) than Merlot in this study (> 50%). This may contribute to differences in data presented here and that for Aki Queen in the aforementioned study. Berries treated during the entire season would have been acclimated to treatments and would not have experienced a comparable shift in temperatures at véraison. Flavonol content was not influenced by treatment; however the proportion of tri-hydroxylated flavonols increased with temperatures in

both experiments and in Phase II of 2006. In general, treatments had little effect on PAs whether initiated before or after véraison in 2007.

Based on the results from 2006 and 2007, we focused on the development of PAs before véraison in 2008. Our objective was to understand discrepancies observed between the first two years, while providing a third consecutive year of data at véraison for all treatments. The results from this study were discussed in Chapter 2. It appears that the accumulation of PAs is tightly coordinated with the developmental stage of berries. Expression of genes related to PA biosynthesis confirms this observation. In 2008, reducing berry temperatures initially inhibited berry development including PA accumulation. After a period of acclimation, cool berries showed an increase in rate of development and PA content increased to the equivalent levels as heat and ambient.

We concluded that considerable shifts in temperature (±6 to8°C) do not significantly affect PA accumulation in grape berries. Across all years of the study we observed small shifts in the partitioning of PAs in skins, with low temperatures favoring tri-hydroxylation of flavan-3-ols in the berry skins. Seed PA accumulation was largely unaffected by temperature treatments as well, although higher temperatures consistently resulted in a higher proportion of gallate esterification of flavan-3-ols as determined in tea leaves (Yao et al., 2005).

Differences observed with regard to the rate of berry development have previously been associated with water stress, solar radiation and temperature. Harris et al. (1968) studied the development of grapes grown at two sites exhibiting comparable differences in temperature profiles to our heat and cool treatments.

Berries were initially smaller at the cooler site (twenty days after anthesis) yet by seventy days after anthesis berry weights were similar at the two sites. They determined that berries at the cool site had slightly lower cell numbers and larger cell volumes. This suggests that cell division was affected early in development and cell enlargement was affected later on. This is a comparable situation to that presented in our study. Hale and Buttrose (1974) also determined that berry growth was most sensitive to temperature before véraison. They succinctly conclude that the effect of temperature on development is dependent on the stage of development. This effect is likely related to the activity of hormones such as cytokinins and auxins, which regulate cell division and elongation (Abel and Theologis, 1996; Kende and Zeevaart, 1997; Stals and Inze, 2001). The temperature dependence of auxin-regulated growth has been shown in *Arabidopsis* and fits with our observations (Gray et al., 1998). The fact that cool berries reach a similar size and composition to that of ambient by véraison suggests some level of acclimation response.

All of the treatments in this study altered the rate of berry development. Although the exact mechanism or relationship between various growth hormones is unclear, this does have consequences for berry development. As berries initially develop at different rates, periods of high or low temperatures (relative to treatment) would coincide with different developmental stages. Berries grown under a reduced diurnal temperature range were more developed by our sampling dates at véraison indicative of a synergistic effect between lower day and higher night temperatures. This suggests they were maintained at more 'optimal' temperatures compared to other treatments. There was not a significant influence on PA accumulation by DTR

compression at any stage of development. It is unclear if the treatments affected the synchronicity of berry ripening within a cluster based on the data we collected. We did not survey individual berries of each experimental cluster to determine inter-cluster variability but this would be of interest in the future. From that data we collected, and visual assessment of clusters, overall hastening of berries was the most noted effect of temperature treatments.

The general conclusion from this study is that berry temperatures before véraison have a small influence on PA partitioning and minimal impact on total accumulation. There is an indication of a temperature effect relative to developmental stage of the berry but this requires additional investigation. Temperatures after véraison alter the partitioning of flavonols and anthocyanins but have little influence on PAs. Inhibitory high and low temperatures are not evident from this study and are still to be determined. It is apparent that grapes are capable of acclimating to long-term temperature shifts without deleterious consequences. The ability of grapes to adapt to transient temperature stress or temperature extremes remains a question.

Future Directions

Future research should address the combined effects of temperature, water and solar radiation. Solar radiation has demonstrated effects on flavonoid metabolism seen as differences in genetic regulation and chemical stability. Water stress has also been shown to alter flavonoid content depending on the timing and severity. As the three climate elements work in concert in nature, it would be valuable to understand combined effects. In scenarios where high temperature and drought stress are

present, availability of sunlight is not likely an issue. In contrast, areas (or seasons) of low temperature are often subject to more rainfall and cloud cover. In this last scenario, the threat of biotic stress (e.g. fungal infection) is often increased adding an additional layer of complexity.

To follow up the investigation of temperature and PA biosynthesis it would be appropriate to investigate higher ranges of temperatures that may be deleterious to berries. In this study, periods of high temperature (35°C) were common however temperatures above 40°C were rare. The effect of transient high and low temperatures should also be investigated to determine if there is a critical developmental stage for PA biosynthesis. Additionally, it would be useful to determine if there is any genetic basis for temperature response (i.e. cultivar sensitivity). This could be valuable in breeding programs and matching cultivars to region or site.

Results from this work have interesting implications with regard to berry development. It would be pertinent to investigate the interaction between temperatures, hormone content and berry development and composition. This would help clarify some of the mechanisms behind the berry ripening process and the regulation of flavonoid biosynthesis. For example: Would application of auxin to *cool* berries cause synchronization with ambient or *heat* berries?

The data presented here may also reflect differences in wine styles based on growing regions or areas of production. This is evident in the many varieties of wine that are produced from different geographic areas. As an example, Cabernet Sauvignon produced in Bordeaux, France, is generally distinct from that produced in

other regions throughout California, Australia, and New Zealand. The types of temperature variations encountered in this study represent the range of differences one may see comparing the climate in these various regions. There is interest in understanding how much of the stylistic differences are related to production methodology versus those related to berry composition at harvest. It is acknowledged that with variations in temperatures at different geographic locations come variations in terroir, precipitation and humidity and light quantity and quality among other environmental factors. This further stresses the need to understand the contribution of each climate factor to grape berry composition and resulting wine production. Areas with compressed DTRs may ripen at the same apparent rate as areas with warmer days, for example, but it would appear that the composition of PAs and other flavonoids would be effected, though not necessarily the total content. This would be an interesting topic to investigate and could be accomplished by distributing a set of potted vines throughout a region based on typical differences in daily temperature profiles.

From a fundamental standpoint, it would be interesting to investigate some of the gene families, such as DFR, within *V. vinifera*. An initial probing of the NCBI database provided a number of 'DFR-type' genes based on amino acid homology. Based on previous work with DFR, it would be interesting to characterize some of these to determine functionality and specificity. A screening of 12 of these genes found that at least 6 were expressed at moderate levels during development of Merlot berries and showed the same general expression pattern as the DFR gene assayed in

Chapter 2. These have not been identified or characterized but it would be interesting to pursue this line of research.

Table 4.1 Summary of skin flavonoids in Merlot berries at véraison and harvest in 2007. Berries from Phase I, Phase II, and full-season experiment. Metabolite content *per berry* (B), percentage of Epigallocatechin as extension unit (EGCx), tri-hydroxylated flavonoids (Tri-OH), and acetyl- and coumaroyl-glucosides of anthocyanins (Acetyl-, Coum- respectively).

		Proanthocyanidin			Anthocyanins				Flavonols	
Treatment		mg/berry	%EGCx	mDP	µg/B	%Tri-OH	Acety-	Coum-	µg/B	%Tri-OH
Phase I	Ambient	0.96	49.2	24.9	[a]-	-	-	-	-	-
	Blower	1.18	52.4	29.0	-	-	-	-	-	-
	Cool	1.20	53.7	26.0	-	-	-	-	-	-
	Heat	1.11	49.2	27.0	-	-	-	-	-	-
	Damp	1.03	49.9	22.4	-	-	-	-	-	-
	[b]*p-value*	*nd*	*nd*	*nd*						
Phase II	Ambient	1.17	49	28.14	[c]928.5b	69.9b	14.6b	4.7c	71.4	17.3c
	Blower	0.94	46.7	24.57	888.1b	70.2b	14.6b	3.7b	66.2	16.1bc
	Cool	0.91	46.3	24.32	685.9a	60.9a	11.4a	2.4a	51.5	10.7a
	Heat	1.03	48.5	26.02	801.6ab	68.4b	13.8b	5.4c	60.9	16.2bbc
	Damp	0.90	45.6	25.00	693.3a	55.9a	10.5a	2.1a	47.2	12.3ab
	p-value	*nd*	*nd*	*nd*	*0.02*	*0.001*	*<0.001*	*<.001*	*nd*	*0.015*
Full Season	Ambient	1.34	50.3	32.55	679.7	77.9b	15.2b	6.6b	61.7	26.6c
	Blower	1.56	51.3	32.54	701.8	75.8b	15.6b	5.5b	58.2	25.4bc
	Cool	1.36	53.4	33.22	800.1	65.4a	10.5a	3.8a	68.5	19.1a
	Heat	1.20	48.6	31.99	601.2	74.3b	14.7b	6.5b	51.0	25.7c
	Damp	1.38	51.2	33.10	801.8	63.6a	11.4a	5.2ab	59.5	21.6ab
	p-value	*nd*	*nd*	*nd*	*nd*	*<.001*	*<.001*	*.03(kw)*	*nd*	*0.004*

[a]Phase I anthocyanins and Flavonols not included.
[b]ANOVA p-values italics; (kw) denotes Kruskal-Wallis p-value.
[c]Values with same letters within week are not different; LSD, α=0.05.

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Appendix

Table A.1 Berry mass, berry volume, skin mass, and percent coloration of Merlot grapes at véraison from 2006 to 2008.

Year	Treatment	Berry Mass (g/berry)	Berry Volume (cm^3/berry)	Skin Mass (mg/berry)	Coloration (%red)
2006	Ambient	[a] 0.50a,x	0.507x	11.97	0.5
	Blower	0.518ab	0.498	11.10	1.0
	Cool	0.597b	0.613	12.44	2.3
	Heat	0.607b	0.610	12.53	4.0
	p-value	*0.051*[b]	*ns*	*ns*	*ns*
2007	Ambient	0.690ab,y	0.700y	11.96	0.0a
	Blower	0.601a	0.601	10.89	2.0ab
	Cool	0.764b	0.749	12.60	16.0b
	Heat	0.687ab	0.701	11.55	12.5b
	p-value	*0.069*	*ns*	*ns*	*0.049*
2008	Ambient	0.635y	0.637y	13.22	0.25a
	Blower	0.602	0.600	11.25	0.0a
	Cool	0.682	0.685	11.34	3.5a
	Heat	0.683	0.680	11.99	21.25b
	p-value	*ns*	*ns*	*ns*	*0.020*[c]

[a] Values with same letters within grouping are not different. Letters x, and y separate Ambient berries across years ($P<0.05$); LSD, $\alpha=0.05$.
[b] ANOVA p-value in italics unless otherwise noted.
[c] Kruskal-Wallis p-value

Berry soluble solids and organic acid analysis

Berry soluble solids and acid content (Table A.2) was analyzed to provide additional information regarding berry development and accumulation of primary metabolites. Briefly, berry juice samples were prepared as described in Chapter 3 for soluble solids analysis. To remove phenolics, 0.1g of insoluble polyvinylpyrrolidone was added per 1ml of juice sample and centrifuged at 16,000g for 15min to remove solids (Keller et al., 1998). Samples were diluted in ultra-pure water to achieve appropriate concentrations for HPLC analysis. Organic acids were separated on two PL Hi-plex H columns in series protected by a guard column of the same material (Varian, Shropshire, UK) at 60°C, using 0.005M H_2SO_4 as per the manufacturer's recommendation. Acids were monitored at 210nm and quantified based on the peak areas of analytical standards (D-(+)-Tartartic and L-Malic acid; Sigma Aldrich, St. Louis, MO).

Table A.2 Total soluble solids, tartrate, and malate content (g/100ml) of juice from Merlot berries sampled at véraison (phase I) and harvest (phase II).

		Treatment	Soluble Solids	Tartrate	Malate	% Malate
Phase I	2006	Ambient	[a]6.4 ± 0.4a	1.7 ± .13ab	1.3 ± .14ab	0.42a
		Blower	6.7 ± 1.1a	2.0 ± .2b	1.4 ± .12b	0.41a
		Cool	7.0 ± 0.4a	1.9 ± .17b	1.7 ± .19c	0.48b
		Heat	7.5 ± 1.3a	1.8 ± .1ab	1.3 ± .2ab	0.41a
		Damp	10.7 ± 0.5b	1.6 ± .07a	1.1 ± .11a	0.41a
		[b]*p-value*	*<0.001*	*0.03*	*0.001*	*0.018*
	2007	Ambient	8.6 ± 1.6a	1.6 ± .16	1.3 ± .12	0.45a
		Blower	7.6 ± 0.8a	1.4 ± .26	1.2 ± .25	0.46a
		Cool	9.1 ± 1.7ab	1.2 ± .19	1.3 ± .36	0.51b
		Heat	9.1 ± 1.3ab	1.5 ± .13	1.2 ± .14	0.45a
		Damp	10.8 ± 0.5b	1.3 ± .08	1.3 ± .08	0.49ab
		p-value	*0.04*	*ns*	*ns*	*0.045*
	2008	Ambient	5.8 ± 0.3a	1.6 ± .15c	1.4 ± .14	0.46
		Blower	6.0 ± 0.7a	1.5 ± .12bc	1.4 ± .03	0.48
		Cool	6.6 ± 1.2a	1.4 ± .02ab	1.5 ± .13	0.52
		Heat	9.7 ± 2.9b	1.4 ± .1ab	1.1 ± .34	0.43
		Damp	10.0 ± 2.9b	1.3 ± .11a	1.2 ± .28	0.47
		p-value	*0.01*	*0.01*	*ns*	*ns*
Phase II	2006	Ambient	20.9 ± 3.9	1.0 ± .09	0.13 ± .02	0.11
		Blower	23.2 ± 2.2	1.0 ± .02	0.13 ± .03	0.11
		Cool	22.8 ± 0.5	0.98 ± .09	0.17 ± .03	0.15
		Heat	24.2 ± 0.5	1.0 ± .02	0.18 ± .05	0.15
		Damp	23.6 ± 1.2	0.96 ± .03	0.17 ± .03	0.15
		p-value	*ns*	*ns*	*ns*	*ns*
	2007	Ambient	25.3 ± 0.5a	1.1 ± .01	0.15 ± .02a	0.12a
		Blower	25.1 ± 0.8a	1.0 ± .03	0.17 ± .02ab	0.15a
		Cool	25.9 ± 0.6ab	1.0 ± .06	0.23 ± .02c	0.18b
		Heat	26.4 ± 0.6b	1.1 ± .05	0.16 ± .02ab	0.13a
		Damp	26.5 ± 0.2b	1.1 ± .09	0.19 ± .02b	0.15a
		p-value	*0.01*	*ns*	*0.002*	*<.001*

[a]Values (± std. dev.) with same letters within week are not different; LSD, α=0.05.
[b]ANOVA p-values italics.