Impact of Temperature Regimes on Flavonoid Accumulation in Merlot (*Vitis vinifera* L.) Grapes

by

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in Plant Science

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Abstract

Flavonoids are an important class of plant secondary metabolites, which impart color and mouthfeel to grapes and wine. Flavonoid accumulation is genetically and environmentally controlled in grapes. Among the environmental factors, temperature is arguably the one that most affects flavonoid accumulation in grape berry. High temperatures (e.g., 30-35 °C) were shown in previous reports to impair anthocyanin accumulation in red grape varieties. Most of the studies compared only two or three temperature regimes and have never investigated the effect of the difference between day and night temperatures on flavonoid accumulation. This study employed controlled-environment (growth chamber) experiments to investigate the effect of temperature regimes and the difference between day and night temperatures on flavonoid accumulation in grapes, with an emphasis on anthocyanins and flavonols. Five temperature regimes were imposed on potted Merlot grapevines during berry ripening in each experiment (day/night, °C: 20/10, 20/15, 25/15, 35/25, 35/30 in Experiment 1; 20/10, 20/15, 25/15, 25/20, 30/20 in Experiment 2). In both experiments, high temperature regimes (i.e., 30/20, 35/25, and 35/30) decreased anthocyanin and flavonol concentrations and increased the relative concentration of 3'4'5'-substituted and methoxylated anthocyanins and flavonols as well as acylated anthocyanins, with comparison to low temperature regimes (i.e., 20/10 and 20/15). The difference between day and night temperatures exerted minor effect when regimes with the same day and different night temperatures were compared. When grapevines were subjected to the same night temperature, a larger difference between day and night temperatures (ΔT = 10 °C, e.g., 25/15) reduced the anthocyanin and flavonol level, with comparison to a smaller temperature difference (ΔT = 5 °C, e.g., 20/15). Gene expression analyses revealed that *VviF3'H* and *VviF3'5'Hs* integrated for the regulation of 3'4'5'-substituted anthocyanin accumulation and *Vvi3AT* controlled the level of

anthocyanin acylation; the down-regulation *VviFLS4* and *VviMybF1* expression in high temperature regimes led to the corresponding reduction of flavonol accumulation. These results indicate that modulation of anthocyanin and flavonol accumulation occurs partially at the transcriptional level. Finally, this study suggests that day temperature plays a more important role than night temperature in determining the concentration and composition of anthocyanins and flavonols in grape berry.

Lay Summary

Flavonoids are an important class of compounds and include anthocyanins, flavonols, and tannins. These compounds contribute to grape and wine color, flavor, and texture. Climate affects the accumulation of these compounds in the grape berry and thus, the quality of wine. This study investigates how temperature affects the accumulation of flavonoids in grapes. It was observed that high temperatures greatly reduce the level of grape anthocyanins (red pigments) and flavonols, indicating that wines produced in hot climates will have lower pigmentation. The study also revealed that night temperatures have less of an effect than day temperatures in modulating the level of these compounds.

Preface

This research was conducted at The University of British Columbia, Vancouver. Grapevines were grown in the UBC Horticulture Greenhouse and the Forestry Growth Chamber Facility. Support in growing the plants and setting up the growth chambers was provided by the staff of the two facilities (e.g., Melina Biron, Jimmy Sung, and Pia Smets).

Grape sample preparation (peeling, de-seeding, grinding) was partially performed by Nolwenn Paugam (Intern in the UBC Wine Research Centre from University of La Salle, France).

The HPLC/MS method used for anthocyanin and flavonol analyses was developed by Lufiani Lina Madilao (the UBC Wine Research Centre).

All other work presented in this thesis represents original, unpublished and independent work of the author, Yifan Yan.

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List of Abbreviations

ABA	abscisic acid
AM3	anthoMATE transporter
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ANR	anthocyanidin reductase
AOMT	anthocyanin 3-O-methyltransferase
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
CHI	chalcone isomerase
CHS	chalcone synthase
Cy	cyanidin
C4H	4-coumarate CoA ligase
DAA	days after anthesis
DFR	dihydroflavonol 4-reductase
Dp	delphinidin
ESI	electrospray ionization
FLS	flavonol synthase
FW	fresh weight
F3H	flavanone 3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
F3'5'H	flavonoid 3'5'-hydroxylase
GST4	glutathione S-transferase
HPLC/MS	high performance liquid chromatography/mass spectrometry
I	isorhamnetin
IPCC	International Panel of Climate Change
K	kaempferol
L	laricitrin
LAR	leucoanthocyanidin reductase
LDOX	leucocyanidin dioxygenase
LSD test	least significant difference test

M	myricetin
Mv	malvidin
PAL	phenylalanine ammonia lyase
PAR	photosynthetically active radiation
Pe	peonidin
PHA	phloroglucinol aldehyde
Pl	pelargonidin
Pt	petunidin
Q	quercetin
qRT-PCR	quantitative real time polymerase chain reaction
RH	relative humidity
S	syringetin
TA	titratable acidity
TF	transcription factor
TSS	total soluble solids
UFGT	UDP-glucose: flavonoid 3-O-glucosyltransferase
VOC	volatile organic compound
WDR	WD40 Repeat (beta-transduction repeat)
2-CEPA	2-chloroethylphosphonic acid
3AT	3-O-glucoside-6"-acyltransferase

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Thanks to my collaborator, Lina Madilao, who aided me with processing HPLC/MS samples. Thanks to Melina Biron for the help in keeping the plants alive in the greenhouse, and to Pia Smets for teaching me setting up the Conviron growth chamber to conduct the experiment.

Thanks to the China Scholarship Council (CSC) and National Sciences and Engineering Research Council (NSERC) for the financial support during the course of the project.

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Last but not least, I would like to thank my family, my parents Xinfa Yan and Huiqing Zhu, my grandparents and my brother, for always being by my side psychologically, and for all your continued support and understanding.

Chapter 1: Introduction

Section 1.1 The British Columbia wine industry

Grapevine is one of the most economically important fruit crops worldwide; and wine consumption is part of the culture of many countries. High-quality grape production is a prerequisite for premium wine production. In British Columbia (BC), grape production occurs in several viticultural regions: the Okanagan Valley, the Similkameen Valley, the Fraser Valley, Vancouver Island, Gulf Islands, Thompson Valley, Shuswap, Lillooet, and Kootenays, with the Okanagan Valley being the largest wine region in BC and the second largest in Canada (https://winebc.com/discover-bc-wine-country/). There are more than 80 grape varieties planted in BC; the major red varieties are Merlot, Pinot noir, Cabernet sauvignon, Syrah, and the major white varieties are Pinot gris, Chardonnay, Gewürztraminer, and Riesling.

According to BC Wine Grape Council Annual Crop Assessment (2018), Merlot was the most widely cultivated red grape variety in BC by tonnage, which accounted for 37.30% of total red varieties (7,957.5 tons). It is also the second most cultivated variety worldwide (Focus OIV, 2017) with 266,000 hectares (ha). The early ripening feature of Merlot grapes has made this variety popular in many wine regions. Merlot grapes are used in blends with other red grape varieties as well as for varietal wines.

Section 1.2 Grape biology and berry ripening

Grape berry growth follows a double-sigmoid pattern where two rapid phases of growth are interrupted by a lag phase during which there is little or no growth in berry size (Matthews et al. 1987a; Robinson and Davies 2000). The first growth phase (Stage I) begins at flowering (i.e., anthesis) and continues until the lag phase (Stage II); the transition from Stage II to the final growth phase (Stage III) is known as veraison, which is the onset of ripening. During Stage I, grape berries increase their

size through both cell division and cell expansion; while in Stage III, berry size increases due only to the cell expansion (Coombe 1976). In viticulture, veraison is regarded as a critical point because, in addition to the resumption of berry growth, numerous ripening processes begin, including berry softening, rapid sugar accumulation and organic acid degradation, and pigmentation in red grape varieties (Castellarin et al. 2016).

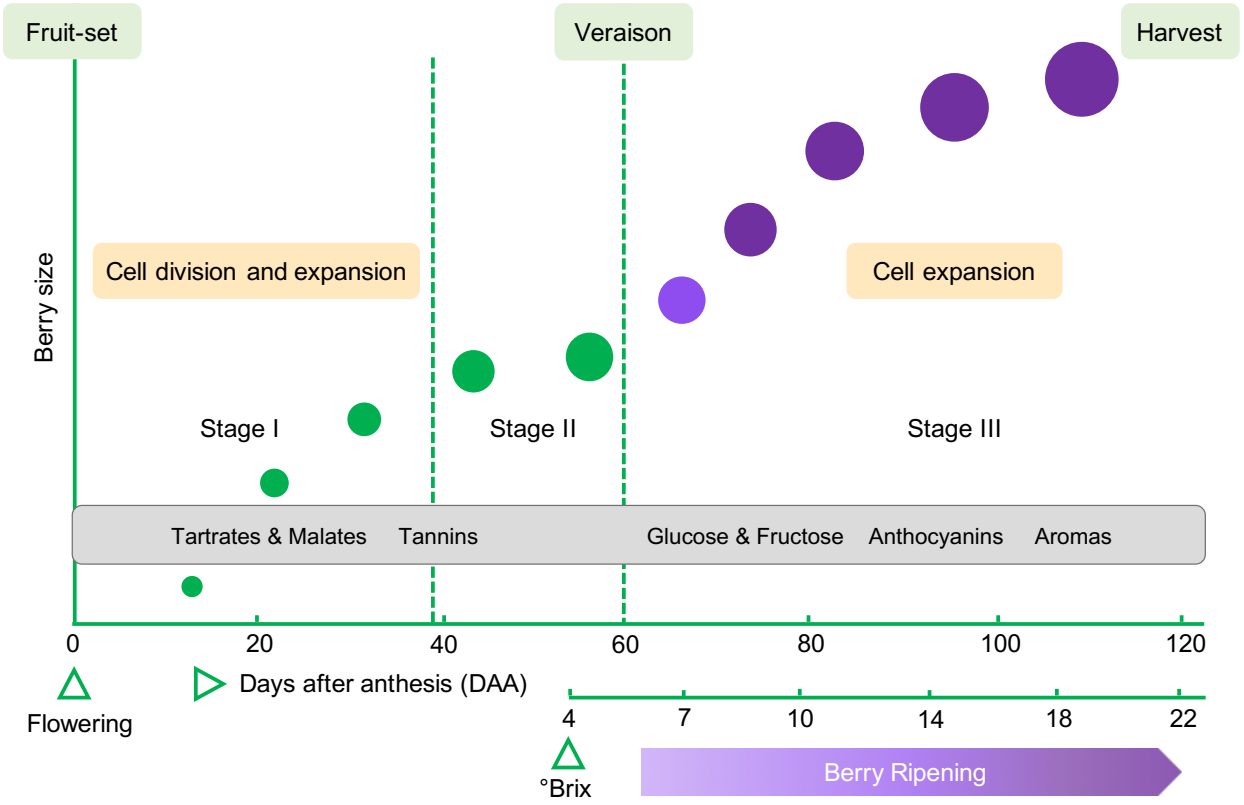


Figure 1.1 Diagram of berry development from berry set to harvest. Relative berry size and color are shown at 10-day intervals after flowering, passing through major developmental events (rounded boxes). Periods of metabolite accumulation and levels of juice total soluble solids (TSS, °Brix) are shown.

Fruit ripening is a complex process which involves many physiological and biochemical processes. On the basis of the ripening behavior, fruits can be classified as climacteric and non-climacteric fruits (Adams-Phillips et al. 2004; Giovannoni 2004). Grapes are non-climacteric fruits,

since there is no burst in respiration rate at the onset of ripening, nor of ethylene accumulation (Coombe and Hale 1973).

Ripening is particularly important for determining grape and wine quality and has major implications for the economic value of the grapes. The initiation of ripening in grapes is accompanied by an increase of the endogenous abscisic acid (ABA) concentration (Deluc et al. 2007; Wheeler et al. 2009; Gambetta et al. 2010; Pilati et al. 2017).

During the ripening stage, a wide variety of metabolites are accumulated in grapes, such as sugars, anthocyanins and flavonols, stilbenoids, and volatile organic compounds (VOCs) including monoterpenes, sesquiterpenes, norisoprenoids, thiols, *etc*. (Adams 2006; Teixeira et al. 2013; Robinson et al. 2014a, b). Sugars play an important role in shaping berry sensory properties, in determining alcohol concentration after fermentation, and as precursors for the synthesis of organic acids, phenolic compounds, and aroma compounds (Dai et al. 2011). *Vitis vinifera* grapes produce almost equal amounts of glucose and fructose during ripening as predominant sugars, together with trace amounts of sucrose (Hawker et al. 1976; Liu et al. 2006; Shiraishi et al. 2010). Anthocyanins, and flavonols are the two major classes of flavonoid compounds synthesized in grapes, and their role in grape and wine will be more elaborately narrated in the next section. Stilbenoids, that include resveratrol, are mostly produced from veraison onward (Gatto et al. 2008). They are phytoalexins and play a role in protection of grapes from wounding and pathogen attack (Downey et al. 2006; Flamini et al. 2013). VOCs determine the aroma of grapes, juice, and wine, particularly when chemical changes are associated with acid and enzymatic modifications of conjugated precursors occur during fermentation and wine aging (González-Barreiro et al. 2015).

Many key compounds for fruit and wine quality are synthesized before veraison and normally exhibit a decrease in their concentration during the ripening period. This is the case for organic acids, proanthocyanidins, hydroxycinnamates, and methoxypyrazines. Tartaric acid and

malic acid are the two major organic acids accumulated in grapes (Kliewer 1966; Kliewer et al. 1967; Shiraishi et al. 2010), which strongly affect the pH of grape juice and wine and contribute to berry quality and wine longevity (Dai et al. 2011). During the ripening stage, the content of tartaric acid remains stable, but the concentration decreases through a dilution effect due to cell expansion (Dai et al. 2011; Regalado et al. 2013). In contrast to tartaric acid, malic acid decreases in both concentration and contents, due both to active metabolization and cell expansion (Sweetman et al. 2009). Proanthocyanidins (also known as tannins) are another important class of flavonoid compounds in grapes and will be discussed in more detail in the next section. Hydroxycinnamic acids, such as *p*-coumaric, caffeic, and ferulic acid are major phenolic acids, which confer bitter and astringent mouthfeel to grape juice and wine (Teixeira et al. 2013). Finally, methoxypyrazines impart the sensory characteristics of bell pepper, asparagus, or pea to certain grapes and wine. These aromas can be perceived as desirable or undesirable depending on grape variety and wine style (Robinson et al. 2014a, b), as well as the concentration of these compounds.

The compounds described above contribute to grape and wine quality features, such as color (Castellarin and Di Gaspero, 2007), flavour (Adams 2006; Robinson et al. 2014b), texture, as well as the antioxidant properties (Teixeira et al. 2013).

Section 1.3 Flavonoids composition and accumulation in grapes

Flavonoids (**Figure 1.2**) are important phenolic compounds in plants, and include flavones, flavanones, flavonols, flavan-3-ols, proanthocyanidins, and anthocyanins. Anthocyanins, flavonols, and proanthocyanidins are the three major classes of flavonoids produced in grapes (Downey et al. 2006). As mentioned in **Section 1.2**, different categories of flavonoids are

synthesized at different developmental stages during grape berry development and are accumulated in different tissues of the grapes.

Section 1.3.1 Major flavonoid compounds in grapes

Anthocyanins are the pigments that confer the color to red grapes and wine. Anthocyanins act as attractors for seed dispersal in grapes (Saito and Harborne 1992; Koes et al. 1994; Shirley 1996; Downey et al. 2006). Anthocyanins are normally synthesized in the skin of red grape varieties, with the exception of the "teinturier" varieties, such as Alicante Bouschet, that accumulate anthocyanins also in their flesh (Castellarin et al. 2011; Falginella et al. 2012). The biosynthesis of anthocyanins commences at veraison and peaks at harvest or close to harvest.

Flavonols are generally considered to act as UV protectants and free-radical scavengers in grapes (Flint et al. 1985; Smith and Markham 1998; Downey et al. 2003; all in Downey et al. 2006). They affect red wine color by non-covalent combination with anthocyanins through a co-pigmentation effect (Oren-Shamir 2009), while directly affecting white wine color (Makris et al. 2006). Flavonols are mostly found in grape skins. Their accumulation initiates at flowering, then the concentration decreases or remains stable during early berry development and increases again from veraison onward (Downey et al. 2003; Castillo-Muñoz et al. 2007; Flamini et al. 2013).

Flavan-3-ols and their polymeric forms, proanthocyanidins, determine sensory and gustatory properties, such as bitterness and astringency in grape seeds and skins, and wine. Due to their bitter and astringent mouthfeel, flavan-3-ols and proanthocyanidins are considered to be feeding deterrents to herbivorous animals in grapes (Downey et al. 2006). In addition, they also improve color stability in wine by forming polymeric complexes with anthocyanins (Timberlake and Bridle 1976; Gawel 1998; Malien-Aubert et al. 2002; Mateus et al. 2002; Vidal et al. 2002).

Proanthocyanidins are found in both skin and seeds. Seed proanthocyanidins are synthesized immediately after fruit set and reach a maximum concentration at around veraison. Skin proanthocyanidin accumulation starts at berry set and continues until 1-2 weeks after veraison (Kennedy et al. 2001; Downey et al. 2003; Bogs et al. 2005).

All the flavonoids described above are antioxidants and have health benefits (Fukumoto and Mazza, 2000), such as cardiovascular disease prevention, obesity control, and diabetes alleviation (Pojer et al. 2013).

Section 1.3.2 Anthocyanin and flavonol types in grapes

Glycosylated pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin are the six most ubiquitous anthocyanin species in plants (**Figure 1.3**, Mazza and Miniati 1993). These anthocyanins vary in color from orange/red to blue. The profiles of anthocyanins vary among plant species or varieties within a species; indicating that the anthocyanin profile is strongly determined by genetic background of plants. For example, cyanidin and delphinidin based anthocyanins are the major anthocyanins in black currant (*Ribes nigrum*), pelargonidin based anthocyanins are the major anthocyanins in strawberry (*Fragariaananassa* cv. Jonsok) (Nyman and Kumpulainen 2001), while bilberry (*Vaccinium myrtillus*) and grape (*Vitis vinifera L.*) lack pelargonidin (Jaakola et al. 2002; Ageorges et al. 2006; Revilla et al. 2009). Even within the same plant species, for example, in *Vitis vinifera* grapes, the composition and distribution of anthocyanins differ greatly among varieties (Castellarin and Di Gaspero 2007).

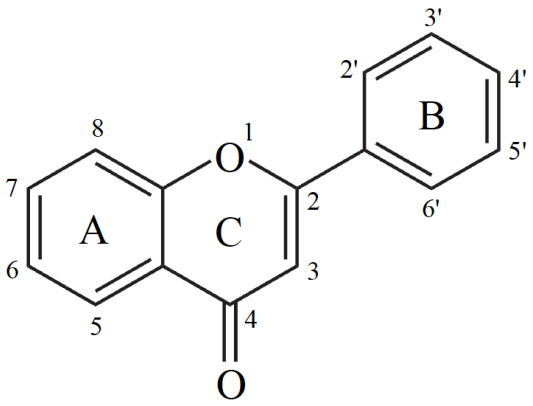


Figure 1.2 Chemical structure of a generic flavonoid (flavone). Letter A, B, C correspond to A-ring, B-ring, and C-ring. Substitution by different moieties at different positions of the C-ring results in the generation of different flavonoid subfamilies (e.g., anthocyanidins, flavonols, flavan-3-ols). Substitution at the B-ring results in different compounds within the same subfamily (pelargonidin, cyanidin, delphinidin, *etc*. in anthocyanidin subfamily or kaempferol, quercetin, myricetin, *etc*. in flavonol subfamily).

In grapes, anthocyanins are conjugated with sugar moieties. Anthocyanin glycosylation with sugars increases their stability (Tanaka et al. 2008). Anthocyanin 3-O-monoglucosides, 3-O-(6''-acetyl)glucosides, 3-O-(6''-caffeyl)glucosides, and 3-O-(6''-*p*-coumaroyl)glucosides are the most common glycosylated forms in *Vitis vinifera* grapes (Mazza and Miniati, 1993). Moreover, anthocyanin 3,5-O-diglucosides are present in American grapes or American hybrids such as Muscadine (*Vitis rotundifolia*) and Concord (*Vitis labrusca*) grapes and their corresponding juice and wine (Wang et al. 2003; Wu and Prior 2005; Huang et al. 2009).

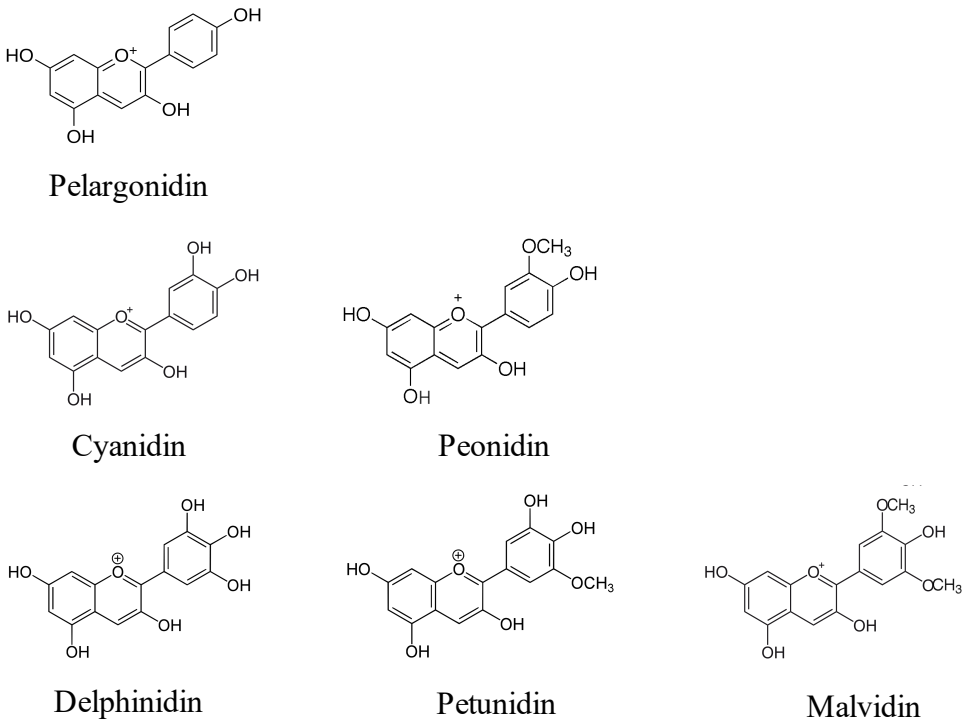


Figure 1.3 Chemical structure of the six most common anthocyanidins produced by plants. These anthocyanidins are normally accumulated in plant tissues as glycosylated forms.

Flavonols constitute another important subfamily of flavonoid compounds whose colors vary from colorless to yellow (Makris et al. 2006). The color is completely masked by anthocyanins in red grapes. In grapes, flavonols exist only as glycosylated forms, with glucose, galactose, and glucuronic acid as major sugar moieties. However, the corresponding aglycones can be found in wines, together with the glycosides, as a result of acid hydrolysis (Castillo-Muñoz et al. 2007). In red grapes, flavonols are present as glycosylated kaempferol, quercetin, myricetin, isorhamnetin, laricitrin, and syringetin, while in white grapes, the major flavonols are glycosylated quercetin, kaempferol, and isorhamnetin (**Figure 1.4**).

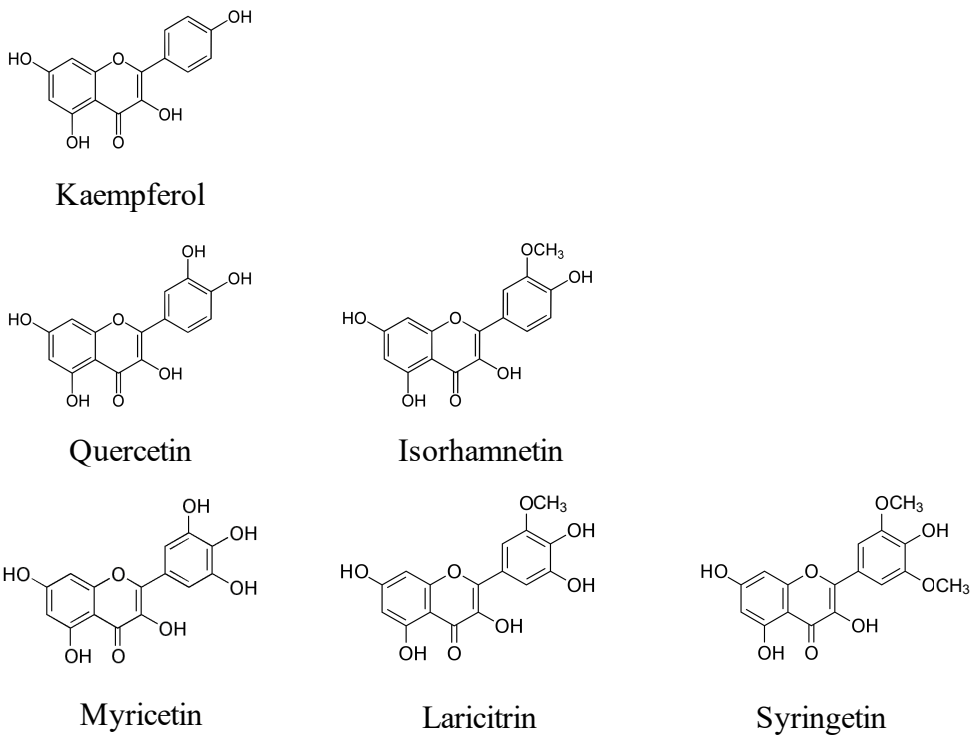


Figure 1.4 Chemical structure of the six most common flavonols produced by plants. These flavonols are normally accumulated in plant tissues as glycosylated forms.

The various anthocyanin and flavonol species are classified into different subfamilies based on different criteria. According to the number of hydroxyl groups on the B-ring (**Figure 1.2)**, there are 4'-substituted anthocyanins (also known as mono-substituted anthocyanins: pelargonidin derivatives) and flavonols (also known as mono-substituted flavonols: kaempferol derivatives), 3'4'-substituted anthocyanins (also known as di-substituted anthocyanins: cyanidin and peonidin derivatives) and flavonols (also known as di-substituted flavonols: quercetin and isorhamnetin derivatives), and 3'4'5'-substituted anthocyanins (also known as tri-substituted anthocyanins: delphinidin, petunidin, and malvidin derivatives) and flavonols (also known as tri-substituted flavonols: myricetin, laricitrin, and syringetin derivatives) (**Figure 1.3 and 1.4**). As mentioned previously, 3'4'5'-substituted flavonols are absent in all white grape varieties, due to the inactivity of the flavonoid 3'5' hydroxylases (F3'5'H), or lack of expression of *VviF3'5'H* transcipts (Mattivi

et al. 2006; Flamini et al. 2013). Based on whether or not the hydrogen(s) in the abovementioned hydroxyl groups at the B ring is/are substituted by methyl group(s), anthocyanins and flavonols can be classified as methoxylated and non-methoxylated. Peonidin, petunidin, and malvidin derivatives are methoxylated anthocyanins, while pelargonidin, cyanidin, and delphinidin derivatives are non-methoxylated anthocyanins (**Figure 1.3)**. Similarly, isorhamnetin, laricitrin, and syringetin derivatives correspond to methoxylated flavonols while kaempferol, quercetin, and myricetin derivatives are non-methoxylated flavonols (**Figure 1.4**).

On the other hand, substitution on the 6" position of the C3-glycoside at the C-ring with an aliphatic or aromatic acyl group (Mazza and Miniati, 1993; Rinaldo et al. 2015) results in the production of acylated anthocyanins (**Figure 1.5**).

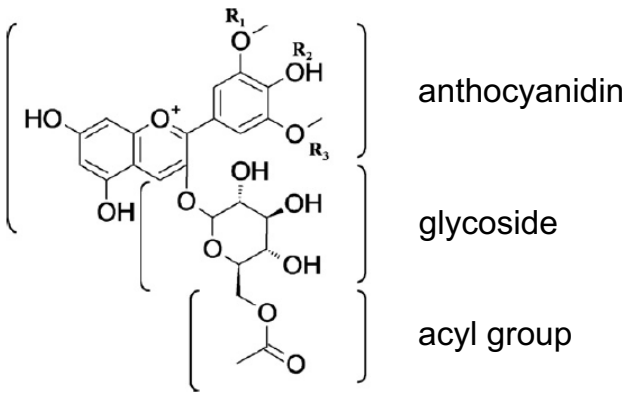


Figure 1.5 The skeletal structure of an acylated anthocyanin (malvidin-3-O-(6"-acetyl)glucoside) (figure from Barnes et al. 2009). R1 = H, OH, or OCH_3; R2 = OH; R3 = H, OH, or OMe, representing for different anthocyanins. In *Vitis vinifera* grapes, acetic acid, *p*-coumaric acid, and caffeic acid are common acyl groups.

Substitution and modification at different levels change the chemical stability as well as color intensity and color hue of the compounds. Substituion of the hydrogen by a hydroxyl group at the C5 position of the B-ring shifts anthocyanin color from red to blue (Castellarin et al. 2006; He et al. 2010; Pastore et al. 2017b; Liu et al. 2018) and increases anthocyanin stability (Mazza and Brouillard 1987). Therefore, 3'4'5'-substituted anthocyanins generally possess a more bluish

color and have higher chemical stabilities. Methoxylation and acylation of anthocyanins increase the chemical and thermal stability of the compounds (Jackman and Smith 1996; Mori et al. 2005b), with the former type of substitution shifting the color to red while the latter shifting the color to blue (Lachman and Hamouz 2005; He et al. 2010; Costantini et al. 2015). Glycosylation at the C3 position of the C-ring increases the stability and color intensity of the compounds in comparison with glycosylation at other carbon positions (Mazza and Brouillard 1987; Liu et al. 2018).

Section 1.3.3 Biosynthesis of flavonoids and transcriptional regulation

The three major classes of flavonoids described above are synthesized through different branches of the same biosynthetic pathway (**Figure 1.6**) (Kuhn et al. 2013). The current study focuses on anthocyanin and flavonol accumulation in grapes and therefore, the biosynthesis of these two types of compounds is highlighted. The precursor of flavonoids, 4-coumaroyl-CoA, is synthesized via the phenylpropanoid pathway and by the catalytic effect of a series of enzymes such as phenylalanine ammonia lyase (PAL) and 4-coumarate CoA ligase (C4H). The phenylpropanoid pathway initiates from the amino acid, phenylalanine, which is a product of the shikimate pathway. The production of chalcone is the first step of the flavonoid pathway and chalcone synthase (CHS) is the first committed enzyme involved in the flavonoid pathway (Sparvoli et al. 1994; Goto-Yamamoto et al. 2003). Chalcone is utilised to produce dihydroflavonols by chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H). Flavonols are synthesized from dihydroflavonols by flavonol synthase (FLS). On the other hand, dihydroflavonol 4-reductase (DFR) competes with FLS for dihydroflavonols as substrates to produce leucoanthocyanidins, which are the substrates of anthocyanidin reductase (ANR) and leucoanhtocyanidin dioxygenase (LDOX, also known as anthocyanidin synthase, ANS) for further

production of flavan-3-ols and anthocyanidins, respectively. UDP-glucose: flavonoid 3'-O-glucosyltransferase (UFGT) glycosylates anthocyanidins to produce anthocyanins that are more stable compared to their aglycones (Ford et al. 1998).

The pathway is divided into two major branches at the upstream level where flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) are responsible for hydroxylation of flavanones (an intermediate product by CHI) and dihydroflavonols which act as substrates to generate precursors of differentially substituted flavonols and anthocyanins (Bogs et al. 2006). Specifically, F3'H is responsible for the production of the precursors of 3’4’-substituted anthocyanins and flavonols, whereas F3'5'H is responsible for the production of the precursors of 3’4’5’-substituted anthocyanins and flavonols.

Anthocyanin 3-O-methyltransferase (AOMT) and 3-O-glucoside-6''-acyltransferase (3AT) are involved in the flavonoid pathway for the post-synthetic modification of anthocyanins. The enzyme AOMT functions in the production of methoxylated anthocyanins while 3AT is responsible for the production of acylated anthocyanins. Recently, several novel O-methyltransferases were reported to be involved in the production for methoxylation of anthocyanins and flavonols (Muzac et al. 2000; Hugueney et al. 2009; Lücker et al. 2010). Anthocyanins are believed to be synthesized at the cytosolic surface of the endoplasmic reticulum in the cell and transported to vacuolar storage later on (Saslowsky and Winkel-Shirley 2001). Glutathione S-transferase (GST) and anthoMATE transporter (AM) act as anthocyanin transporters in grapes (Conn et al. 2008; Gomez et al. 2009, 2011).

The control of flavonoid structural genes via specific transcription factors (TFs) is highly conserved in plants. Members of three protein families, namely R2R3-MYB TFs, basic helix-loop-helix (bHLH) family, and WD40 Repeat (WDR, also known as beta-transduction repeat) proteins,

are major transcriptional regulators for the expression of structural genes of the flavonoid pathway. These TFs interact with each other to form a MYB-bHLH-WDR (MBW) complex that modulates flavonoid biosynthesis (Zhang et al. 2014). The first MYB TF regulating flavonoid biosynthesis was identified in maize in 1987 (Paz-Ares et al. 1987). To date, specific MYB TFs have been identified in many plant species such as arabidopsis (Borevitz et al. 2000; Gonzalez et al. 2008), strawberry (Aharoni et al. 2001), grapevine (Kobayashi et al. 2002; Deluc et al. 2006, 2008; Walker et al. 2007; Cutanda-Perez et al. 2009), tomato (Mathews et al. 2003; Ballester et al. 2010), petunia (Quattrocchio et al. 2006; Albert et al. 2009), apple (Espley et al. 2007, 2009), potato (Mano et al. 2007; Jung et al. 2009), pear (Feng et al. 2010), *etc*.

In *Vitis vinifera* grapes, *VviMybA* genes are functionally important for berry pigmentation (Kobayashi et al. 2004; Walker et al. 2007; Yakushiji et al. 2006). *VviMybAs* act as the specific TF for *VviUFGT* which is responsible for stable anthocyanin production (Petroni and Tonelli 2011). Inactivation of *VviMybAs* results in no coloration in grape skin. In addition, *VviMybAs* is also an activator of other genes involved in the anthocyanin pathway of biosynthesis, modification, and transport, such as *VviF3'5'Hs* (Rinaldo et al. 2015), *VviAOMTs* (Hugueney et al. 2009; Lücker et al. 2010), *Vvi3AT* (Rinaldo et al. 2015), *VviGST4* and *VviAM3* (Conn et al. 2008; Gomez et al. 2011).

Ectopic expression of *VviMybPA1* and *VviMybPA2* indicated that the two TFs exclusively activate genes involved in flavan-3-ol and proanthocyanidin synthesis, such as *VviANR* and *VviLAR (leucoanthocyanidin reductase)* (Bogs et al. 2005, 2007; Deluc et al. 2006, 2008; Terrier et al. 2009). *VviMybF1* was identified to be the key regulator for *VviFLS* genes, and specifically modulates the production of flavonols (Czemmel et al. 2009). *VviMyb5a* and *VviMyb5b* were identified as general regulators for common flavonoid pathway controlling the synthesis of

anthocyanins and proanthocyanidins (Bogs et al. 2007; Terrier et al. 2009). Recently, two MYB TFs, *VviMYB4a* and *VviMYB4b*, have been characterized as negative regulators for genes involved in general phenylpropanoid pathway (e.g. *VviPAL* and *Vvi4CL*, Cavallini et al. 2015).

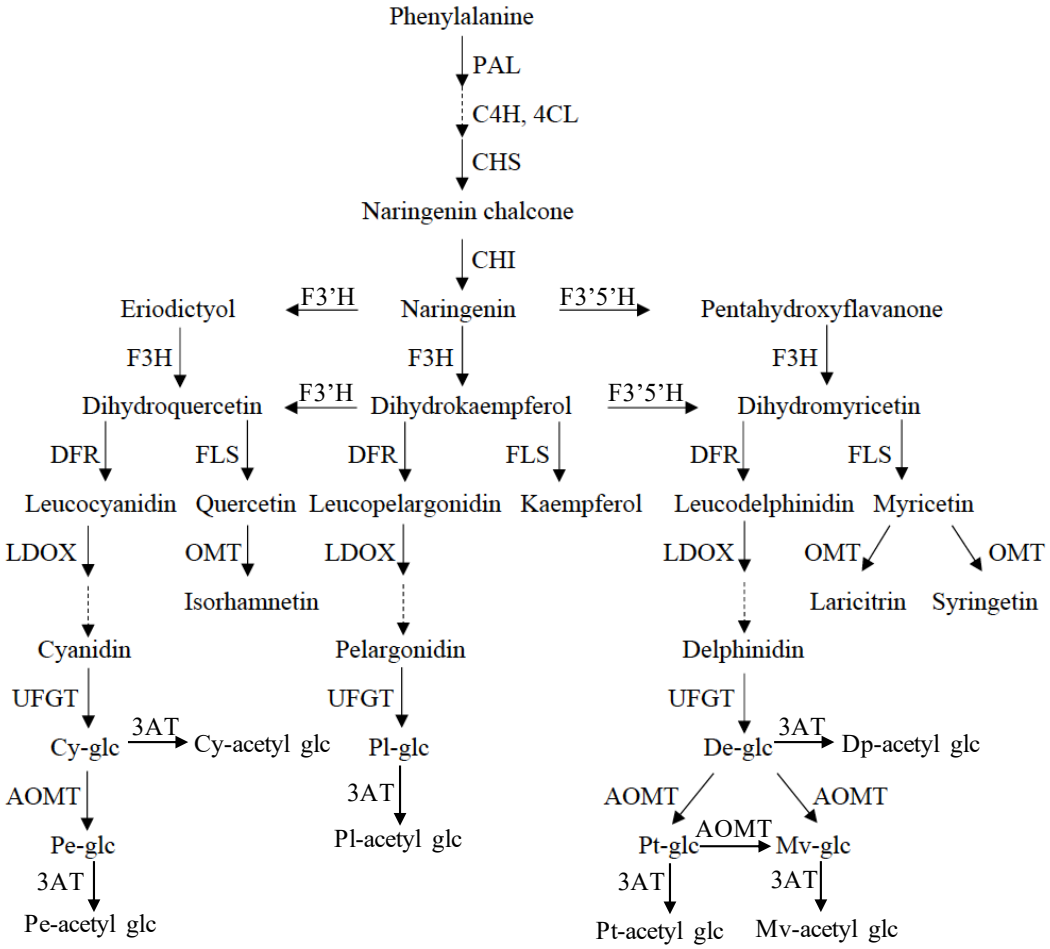


Figure 1.6 Flavonol and anthocyanin biosynthetic pathway in plants. Solid arrows represent confirmed steps, while dash arrows represent unconfirmed steps in the pathway. Compounds besides the arrows are enzymes involved in the specific steps of the pathway. Cy, Pl, De, Pe, Pt, and Mv stand for cyanidin, pelargonidin, delphinidin, peonidin, petunidin, and malvidin, respectively; glc and acetyl glc stand for glucoside and acetyl glucodise, respectively.

Besides these major MYB TFs, a bHLH TF, *VviMYC1* (also known as *VviMYCA1*), was reported to be involved in the regulation of anthocyanin and proanthocyanidin accumulation during berry development (Hichri et al. 2010; Matus et al. 2010). A WDR member, *VviWDR1*,

was identified by Matus et al. (2010), as a modulator of anthocyanin accumulation. Additionally, three basic leucine zipper (bZIP) TFs, *VviHY5*, *VviHYH*, and *VvibZIPC22*, were characterized as regulators of *VviFLS* expression and flavonol biosynthesis (Malacarne et al. 2016; Loyola et al. 2016).

Section 1.4 Temperature effects on grapevine growth and berry development

Many abiotic factors are reputed to affect grapevine vegetative and reproductive growth, including solar radiation, temperature, water status, soil nutrients, *etc*. Among these factors, temperature is one of the most important since it affects a series of physiological and biological processes such as photosynthesis and transpiration, as well as primary and secondary metabolite accumulation during grapevine growth and berry development. The effect of day and night temperatures, applied at different developmental stages, on grapevine growth, berry development and quality, and wine quality has been investigated since the last century. However, several physiological responses of grapevines to temperature remain unclarified. Worldwide climate change is expected to have a great impact on the wine industry (Jones et al. 2005), and the grapevine response to different temperature regimes remains one of the major topics in viticulture.

Section 1.4.1 Temperature effects on grapevine vegetative growth

The temperatures at which most physiological processes occur in plants range from 10-40 ℃ (Went, 1953). In grapes, physiological processes are greatly reduced or even inhibited when the temperature goes below 10 ℃ or over 30 ℃ (Coombe, 1987). To some extent, warmer temperatures enhance grapevine growth within a specific range of temperatures, while too high or too low temperatures inhibited the growth. Based on early studies, physiological parameters

related to vegetative growth, such as shoot length, leaf area, number of lateral shoots, and number of leaves, were maximised when measured at 25 ℃, rather than at 20 or 30 ℃ (Buttrose, 1968), while fruitfulness was maximized between 30 and 35 ℃ (Buttrose 1969). Hendrickson et al. (2004) identified that between two sites within a vineyard that had a air temperature variation of 1-3 ℃ (average daily temperature 16-17 ℃ versus 14-15 ℃ during the growing season), the rate of shoot growth was 34-63% higher in the warmer microsite than in the cooler one. On the other hand, soil temperature plays a key role in the regulation of root growth, with warmer soil temperatures stimulating root starch accumulation and nutrient uptake (Clarke et al. 2015). However, excessive high temperatures (e.g., tempereature >35 ℃ constantly during the growing season) may have detrimental effects on grapevine vegetative growth (Keller 2010).

Section 1.4.2 Temperature effect on grapevine leaf gas exchange

Plant photosynthesis was reported not to be significantly affected by temperature as long as the plants are not subjected to extreme conditions (e.g., temperature >35 ℃), due to leaf plasticity in photosynthesis (Jackson 2008). Kliewer (1973) indicated that photosynthesis was always operating at 90 to 100% efficiency when grapevines were exposed to 18-33 ℃ but was inhibited markedly as temperatures went below or above this range. Kriedemann (1968) and Kriedemann and Smart (1971) suggested a narrower range for maximum rate of photosynthesis (25-30 ℃) and that a higher temperature (33-41 ℃) resulted in an approximately 40% reduction in photosynthetic rate. Ferrini et al. (1995) reported 20-27.5℃ as the optimum temperature range for grapevine net photosynthesis. Generally, grapevines maintain their photosynthetic rate at a maximum level within 20-30 ℃ depending on the variety.

Section 1.4.3 Temperature effects on blooming and fruit set

Temperature has critical effects on both the duration of blooming and rate of fruit set in grapes. Blooming does not occur in grapes until the average daily temperature reaches 20 °C (Winkler 1974; Weaver 1976; Vasconcelos et al. 2009). Cold temperatures reduce the overall pollen viability, delay the fertilization and cause the abortion of ovules. Studies on blooming and fruit set under different temperature conditions in Chardonnay and Shiraz grapes showed that low temperature treatments (12/9 °C or 17/4 °C compared to 25/20 °C, day/night temperature) before blooming and fruit set reduced the number of pollen tubes and retarded pollen-tube growth, pollen germination, pollen release, and subsequent fruit set (Ebadi et al. 1995a, b). On the other hand, excessive high temperatures (e.g., 35-40 °C) during bloom-set are also detrimental to ovule fertility and fruit set, which affect berry development later on (Hale and Buttrose 1974; Kliewer 1977a). Overall, in grapes, temperatures between 20 and 28 °C during bloom-set are beneficial to ovule fertility, pollen activity, pollen tube growth, fruit set, and berry growth.

Section 1.4.4 Temperature effects on sugar and acid levels in the berry

Grape ripeness, based on sugar and organic acid levels in berries at harvest, is considered to be optimal when grapevines are exposed to temperatures between 20 and 25 °C during the berry growing season (Dokoozlian, 2000). However, temperature effects on sugar accumulation, when expressed as total soluble solids (TSS), are still controversial. Early studies indicated that warmer temperatures (e.g., 32 °C during the day in comparison to 22 °C) during ripening resulted in berries that had a higher level of TSS (Aljibury et al. 1975; Kliewer 1977b). Nevertheless, several recent studies reported that temperatures during berry ripening had little effect on TSS at harvest (Mori et al., 2005a, b; Sadras et al., 2013; Shinomiya et al., 2015; De Rosas et al. 2017). Sadras and

Moran (2012) suggested that higher temperatures (1-3 ℃ higher than control treatment) during fruit development accelerated the onset of ripening and hence, enhanced sugar accumulation during ripening but had no effect on the rate of sugar accumulation.

On the other hand, organic acids, as expressed by titratable acidity (TA), were consistently decreased by increased temperatures (Bergqvist et al. 2001; Mori et al. 2005a; Sadras et al. 2013; Rienth et al. 2016).

Section 1.4.5 Temperature effects on flavonoid accumulation in grapes

It is well documented that relatively lower temperatures (e.g., 20-25 °C in comparison to 30-35 °C) after veraison are preferable for anthocyanin and flavonol accumulation (Kliewer and Torres 1972; Kliewer 1977b; Mori et al. 2005a, b; 2007a, b). Many metabolic processes during ripening are significantly inhibited or totally stopped at high temperatures (e.g., >30 °C) in grapes (Coombe 1986, 1992). Empirical experience also indicates that grapevines grown in relatively cooler areas produce more pigments and aromas. Kliewer and Torres (1972) investigated the effect of separated day and night temperature as well as temperature regimes on grape coloration in different grape varieties and showed that high day temperature (e.g., 35 °C) strongly reduced grape coloration, which could not be reverted by any of the night temperatures tested. The effect of night temperature was more complex and not as evident as the effect of day temperature. Cool (e.g., 15 or 20 °C) rather than warm (e.g., 25 °C) night temperature was preferable for coloration combined with cool or warm (e.g., 15 or 25 °C) day temperature; however, in the case of hot day temperature (35 °C), warm (e.g., 25 °C) night temperature enhanced the coloration compared to cool night temperature (e.g., 15 or 20 °C). Kliewer (1977b) indicated that constant high day and night temperatures (37/32 ℃, day/night temperature) completely inhibited anthocyanin accumulation in

Emperor grape skin. Mori et al. (2005a, b; 2007a, b) investigated the effect of high day and night temperature on the accumulation and composition of anthocyanins, as well as flavonols. High temperatures (e.g., 30 or 35 °C) strongly decreased the anthocyanin levels, but improved the relative concentration of 3'4'-substituted (cyanidin and peonidin based) anthocyanins. On the other hand, high night temperature decreased the relative concentration of non- and low-methoxylated anthocyanins (cyanidin, delphinidin, and petunidin based), indicating potentially different regulatory mechanisms involved in anthocyanin accumulation under high day and high night temperature conditions. Later on, Tarara et al. (2008) and De Rosas et al. (2017) also found decreased total anthocyanin concentrations and altered anthocyanin profiles (increased proportion of 3'4'5'-substituted, methoxylated and acylated anthocyanins) in different grape varieties (Merlot, Malbec, and Bonarda) exposed to high temperatures. Other than that, temperature effects also depend on the developmental stages when the treatments are applied. Yamane et al. (2006) suggested that the most sensitive stage of berry development was 1-3 weeks after veraison in grapes. High temperature during this stage strongly reduced anthocyanin level in grape skin. However, in a recent study by Gaiotti et al. (2018), cool night temperature (~10 °C) applied at veraison enhanced anthocyanin accumulation in comparison with a higher night temperature (~20 °C), while post-veraison low night temperature treatment had no effect in promoting anthocyanin accumulation.

Studies on flavonol accumulation under different temperature regimes have also been conducted (reviewed by Gouot et al. 2018). Spayd et al. (2002) reported that post-veraison temperatures had little effect on flavonol accumulation which in contrast, was substantially affected by solar radiation. Similar results were observed by Mori et al. (2005b), Cohen et al. (2008), and Tarara et al. (2008). Only recently, studies showed that extremely high temperatures

during post-veraison stages exerted negative effects on flavonol accumulation (Degu et al. 2016; Pastore et al. 2017b). The profiles of flavonols were also greatly shifted under high temperature conditions (6 or 9 °C higher than control condition), with the most affected compounds being kaempferol 3-O-glucoside (no-longer detectable), quercetin 3-O-glucoside (85% loss), unidentified flavonols (70% loss), and myricetin 3-O-glucoside (65% loss) (Pillet et al. 2011, reviewed by Gouot et al. 2018).

It was also reported that the expression of grape phenylpropanoid and flavonoid genes, including *VviPAL*, *VviCHS*, *VviCHI*, *VviUFGT*, *etc*., and the transcription factors *VviMybA*, *VviMybA2*, *VviMybPA1*, was down-regulated by high temperatures (e.g., 30-35 °C in comparison to 15-20 °C) (Yamane et al. 2006; Azuma et al. 2012; Movahed et al. 2016; De Rosas et al. 2017; Gaiotti et al. 2018). However, the expression of *VviUFGT* and *VviMybA* was reported not to be affected by temperature in Mori et al. (2007b); whereas an increased UFGT enzyme activity was observed under high temperature in the same study. Interestingly, Rienth et al. (2014b) observed an up-regulation of *VviUFGT* under high temperature condition (37 °C in comparison to 22 °C) while a down-regulation of *VviMybA* expression.

Section 1.5 Impact of other exogenous factors on flavonoid accumulation in grapes

Besides the effect of temperature, that was discussed in **Section 1.4.5**, the accumulation of anthocyanins and flavonols is also affected by other abiotic factors, such as solar radiation, water and nutrient availability, and the exogenous applications of plant growth regulator, *etc*.

Light/solar radiation affects anthocyanin and flavonol biosynthesis and composition in grape skin. On the one hand, flavonol biosynthesis has long been known to be extremely sensitive

to light conditions (Price et al. 1995; Keller and Hrazdina 1998; Downey et al. 2004; Tarara et al. 2008; Czemmel et al. 2009; Azuma et al. 2012; Liang et al. 2012; Figueiredo-González et al. 2013; Flamini et al. 2013), and exposure to sunlight can increase total flavonol content by more than ten times (Carbonell-Bejerano et al. 2014). On the other hand, anthocyanins are less sensitive to light than flavonols. Nevertheless, anthocyanin levels were induced as light intensity increased in several grape varieties (Smart et al. 1985; Dokoozlian and Kliewer 1996; Downey et al. 2004; Jeong et al. 2004;). The interrelationships between temperature and light are not negligible in most cases, since the exposure of the berry to direct solar radiation also increases the berry temperature. Azuma et al. (2012) investigated the separate effects of temperature and light as well as their dual effects on anthocyanin and flavonol accumulation and demonstrated a synergistic effect of high temperature (35 °C) and dark conditions on impairing anthocyanin and flavonol accumulation.

Plant water status, which can be manipulated by different irrigation strategies, is known to be a regulator of flavonoid biosynthesis in the berry (Matthews et al. 1987b; Deloire et al. 2001; Castellarin et al. 2007a, b). Deficit irrigation, which implies irrigating grapevines at sub-optimal conditions in order to keep them under moderate water deficit, enhances flavonoid, particularly anthocyanin accumulation in grapes (Kennedy et al. 2001; Castellarin et al. 2007a, b; Bucchetti et al. 2011; Ollé et al. 2011; Zarrouk et al. 2012). Deficit irrigation affects the anthocyanin profile as well. Castellarin et al. (2007a, b) showed that the relative concentration of 3’4’5’-substituted anthocyanins in grapes was induced when the vines were subjected to deficit irrigation.

Soil nutrient availability, and particularly nitrogen availability, is essential for grapevine development. However, excessive high or low levels of nitrogen exerted negative effects on grape coloration (Kliewer 1977b; Keller and Hrazdina 1998; Delgado 2004, 2006). High potassium

levels significantly reduced anthocyanin concentration in grapes (Jackson and Lombard 1993; Delgado et al. 2006).

Finally, exogenous applications of plant growth regulators, a viticulture practice mostly used in table grape production, also modulate anthocyanin and flavonol accumulation in grape skin (Flamini et al. 2013). Exogenous ABA application stimulates anthocyanin biosynthesis (Peppi et al. 2008; Wheeler et al. 2009; Davies and Böttcher 2009; Gambetta et al. 2010; Koyama et al. 2018). El-Kereamy et al. (2003) showed that the application of the ethylene-releasing compound, 2-chloroethylphosphonic acid (2-CEPA), hastened anthocyanin accumulation in grape skin as well. The application of auxins to grapes inhibited anthocyanin accumulation (Ozeki and Komamine 1981, 1986; Mori et al. 1994).

Section 1.6 Rationale for the study

Grape development, especially the ripening process, is very sensitive to climate and in particular, temperature. Temperature regimes greatly affect berry quality and wine production through a series of physiological and biological processes. Anthocyanin and flavonols are key determinants of grape and wine quality in red grape varieties. Understanding the optimum temperature regimes for anthocyanin and flavonol production is important for viticulture site selection of red grape production in BC as well as other viticultural regions worldwide. Moreover, the increasing temperature due to climate change might strongly affect wine regions, potentially shifting the regions of grape production toward northern areas (Hannah et al. 2013). Temperature of the berry can also be affected by viticultural practices such as training system selection and canopy management. A comprehensive understanding of temperature effects on berry composition is needed for growers to make more educated choices in adapting viticulture to climate change.

Although several studies have investigated the temperature effects on anthocyanin accumulation in grapes, there is still a gap in knowledge on the role of night temperatures. Moreover, anecdotally, it is believed that the difference between day and night temperatures regulates anthocyanin production, with a larger difference between day and night temperatures stimulating the accumulation of anthocyanins in the berry. According to the IPCC (International Panel of Climate Change, 2013), the elevation of night temperatures was faster than that of day temperatures in the last half century, indicating that there will be a further decrease in the difference between day and night temperatures in future years with potential implications for anthocyanin production in grapes. Therefore, a better understanding of the effect of the difference between day and night temperatures on anthocyanin accumulation in grapes will help elucidate how future climates might impact the composition of wine grapes.

On the other hand, flavonol biosynthesis is proposed to be slightly inhibited by an increase in temperature (Downey et al. 2004; Fujita et al. 2006) but it remains controversial whether and how temperature regimes and the difference between day and night temperatures affect flavonol accumulation in grapes. Furthermore, the modulation of the flavonoid pathway under different temperature regimes needs further elucidation as well.

Section 1.7 Objectives of the study

The objectives of this study were to i) investigate the influence of temperature regimes on the accumulation of anthocyanins and flavonols during grape ripening, ii) elucidate whether and how the difference between day and night temperatures affects the anthocyanin and flavonol accumulation, and iii) understand the response of flavonoid genes to these temperature regimes. Regimes with day and night temperature from low to high were considered, including regimes that

had the same day or night temperature but different night or day temperatures in order to assess the effect on berry anthocyanins and flavonols of low, medium, and high temperature regimes, of the difference between day and night temperatures.

Chapter 2: Material and methods

Section 2.1 Experimental design and plant material

Two experiments were conducted. Experiment 1 was conducted in 2017 and Experiment 2 was conducted in 2018. Both experiments considered the same grapevine variety (Merlot) and utilised five growth chambers; however, differences in the grapevine training and temperature regimes tested occurred between the two experiments as is reported below.

Experiment 1. In 2017, Merlot (Clone 347) fruiting cuttings were obtained according to Mullins and Rajasekaran (1981) with some modifications. Three-node mature canes were collected during the dormant season in 2017 (Okanagan, Canada). Rooting was induced using Roots Liquid Stimulator (Wilson, Canada) in a heated container (25 °C at the base) with Greenhouse Potting Mix, perlite, and sand (v/v, 3: 1: 0.5). The container with the cuttings was stored in a cold room (+4 °C) in darkness for four weeks. After then, rooted cuttings were planted into 3.75 L pots containing Greenhouse Potting Mix and transferred into the UBC Horticulture greenhouse. The temperature in the greenhouse was set at 22.5 ± 0.9 °C during the day and 19.8 ± 0.5 °C at night; relative humidity (RH) was 54.5 ± 1.6%. Environmental solar radiation was supplemented with Green Power LED (Phillips, Canada) top lighting in order to guarantee 200 μmol m^{-2} s^{-1} of Photosynthetically Active Radiation (PAR) at the table surface for 16 hours per day. Plants were irrigated every other day, and concomitantly they were supplemented with a nutrient solution (Relab Den Haan, Hoorn, Netherlands; containing NO_3^-, 1.1; Cl^-, 0.6; SO_4^{2-}, 2.9; HCO_3^-, <0.2; PO_4^{3-}, 0.6; NH_4^+, 0.2; K^+, Na^+, 0.3; Ca^{2+}, 2.8; Mg^{2+}, 1.6; Si^{2+}, 0.12, in mmol/L; and Fe^{2+}, 1.0; Mn^{2+}, 3.4; Zn^{2+}, 0.7; BO_3^{3-}, 5.0; Cu^{2+}, <0.2; MoO_4^{2-}, <0.2, in μmol/L). After bud-break, plants were transferred into a 7.5 L pots filled with Greenhouse Potting Mix. Shoot tips were topped after

inflorescence development; one cluster was selected to be maintained for each plant after fruit set. In the meantime, one lateral shoot was left to feed the cluster.

Figure 2.1 Merlot grapevine plants grown in the growth chambers of the UBC Faculty of Forestry in Experiment 1.

At 60 days after anthesis (DAA), when 50% of the berries showed partial or full red pigmentation (veraison), 60 grapevines were randomly selected and used for the growth chamber experiment. Five Conviron E15 (Conviron, Winnipeg, Canada) growth chambers were used for the experiment. Before the grapevines were moved to the growth chambers, grapevine shoot tips were cut to maintain 20 leaves on each shoot. Newly grown lateral shoots were removed during the experiment. Each temperature regime was imposed on 12 grapevines divided in groups of three grapevines per biological replicate, for a total of four biological replicates. Five temperature

regimes were set up: i) 20/10 (day/night temperature, °C); ii) 20/15; iii) 25/15; iv) 35/25; v) 35/30, in order to expose grapevines to low (i.e., 20/10 and 20/15), medium (i.e., 25/15), and high (i.e., 35/25 and 35/30) temperatures and two temperature differences between day and night: ΔT = 5 °C (for 20/15, 35/30) and ΔT = 10 °C (for 20/10, 25/15, 35/25). Temperature increased from night to day temperature and decreased from day to night temperature during two transition stages of one hour each to simulate natural conditions at sunrise and sunset. The day/night cycle was 14 h/8 h (plus 2 hours of transition stages). Mean PAR during the day cycle was set at 400 µmol m^{-2} s^{-1} in all chambers at the pot level, while during the transition stage was set at 100 µmol m^{-2} s^{-1}. Growth chamber conditions were monitored manually. Light intensity around the clusters was measured with a PAR sensor (Kipp and Zonen, Delft, Holland). Relative humidity and air temperature were measured with a temperature and humidity probe (Campbell Scientific, Edmonton, Canada). Berry surface temperature was measured with an infrared thermal gun (NAPA, Canada). During the growth chamber experiment, plants were irrigated every other day, and were supplemented with a nutrient solution (same as described above) twice per week.

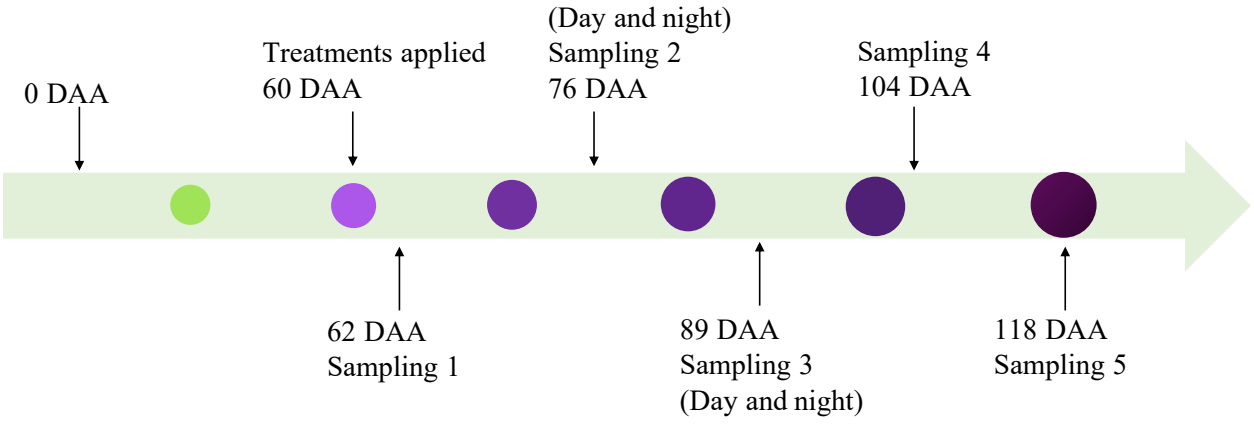


Figure 2.2 Schematic of the progression of ripening, indicated by relative berry size and color, of Merlot grapes sampled during Experiment 1. The time of samplings, indicated as day after anthesis (DAA), are shown.

Berry sampling was carried out at five time-points throughout ripening (**Figure 2.2**). Eight berries per biological replicate were randomly collected at 62, 76, 89, 104, and 118 DAA (harvest date) at 4 PM for metabolite and gene expression analyses. Additionally, at 76 and 89 DAA (in the middle of berry ripening), berry samples were also collected at 4 AM, in order to investigate whether diurnal or nocturnal conditions would affect the accumulation of flavonoid compounds and the expression of genes involved in flavonoid biosynthesis. Berries were removed from clusters by carefully cutting the pedicel, then berries were flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

Table 2.1 Definition and description of temperature regimes in Experiment 1 and 2

Experiment	Temperature regimes (day/night temperature, °C)	Day temperature (°C)	Night temperature (°C)	Difference between day and night temperatures (°C)	Mean daily temperature	Definition
Experiment 1	20/10	20	10	10	15	Low temperature regime
	20/15	20	15	5	17.5	Low temperature regime
	25/15	25	15	10	20	Intermediate temperature regime
	35/25	35	25	10	30	High temperature regime
	35/30	35	30	5	32.5	High temperature regime
Experiment 2	20/10	20	10	10	15	Low temperature regime
	20/15	20	15	5	17.5	Low temperature regime
	25/15	25	15	10	20	Intermediate temperature regime
	25/20	25	20	5	22.5	Intermediate temperature regime

	30/20	30	20	10	25	High temperature regime

Experiment 2. In 2018, 20 one-year Merlot rooted cuttings obtained from the previous season as described above were pruned to 5-6 dormant buds and grown in 11.25 L pots filled with Greenhouse Potting Mix. The plants were conserved in the greenhouse from bud-break to veraison (69 DAA) under the same conditions as for Experiment 1. Two shoots with 2-3 clusters per shoot were maintained in each plant after fruit-set.

Figure 2.3 Merlot grapevine plants grown in the growth chambers of the UBC Faculty of Forestry in Experiment 2.

At veraison (69 DAA), grapevines were transferred into the growth chambers. Before moving to the growth chamber, the shoots were topped to maintain 20 leaves. No pruning was conducted in the growth chamber and lateral shoots were maintained until harvest. Four plants per treatment, one plant per biological replicate, four biological replicates in total, were grown into five growth chambers until harvest (135 DAA). In Experiment 2, the two high temperature regimes of Experiment 1 (i.e., 35/25 and 35/30) were substituted with two milder temperature regimes (i.e., 25/20 and 30/20). Therefore, the five treatments considered were: i) 20/10 (day/night temperature, °C); ii) 20/15; iii) 25/15; iv) 25/20; v) 30/20. Day and night cycles and light intensities were set as described for Experiment 1. Growth chamber conditions were monitored manually. Light intensity around the clusters was measured with a PAR sensor (Kipp and Zonen, Delft, Holland). Relative humidity and air temperature were measured together using a temperature and humidity probe (Campbell Scientific, Edmonton, Canada). Berry surface temperature was not measured due to the in availability of the infrared thermal gun for measurements. During the growth chamber experiment, plants were fully irrigated, and were supplemented with a nutrient solution (same as described above) twice per week.

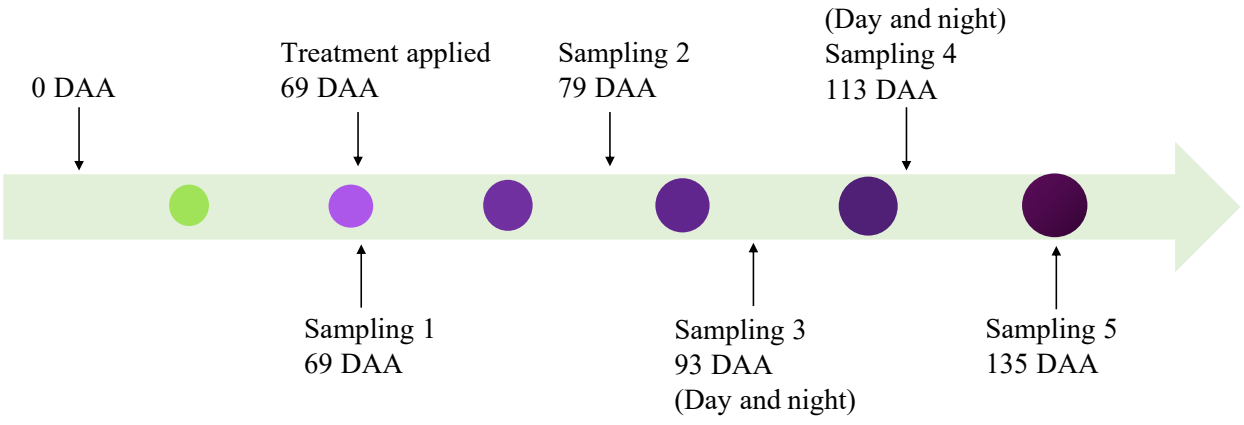


Figure 2.4 Schematic of the progression of ripening, indicated by relative berry size and color, of Merlot grapes sampled during Experiment 2. The time of samplings, indicated as day after anthesis (DAA), are shown.

Five biological replicates with 25 berries per replicate were randomly collected from 4 out of 20 grapevines at 69 DAA, before the grapevines were moved into the growth chambers. During the growth chamber experiment, berry samples (25 berries per sample) were randomly collected from each biological replicate at 79, 93, 113, and 135 DAA at 4 PM (**Figure 2.3**). Additional samples were collected at 93 and 113 DAA at 4 AM for the analysis of metabolite accumulation. Berry samples were flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

Section 2.2 Physiological measurements

Once per experiment, grapevine leaf water potential (Ψ) was tested at 18 and 24 hours after irrigation in Experiment 1 and 2, using a Scholander pressure chamber (Model 615, Albany, USA). Two healthy, mature leaves from the primary shoot were selected for the measurement. The leaf was first covered with a plastic bag and then immediately cut at the petiole and sealed into the pressure chamber for the determination of the balancing pressure analyzed (Matthews et al. 1987b).

Leaf photosynthesis, stomatal conductance, and transpiration rate were measured at 78 and 107 DAA in Experiment 1 and at 83, 104, and 118 DAA in Experiment 2 using an LI-6400 portable photosynthesis system (Li-COR Inc, Nebraska, USA). Two fully developed leaves from the ninth and tenth nodes were selected for the measurement (leaves from the eighth or eleventh nodes were used alternatively if the ninth or tenth were destroyed).

Total leaf area was measured at harvest in both experiments (118 DAA in Experiment 1 and 135 DAA in Experiment 2), by a destructive method using an LI-3100C Area Meter (Li-COR Inc, Nebraska, USA) (Herrera et al. 2015). At harvest, cluster weight was measured, grapevine yield was recorded, and the ratio of total leaf area to grapevine yield was then calculated.

In Experiment 1, one grapevine per biological replicate was randomly selected and used for leaf water potential, leaf gas exchange, total leaf area, and cluster weight measurement during the experiment. In Experiment 2, all grapevines from each temperature regime were used.

Section 2.3 Berry sample preparation

The frozen berry samples were weighed, peeled, and deseeded using a scalpel and tweezers. Berry tissues were kept frozen by using liquid nitrogen. Skin, flesh, and seed weights were recorded. Skin and flesh tissues were ground under liquid nitrogen into a fine powder using a mortar and pestle and an electronic mill (IKA A11 S001, USA), respectively. Skin powder was used for the quantification of anthocyanins and flavonols, and the gene expression analyses; flesh powder was used for the TSS and TA measurement.

Section 2.4 Analyses of berry total soluble solids, titratable acidity, and pH

An aliquot of 2 g flesh powder was thawed at room temperature for 20 min and the juice was collected by centrifugation at 10,000 × g for 10 min. TSS was measured with a digital refractometer (Reichert A2R200, Reichert GmbH, Seefeld, Germany) and reported as °Brix. TA was measured by titration with 0.1 N NaOH. An aliquot of 1 mL of fruit juice was diluted by 10 times in a 25 mL conical flask and titrated until pH 8.2 using a 50 mL alkali burette. The titratable acidity was expressed as tartaric acid equivalents (g/L). The juice pH was measured with a pH meter (AR20 pH/Conductivity Meter, Thermo Fisher Scientific, Waltham, USA). For both experiments, additional 50 berries for each treatment and biological replicate were collected at harvest and processed freshly to determine TSS, TA, and pH as reported in Herrera et al. (2015), in order to assess if freezing of the tissue generated tartaric acid precipitates and decreased the TA

level, as well as the level of TSS and pH. The statistical comparison between the data obtained from fresh samples and frozen-thawed samples yielded no significant difference for TSS and pH measurements, but showed a ~10% higher TA level for the fresh samples. However, due to the limitation of the plant and cluster size in the growth chamber, the decision was taken to use the data from frozen-thawed flesh powder.

Section 2.5 Anthocyanin and flavonol analyses

Anthocyanins and flavonols were extracted with an extraction solution containing acidified methanol (v/v, 49.5: 49.5: 1, methanol: water: formic acid) and analyzed by high performance liquid chromatography/mass spectrometry (HPLC/MS). The extractions and HPLC/MS analysis were performed according to Downey and Rochfort (2008) with some modifications. An aliquot of 0.15 g of skin powder was extracted in 1.5 mL of the extraction solution followed by 20-min sonication at room temperature and 10-min centrifugation at 13,000 × g at 20 °C. The supernatant was collected, filtered, and transferred into an amber vial (Agilent Technologies, Mississauga, Canada) using a syringe (Luer-Lok Tip Syringe, Sigma-Aldrich, Oakville, Canada) and filter (0.22 μm × 13 mm, PVDF Millex Filter, Sigma-Aldrich, Oakville, Canada). Afterwards, the pellet was extracted for a second time with the same procedure described above. The two fractions of supernatant were combined. Five μL of extract was injected into an Agilent 1100 Series LC coupled to an MSD Trap XCT Plus System (Agilent Technologies, Mississauga, Canada) and equipped with a Diode Array Detector (DAD). Chromatographic separation was carried out by an Agilent ZORBAX SB-C18 Column (1.8μm, 4.6 × 50 mm) (Agilent Technologies, Mississauga, Canada). The temperature of the column was maintained at 67.0°C. The mobile phases were 2% formic acid (v/v with water; Solvent A) and acetonitrile/formic acid (98: 2, v/v; Solvent B). The

LC separation used a binary solvent gradient, with a flow rate of 1.20 mL/min. The gradient conditions were 0.20 min, 5.0% solvent B; 6.00 min, 20.0% solvent B; 9.00 min, 80.0% solvent B; 10.00 min; 90.0% solvent B; 10.10 min, 90.0% solvent B; 11.00 min, 5.0% solvent B; and stopped at 11.50 min. Mass spectra were generated via electrospray ionization (ESI) in both positive and negative modes. UV-vis data were collected at wavelengths 520 nm and 353 nm, for anthocyanins and flavonols, respectively. Compound identification was conducted firstly by matching the mass spectra of identified peaks with anthocyanin and flavonol compounds retrieved from published papers and comparing their elution order. Uncertain peaks were then verified by MS/MS analysis. Anthocyanin and flavonol quantification were based on UV-vis spectra and expressed as malvidin 3-O-glucoside and quercetin 3-O-glucoside equivalents, respectively. Calibration curves were constructed using 5 solutions with gradient concentrations of malvidin 3-O-glucoside (1, 5, 50, 100, 250, in μg/mL) and quercetin 3-O-glucoside (0.5, 1, 2.5, 5, 10, in μg/mL).

Section 2.6 RNA extraction and gene expression analyses

Samples collected in Experiment 1 at 76 and 89 DAA were used for transcript analysis. Total RNA was extracted from 0.2 g of grape skin using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Oakville, Canada) according to the manufacturer's instructions. RNA quality was assessed by a spectrophotometer (NanoDrop-1000, Thermo Fisher Scientific, Waltham, USA) and RNA integrity was assessed by running the samples in an agarose gel, respectively. RNA was treated with 1 U/μL of DNase I (# EN0521, Thermo Fisher Scientific, Waltham, USA) to remove any genomic DNA. An aliquot of 1 μg of RNA was used for first strand cDNA synthesis using the

Invitrogen™ SuperScript™ IV Vilo™ Master Mix (Thermo Fisher Scientific, Waltham, USA) following manufacturers' instructions.

Fourteen genes involved in the anthocyanin and flavonol biosynthesis, modification, and transport were selected for expression analyses, including *VviCHI1*, *VviF3'Ha*, *VviF3'5'Hi*, *VviF3'5'Hf*, *VviLDOX*, *VviUFGT*, *VviFLS4*, and *VviFLS5* for anthocyanin and flavonol biosynthesis; *VviAOMT*, *Vvi3AT*, *VviGST4*, and *VviAM3* for post-synthetic modification and transport from cytosol into vacuole, and the TFs, *VviMybA* and *VviMybF1*, which are known to regulate the expression of the structural genes. These genes were selected based on several criteria: i) they are expressed during berry ripening (Falginella et al 2012); ii) they codify for enzymes that act across the flavonoid pathway, from upstream and intermediate stages (*VviCHI1*, *VviF3Ha*, *VviLDOX)*, to specific branching points (*VviF3'H*, *VviF3'5'Hi*, *VviFLS4*, *VviFLS5*, *VviUFGT*, *VviAOMT*, *Vvi3AT*); iii) they codify for key anthocyanin transporters (*VviGST4* and *VviAM3*); iii) they codify for key transcription factors related to flavonol and anthocyanin biosynthesis (*VviMybF1*, *VviMybA*). Specific primers for flavonoid pathway genes were retrieved from previous studies and are listed in **Table 2.2**.

Transcript abundance was assessed by quantitative real time polymerase chain reaction (qRT-PCR) using 7500 Real-Time PCR Systems (Applied Biosystems™, ThermoFisher Scientific, Waltham, USA). Each reaction (10 μL) contained 0.4 μL (10 μM) of each primer, 2 μL of cDNA, 5 μL of master mix (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems™, Thermo Fisher Scientific, Waltham, USA), and 2.2 μL of ddH_2O. Thermal cycling conditions were 95 °C for two minutes of initial denaturation, followed by 40 cycles of 15 seconds denaturation at 95 °C, 20 seconds of annealing at 56 °C, and 60 seconds of extension at 72 °C, followed by a melting curve from 56 to 95 °C. Each cDNA sample was run in duplicate. *VviUbiquitin* was used as a

reference gene. Gene expression was quantified upon normalization to the expression of *VviUbiquitin* by comparing the cycle threshold (C_T) of target gene with *VviUbiquitin* (Bogs et al. 2005).

Section 2.7 Statistical analysis

Statistical analyses were performed using SPSS version 23.0 (IBM, New York, USA). One-way analysis of variance (ANOVA) test was used to evaluate the differences among temperature regimes of all the parameters assessed within each specific sampling point. Separation of means was performed using a least significant difference (LSD) test. A two-way ANOVA test was used to evaluate the effect of temperature regimes, sampling time (day or night), and temperature regimes × sampling time (day or night) interaction on the gene expression level. Linear regression analysis was conducted between TSS and total anthocyanin concentration (expressed as μg/g skin FW) to test the relationship between sugar and anthocyanin accumulation during berry ripening. Analysis of covariance (ANCOVA) test was conducted in order to assess the significant difference between the coefficients of the linear regression curves under different temperature regimes.

Table 2.2 Forward (for) and reverse (rev) primers of genes selected for quantitative real time PCR (qRT-PCR)

Gene name	Primer sequence_for	Primer sequence_ rev	Reference
VviUbiquitin1	GTGGTATTATTGAGCCATCCTT	AACCTCCAATCCAGTCATCTAC	Bogs et al. (2006)
VviCHI1	CAGGCAACTCCATTCTTTTC	TTCTCTATCACTGCATTCCC	Jeong et al. (2004)
VviF3'Ha	GGCGGAAGGTTTCCTTGAT	GCACGTTGATCTCGGTGAG	Falginella et al. (2010)
VviF3'5'Hf	TGTACCAACGACCCCAAAAT	GAACCTTCCTCGTGTCTCAG	Falginella et al. (2010)
VviF3'5'Hi	GCCAGAGACCACTCGATTAC	ACCCAGATTTTCTGGACGTG	Falginella et al. (2010)
VviFLS4	AAACCACCTACTTACAGAGC	ACCTAACCCCAGTGACAGAC	Fujita et al. (2006)
VviFLS5	AACCAAGATGACTAAGAACC	CTTCTGTGACTTCCCTGTAG	Fujita et al. (2006)
VviLDOX	AGGGAAGGGAAAACAAGTAG	ACTCTTTGGGGATTGACTGG	Jeong et al. (2004)

VviUFGT	AATCTGAGAGCCCTAAGAGA	GGTGGTACAAGCAACAGTTC	Goto-Yamamoto et al. (2002)
VviAOMT	CTCTGCAGGCGCCTCTATTA	CCCAAAACAGAGTCTGGACA	Hugueney et al. (2009)
Vvi3AT	AGTGAGTCGCGAGGATGTGTTGT	TCCAAGCAGGATTTCCCCAACCA	Rinaldo et al. (2015)
VviGST4	ACTTGGTGAAGGAAGCTGGA	TTGGAAAGGTGCATACATGG	Terrier et al. (2005)
VviAM3	GCAAACAACAGAGAGGATGC	AGACCTCGACAATGATCTTAC	Gomez et al. (2009)
VviMybA	GAGGGTGATTTTCCATTTGAT	CAAGAACAACTTTTGAACTTAAACAT	Walker et al. (2007)
VviMybF1	GGAGGTTGAGGGGTTGTG	AAGTTGGGGAAGAGCAGGAG	Czemmel et al. (2009)

Chapter 3: Results

Section 3.1 Temperature effects on canopy growth and berry development

At harvest, grapevines exposed to high temperature regimes (i.e., 30/25, 30/20, 25/15) had a higher leaf area than the ones exposed to low temperature regime (i.e., 20/10) in Experiment 1 (**Table 3.1**). Leaf area at harvest was not affected by temperature regimes in Experiment 2. Grapevine yield at harvest was higher in 20/15 regime than in 35/30 and 35/25 regimes in Experiment 1, while no differences in the yield were observed in Experiment 2. The leaf area to grapevine yield ratio was lower in 20/10 and 20/15 regimes than in 35/25 and 35/30 regimes in Experiment 1. In Experiment 2, the leaf area to cluster weight ratio was not affected by temperature regimes.

Table 3.1 Temperature effects on grapevine canopy and berry development at harvest in Experiment 1 and 2.

Experiment	Temperature regimes (day/night temperature, °C)	Total leaf area at harvest (cm^2)	Grapevine yield (g)	Total leaf area/grapevine yield (cm^2/g)	Average berry weight (g)	Average skin weight (mg)	Average seed weight (mg)	Skin to berry weight ratio	Seed to berry weight ratio
Experiment 1	20/10	2407.8 ± 273.7 b	87.8 ± 15.5 ab	29.8 ± 5.5 b	1.09 ± 0.07 ab	98.84 ± 7.17 a	38.74 ± 2.97	0.091 ± 0.003	0.081 ± 0.003 ab
	20/15	2627.5 ± 187.7 ab	107.9 ± 31.3 a	31.5 ± 10.2 b	1.19 ± 0.07 ab	97.31 ± 8.72 a	38.33 ± 2.22	0.082 ± 0.006	0.072 ± 0.006 ab
	25/15	2933.1 ± 314.3 a	88.0 ± 23.7 ab	41.9 ± 12.7 ab	1.29 ± 0.09 a	85.06 ± 7.64 b	41.09 ± 1.22	0.066 ± 0.005	0.068 ± 0.005 b
	35/25	2886.8 ± 138.2 a	54.4 ± 9.9 b	55.0 ± 8.6 a	0.96 ± 0.06 b	85.09 ± 10.79 b	36.97 ± 2.32	0.086 ± 0.011	0.079 ± 0.003ab
	35/30	3121.1 ± 80.5 a	58.9 ± 13.0 b	59.0 ± 9.3 a	0.93 ± 0.13 b	64.88 ± 2.94 c	39.82 ± 2.88	0.069 ± 0.012	0.088 ± 0.008 a
Experiment 2	20/10	3980.4 ± 245.2	326.5 ± 88.2	17.7 ± 7.7	1.15 ± 0.13	110.40 ± 4.68 a	39.37 ± 1.67	0.097 ± 0.008 a	0.078 ± 0.008 a
	20/15	3892.7 ± 429.3	236.8 ± 71.1	21.1 ± 6.6	0.98 ± 0.05	97.33 ± 4.58 ab	37.74 ± 2.09	0.099 ± 0.006 a	0.073 ± 0.003 ab
	25/15	3905.0 ± 276.9	158.7 ± 49.8	24.2 ± 5.2	1.28 ± 0.013	97.61 ± 2.16 ab	37.91 ± 2.01	0.076 ± 0.002 b	0.056 ± 0.002 b
	25/20	4013.5 ± 232.8	209.5 ± 57.0	23.3 ± 5.4	1.28 ± 0.19	100.94 ± 4.12 ab	37.52 ± 2.54	0.079 ± 0.001 b	0.070 ± 0.008 ab
	30/20	3916.6 ± 168.4	284.7 ± 60.5	16.0 ± 3.5	1.12 ± 0.12	77.87 ± 3.46 b	37.20 ± 1.31	0.070 ±0.003 b	0.071 ± 0.007 ab

Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each column in each experiment according to an LSD test ($p \leq 0.05$).

Berry weight at harvest was generally not affected by temperature regimes, except for 25/15 regime in Experiment 1, that had a larger berry than 35/25 and 35/30 regimes (**Table 3.1**). In Experiment 1, skin weight was higher in 20/10 and 20/15 regimes than in any other regimes. In Experiment 2, skin weight was higher in 20/10 than in 30/20 berries. Seed weight at harvest was not affected by temperature regimes in both experiments. At harvest, no difference in the skin to berry weight ratio was found in Experiment 1 while a higher ratio was found for 20/10 and 20/15 berries than for 25/15, 25/20, and 30/20 berries in Experiment 2. The seed to berry weight ratio at harvest was affected by temperature regimes in both experiments. In Experiment 1, 25/15 berries had lower seed to berry weight ratio than 35/30 berries. In Experiment 2, 25/15 berries had lower seed to berry weight ratio than 20/10 berries.

The difference between day and night temperatures (ΔT = 5 or 10 °C) did not result in differences in leaf area, grapevine yield, and berry weight. The only difference observed was in the skin to berry weight ratio in Experiment 2. With the same night temperature (15 °C) applied, berries exposed to the regime with a smaller difference between day and night temperatures (i.e., 20/15, ΔT = 5 °C) had a higher ratio of skin to berry weight than the berries exposed to the regime with a larger difference (i.e., 25/15, ΔT = 10 °C).

Section 3.2 Temperature effects on grapevine leaf gas exchange

Grapevine photosynthesis rate (A), stomatal conductance (g_s), and transpiration rate (E) showed similar trends among different temperature regimes in Experiment 1 (**Appendix 4**). The lowest A was found in the highest temperature regime 35/30 at 78 DAA; other than that, no differences were observed. A similar trend for g_s and E was found as for A at 78 DAA. At 107

DAA, A, g_s, and E were lower in 20/10 and 20/15 regimes than any other regimes. The 35/30 regime showed a higher rate of E than 25/15 and 35/25 regimes, but not for A or g_s.

In Experiment 2, A was not affected by temperature regimes at 83 and 118 DAA. At 104 DAA, high temperature regime (i.e., 30/20) showed lower A than low temperature regimes (i.e., 20/10 and 20/15) (**Appendix 5**). The g_s and E showed similar trends at all stages measured but differences among temperature regimes were observed only at 83 DAA, when 25/15 regime had the highest g_s and E and the high temperature regime 30/20 had the lowest; and at 118 DAA, when 25/15 and 25/20 regimes had higher E than 20/10 and 20/15 regimes.

Section 3.3 Temperature effects on sugar and acid accumulation

At harvest, TSS accumulation in Merlot berries was not affected by either temperature regimes or by the difference between day and night temperatures in Experiment 1, while showed a higher level in 20/15 berries than 25/20 berries in Experiment 2 (**Table 3.2**). During berry ripening, 20/15 berries had higher TSS than 35/30 berries at 104 DAA in Experiment 1 (**Figure 3.1 A**), while in Experiment 2, 20/10 and 20/15 berries had higher TSS than 25/20 berries at 113 DAA (**Figure 3.1 B, Table 3.2**).

Table 3.2 Temperature effects on berry total soluble solids, titratable acidity, and pH at harvest in Experiment 1 and 2

Experiment	Temperature regimes (day/night temperature, °C)	TSS (ºBrix)	TA (g/L)	pH
Experiment 1	20/10	19.26 ± 0.55	6.34 ± 0.16 a	3.55 ± 0.03 d
	20/15	20.64 ± 1.01	6.14 ± 0.15 a	3.72 ± 0.04 c
	25/15	19.85 ± 1.17	5.47 ± 0.12 b	3.86 ± 0.04 b
	35/25	19.38 ± 0.79	4.80 ± 0.02 c	4.22 ± 0.01 a
	35/30	17.33 ± 0.83	4.75 ± 0.27 c	4.26 ± 0.06 a
Experiment 2	20/10	20.65 ± 0.85 ab	7.02 ± 0.11 a	3.45 ± 0.07 c
	20/15	20.78 ± 0.84 a	5.98 ± 0.16 b	3.69 ± 0.07 bc
	25/15	20.50 ± 0.16 ab	4.70 ± 0.24 c	3.91 ± 0.01 ab
	25/20	18.18 ± 1.14 b	5.05 ± 0.50 c	3.92 ± 0.01 ab
	30/20	19.11 ± 0.52 ab	4.63 ± 0.35 c	4.14 ± 0.02 a

Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$).

Titratable acidity was significantly and consistently affected by temperature regimes in both experiments. Soon after treatment application (16 days in Experiment 1, 1.e., 76 DAA; 10 days in Experiment 2, i.e., 79 DAA), low temperature regimes (i.e., 20/10 and 20/15) displayed the highest berry TA levels until harvest (**Figure 3.1 C and D, Table 3.2**). The difference between day and night temperatures only influenced TA in low temperature regimes. In Experiment 1, at 20°C day temperature, a larger difference between day and night temperatures (i.e., 20/10, ΔT = 10 °C) determined a higher TA than a smaller delta (i.e., 20/15, ΔT = 5 °C) at 76 and 89 DAA. The same result was found at harvest in Experiment 2. While at 15°C night temperature, TA was constantly higher in the regime with a smaller delta (i.e., 20/15, ΔT = 5 °C) than the one with a larger delta (i.e., 25/15, ΔT = 10 °C) in both experiments.

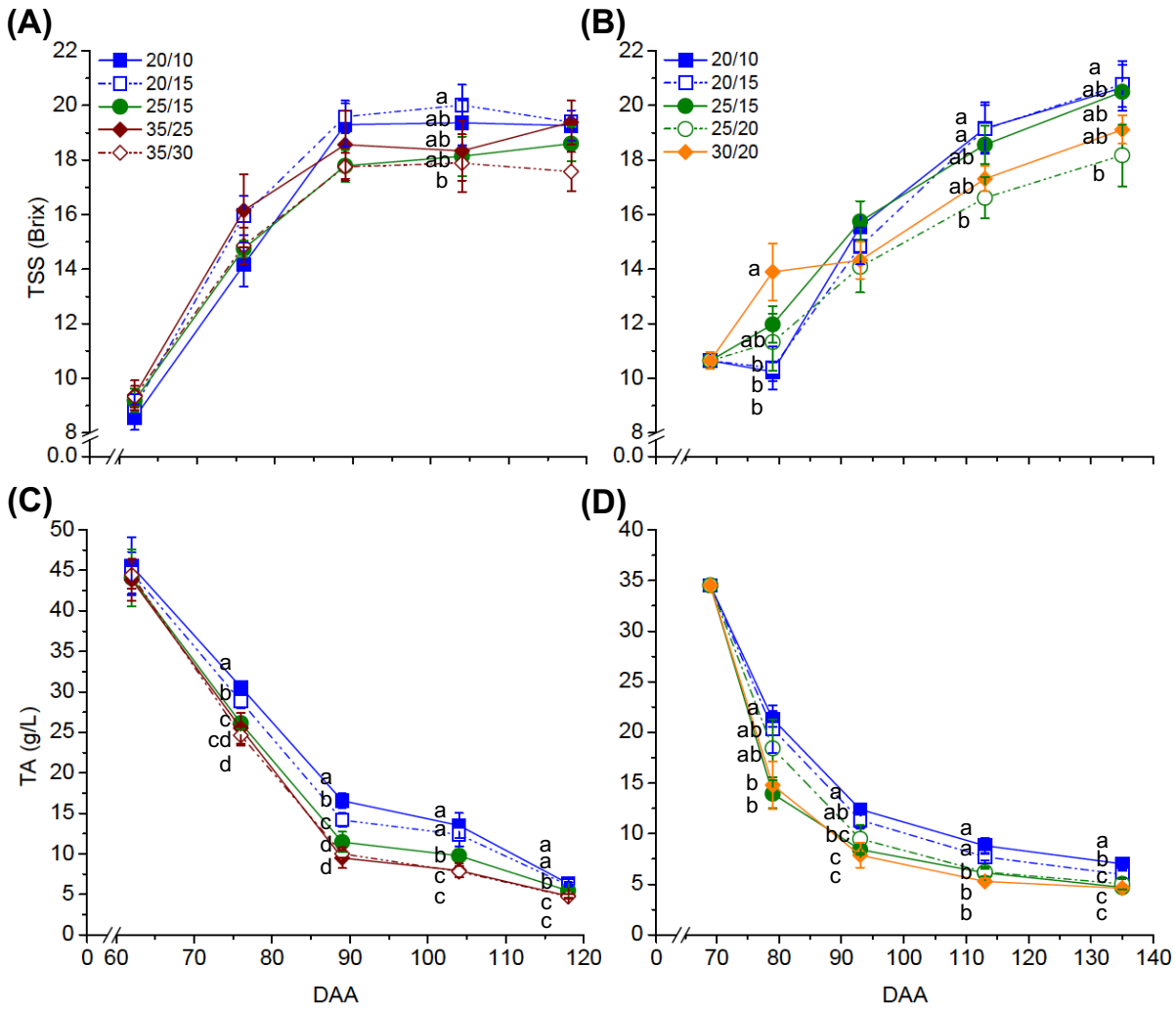


Figure 3.1 Temperature effects on total soluble solids (A and B) and titratable acidity (C and D) in Merlot grapes in Experiment 1 (A and C) and 2 (B and D). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A and C; legends in B indicated the treatments in B and D.

The response of juice pH to temperature regimes mostly reflected what has been observed for TA (**Table 3.2**). In Experiment 1, high temperature regimes (i.e., 35/25 and 35/30) determined the highest pH values from 76 DAA onward, with no observed effects of the difference between day and night temperatures when grapevines were exposed to the same day temperature (**Figure 3.2 A**). The low temperature regimes (i.e., 20/10 and 20/15) had the lowest pH, and no differences between the two treatments were observed except for 89 DAA, when 20/15 berries had higher a pH than 20/10 berries. When regimes with 15°C night temperature were compared, the difference

between day and night temperatures exerted a significant effect on pH with constantly higher pH values in regimes with a larger temperature difference (i.e., 25/15, ΔT = 10 °C) than a smaller temperature difference (i.e., 20/15, ΔT = 5 °C). In Experiment 2, similar trends were observed, since increases in berry pH paralleled the increases in average daily temperatures (**Figure 3.2 B**). The difference between day and night temperatures did not affect berry pH when the same day temperatures were considered (e.g., 20/10 versus 20/15; 25/15 versus 25/20, **Figure 3.2 B**). When regimes with 15°C night temperature were compared, pH values were higher in regimes with a larger temperature difference (i.e., 25/15, ΔT = 10 °C) than a smaller temperature difference (i.e., 20/15, ΔT = 5 °C), but irrespective to Experiment 1, the differences were not statistically significant.

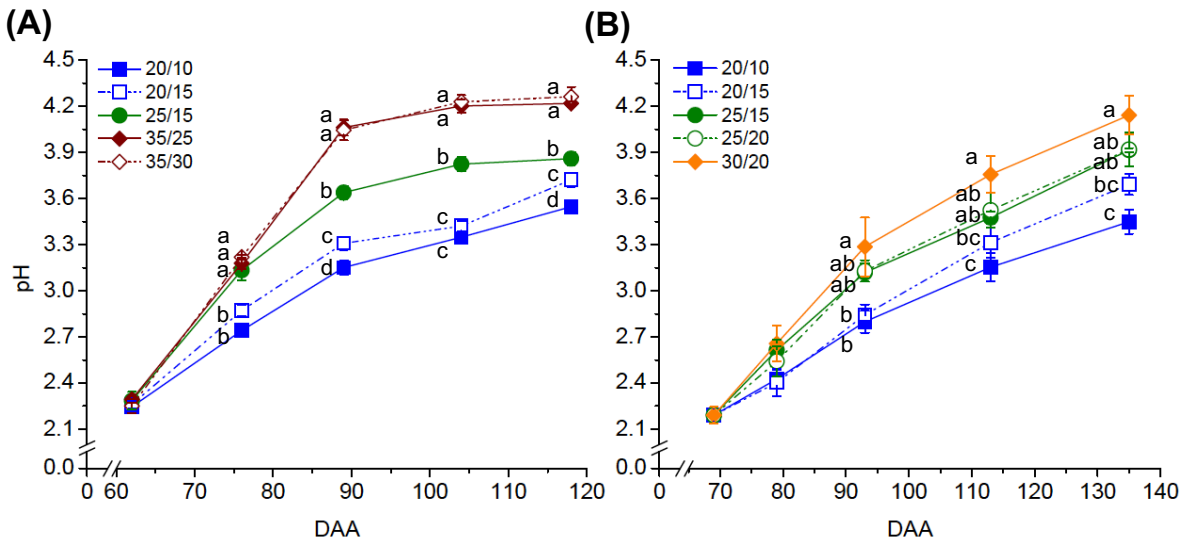


Figure 3.2 Temperature effects on pH in Merlot grapes in Experiment 1 (A) and 2 (B). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$).

Section 3.4 Temperature effects on anthocyanin accumulation

Anthocyanin concentration (expressed as µg/g skin FW) were greatly affected by temperature regimes (**Figure 3.3 A and B**). In both experiments, anthocyanin concentration was

maximised at low temperature regimes (i.e., 20/10 and 20/15). Differences between these two temperature regimes and the others were observed from 104 DAA to harvest in Experiment 1 and from 113 DAA to harvest in Experiment 2. Throughout ripening, no significant differences were observed between the anthocyanin concentration in 20/10 and 20/15 berries. In Experiment 1, the regimes with a high day temperature (i.e., 35/25 and 35/30) demonstrated the lowest anthocyanin concentration in the berries (-62.1% relative to 20/10 and 20/15 regimes at harvest, on average); differences with the other regimes were observed from 89 DAA for 35/25 regime and from 76 DAA for 35/30 regime. In Experiment 2, 30/20 berries had the lowest anthocyanin concentration in comparison to the other four temperature regimes; differences were observed from 113 DAA to harvest. In both experiments, temperature regimes with intermediate day temperature (i.e., 25/15 in Experiment 1; 25/15 and 25/20 in Experiment 2) had intermediate levels of anthocyanin concentration in comparison to regimes with a higher or lower day temperature. Interestingly, at the first sampling date after the vines were placed into the growth chambers, a transient higher anthocyanin concentration was observed in 35/25 regime than 20/10 regime in Experiment 1 and in 30/20 and 25/20 regimes than in 20/10, 20/15, and 25/15 regimes in Experiment 2.

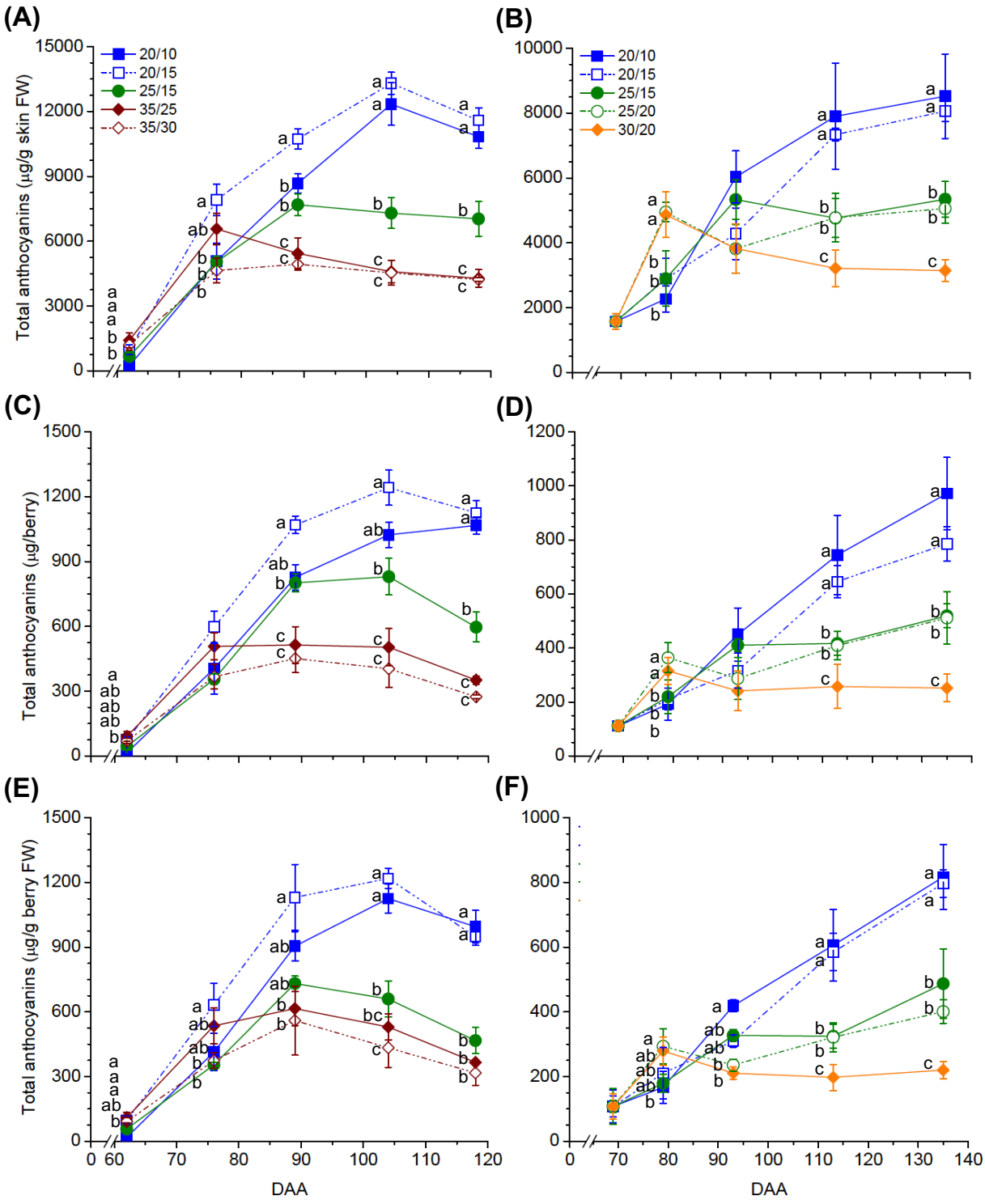


Figure 3.3 Temperature effects on anthocyanin concentration (µg/g skin FW, A and B; µg/g berry FW, E and F) and content (µg/berry, C and D) in Merlot grapes in Experiment 1 (A, C, and E) and 2 (B, D, and F). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significant different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A, C, and E; legends in B indicated the treatments in B, D, and F.

When the temperature regimes with the same day temperature but different night temperatures were compared (i.e., 20/10 versus 20/15, 35/25 versus 35/30 in Experiment 1; 20/10 versus 20/15, 25/15 versus 25/20 in Experiment 2), no differences in anthocyanin concentration were observed, except for 76 DAA in Experiment 1, when the concentration was lower in 20/10 berries than in 20/15 berries. When regimes with the same night temperature were compared, significant differences in the anthocyanin concentration were observed; a smaller difference between day and night temperatures (i.e., 20/15, $\Delta T = 5$ °C versus 25/15, $\Delta T = 10$ °C in Experiment 1; 20/15, $\Delta T = 5$ °C versus 25/15, $\Delta T = 10$ °C; 25/20, $\Delta T = 5$ °C versus 30/20, $\Delta T = 10$ °C in Experiment 2) determined a higher anthocyanin concentration. Differences between these regimes were remarkable; for example, in Experiment 1, anthocyanin concentration in 20/15 berries was 39.3% higher than in 25/15 berries at harvest. Similarly, in Experiment 2, the 20/15 berries had 33.7% higher anthocyanin concentration than 25/15 berries, while 25/20 berries had 37.9% higher concentration than 30/20 berries at harvest.

Similar effects of the temperature regimes and the difference between day and night temperatures were observed in anthocyanin content expressed as per berry weight (µg/berry, **Figure 3.3 C and D**) and in anthocyanin concentration expressed as per gram of berry FW (µg/g berry FW, **Figure 3.3 E and F**).

Section 3.5 Temperature effects on anthocyanin profiles

At each sampling point, individual anthocyanins were quantified (**Appendix 8-14**). Fifteen anthocyanins were profiled in Merlot berry skin (**Table 3.3**, calculated by concentration, µg/g skin FW; **Appendix 6**, calculated by content, µg/berry; **Appendix 7**, calculated by concentration, µg/g berry FW).

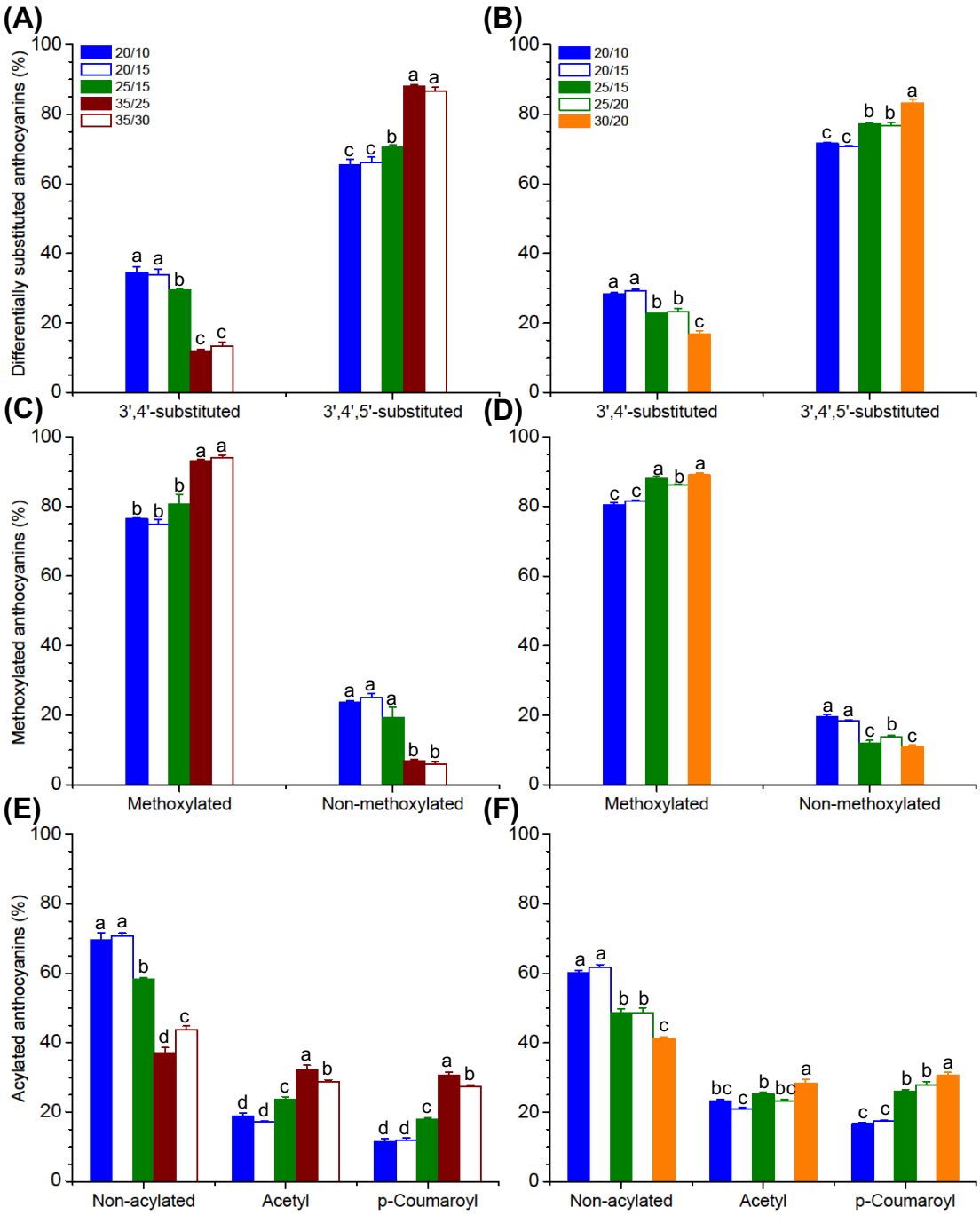


Figure 3.4 Temperature effects on the relative level of substitution, methoxylation, and acylation of anthocyanins at harvest in Experiment 1(A, C, and E) and 2 (B, D, and F). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate different means according to an LSD test ($p \leq 0.05$). Legends in A indicate the treatments in A, C, and E; legends in B indicate the treatments in B, D, and F. Non-acylated anthocyanins represent the sum of 3-O-monoglucosides; Acetyl anthocyanins represent the sum of 3-O-(6''-acetyl)glucosides; *p*-Coumaroyl anthocyanins represent for the sum of 3-O-(6''-*p*-coumaroyl)glucosides.

Temperature regimes greatly affected the relative concentration of various anthocyanin subfamilies; the proportion of 3'4'5'-substituted, methoxylated, and acylated (acetyl- and *p*-coumaroyl-) anthocyanins increased in high temperature regimes at harvest (**Figure 3.4**). In detail, among the fifteen anthocyanins profiled, the relative contribution of 3'4'5'-substituted anthocyanins (delphinidin, petunidin, and malvidin) at harvest was higher in the 35/25 and 35/30 berries than in the berries of any other temperature regimes in Experiment 1 and in the 30/20 berries than in the berries of any other temperature regimes in Experiment 2. Differences among treatments in 3'4'5'-substituted anthocyanin proportion were remarkable; in Experiment 1, the proportions of 3'4'5'-substituted anthocyanins in 35/25 and 35/30 berries were 88.1% and 86.7%, respectively, while in 20/10 and 20/15 berries were 65.5% and 66.1%, respectively (**Figure 3.4 A**). Similarly, in Experiment 2, 30/20 berries had the highest relative concentration of 3'4'5'-substituted anthocyanins (83.3%), 25/15 and 25/20 berries had intermediate relative concentrations (77.0% on average), and 20/10 and 20/15 berries had the lowest (70.1% on average) (**Figure 3.4 B**).

In Experiment 1, the relative contribution of methoxylated anthocyanins was higher in 35/25 and 35/30 berries (93.1% and 94.1%, respectively) than in the berries from other three regimes, where comparable levels of methoxylated anthocyanin proportion among them (77.3% on average) (**Figure 3.4 C**). In Experiment 2, this trend was less obvious, although 25/15, 25/20, and 30/20 berries had higher relative concentrations of methoxylated anthocyanins than 20/10 and 20/15 (**Figure 3.4 D**).

As expected, anthocyanins were detected as 3-O-monoglucosides, 3-O-(6"-acetyl)glucosides, and 3-O-(6"-*p*-coumaroyl)glucosides, which accounted for 55.9%, 24.2%, and 19.9% of the total anthocyanins at harvest in Experiment 1, respectively, and 52.1%, 24.2%, and

23.7% of total anthocyanins at harvest in Experiment 2, respectively (**Figure 3.4 E and F**). Temperature regimes also affected the level of anthocyanin acylation (**Figure 3.4 E and F**). In both the experiments, the relative concentration of acetyl and *p*-coumaroyl anthocyanins increased progressively with the increase in average daily temperatures. The only exception was the acetyl fraction in Experiment 2, where differences among temperature regimes with day temperature 20 and 25 °C (i.e., 20/10, 20/15, 25/15, and 25/15) were much less pronounced.

In Experiment 1, differences in the proportion of 3'4'5'-substituted (**Appendix 31 A**), methoxylated (**Appendix 31 C**), and acylated (**Appendix 30 A and C**) anthocyanins among temperature regimes were observed from 76 or 89 DAA onwards. In summary, high temperature regimes (i.e., 35/25 and 35/30) showed a higher proportion of 3'4'5'-substituted, methoxylated, and acylated anthocyanins throughout ripening. In Experiment 2, the predominance of 3'4'5'-substituted, methoxylated, and acylated anthocyanin proportion in the highest temperature regime (i.e., 30/20) was observed only at harvest for 3'4'5'-substituted (**Appendix 31 B**) and methoxylated anthocyanins (**Appendix 31 D**) and since 113 DAA for the acylated anthocyanins (**Appendix 30 B and D**).

As was observed for total anthocyanins levels, when subjected to the same day temperature, the difference between day and night temperatures (ΔT = 5 °C or 10 °C) showed little effect on the proportions of 3'4'5'-substituted (**Figure 3.4 A and B**), methoxylated (**Figure 3.4 C and D**), and acylated (**Figure 3.4 E and F**) anthocyanins at harvest, with two exceptions observed. The proportion of acylated anthocyanins was higher in 35/25 regime than in 35/30 regime in Experiment 1 and the proportion of methoxylated anthocyanins was higher in 25/15 regime than in 25/20 regime in Experiment 2. When regimes with the same night temperature but different day temperatures were compared, the relative concentrations of 3'4'5'-substituted, methoxylated, and

acylated anthocyanins were higher in temperature regimes with a larger difference between day and night temperatures (e.g., 25/15, ΔT = 10 °C versus 20/15, ΔT = 5 °C in Experiment 1; 25/15, ΔT = 10 °C versus 20/15, ΔT = 5 °C and 30/20, ΔT = 10 °C versus 25/20, ΔT = 5 °C in Experiment 2). The only exception was the methoxylated anthocyanin proportion observed in Experiment 1 that was not affected.

Table 3.3 Temperature effects on anthocyanin accumulation (concentration, μg/g skin FW) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡									
						Experiment 1					Experiment 2				
						20/10	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	909.4 ± 56.2 b †	1260.5 ± 45.7 a	551.7 ± 134.9 c	123.9 ± 2.6 d	117.1 ± 22.2 d	585.6 ± 93.5 a	365.0 ± 9.5 b	187.9 ± 34.9 bc	271.5 ± 13.3 bc	88.5 ± 8.3 c
2	Cy 3-O-glc	2.9	449	162	287	975.2 ± 49.6 a	981.0 ± 194.2 a	390.9 ± 93.4 b	44.0 ± 4.8 c	50.5 ± 3.6 c	415.2 ± 76.2 a	440.5 ± 31.5 a	106.0 ± 23.3 b	154.5 ± 13.5 b	105.5 ± 15.1 b
3	Pt 3-O-glc	3.4	479	162	317	964.6 ± 86.5 a	847.7 ± 71.2 a	448.3 ± 36.2 b	151.6 ± 17.7 c	151.9 ± 17.2 c	455.6 ± 105.4 a	454.4 ± 38.4 a	228.4 ± 47.4 b	205.2 ± 15.6 b	66.1 ± 21.1 c
4	Pe 3-O- glc	3.9	463	162	301	1944.6 ± 79.4 a	2064.2 ± 106.8 a	977.3 ± 47.4 b	266.9 ± 25.4 c	338.0 ± 39.5 c	1205.0 ± 143.9 a	1222.6 ± 55.7 a	627.5 ± 49.9 b	560.4 ± 33.0 b	208.2 ± 52.3 c
5	Mv 3-O- glc	4.4	493	162	331	2733.4 ± 80.4 a	3044.9 ± 58.0 a	1728.3 ± 139.1 b	995.4 ± 92.5 c	1193.6 ± 17.1 c	2438.8 ± 309.5 a	2481.5 ± 48.0 a	1465.2 ± 170.7 b	1259.2 ± 92.1 b	831.3 ± 59.8 c
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	328.0 ± 45.1 a	299.3 ± 13.8 a	235.9 ± 76.0 a	32.4 ± 2.0 b	19.7 ± 1.6 b	281.6 ± 86.7 a	208.0 ± 9.2 a	54.8 ± 13.6 b	35.8 ± 8.7 b	20.7 ± 6.2 b
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	140.9 ± 9.4 a	150.3 ± 29.9 a	95.1 ± 32.3 a	27.4 ± 3.2 b	18.7 ± 0.8 b	81.2 ± 24.8 a	57.6 ± 7.0 ab	24.6 ± 4.9 b	21.2 ± 3.2 b	15.9 ± 3.9 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	259.8 ± 41.4 a	176.0 ± 18.6 b	146.7 ± 24.4 b	45.7 ± 2.8 c	29.8 ± 4.4 c	185.7 ± 60.4 a	100.8 ± 6.1 ab	57.4 ± 10.9 b	36.8 ± 8.8 b	23.2 ± 4.8 b
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	304.3 ± 4.3 a	333.2 ± 23.5 a	245.3 ± 40.6 a	71.4 ± 12.7 b	65.9 ± 7.8 b	389.5 ± 48.4 a	323.8 ± 20.7 a	180.4 ± 9.3 b	125.8 ± 25.7 b	20.7 ± 3.1 c
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	1018.5 ± 111.5	1043.9 ± 62.3	961.1 ± 69.1	1205.2 ± 118.8	1081.1 ± 41.1	1056.1 ± 128.9	1003.6 ± 67.9	1022.2 ± 64.5	964.6 ± 76.6	794.8 ± 31.1
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	115.0 ± 21.6 a	103.7 ± 10.7 a	86.8 ± 17.3 ab	47.1 ± 9.7 bc	32.6 ± 3.4 c	276.4 ± 29.6 ab	366.7 ± 33.0 a	249.3 ± 20.4 b	181.4 ± 15.6 bc	103.5 ± 20.7 c
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	93.0 ± 7.9 ab	119.7 ± 14.7 a	70.8 ± 14.8 b	14.6 ± 1.6 c	9.9 ± 1.0 c	46.9 ± 10.3 ab	50.1 ± 6.7 a	30.8 ± 6.8 ab	38.2 ± 9.0 ab	14.5 ± 3.7 b
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	98.0 ± 18.5	88.4 ± 7.0	57.7 ± 6.1	90.9 ± 16.4	60.4 ± 5.4	73.7 ± 12.8 a	54.3 ± 2.2 ab	43.0 ± 6.6 ab	35.4 ± 9.3 b	28.9 ± 5.4 b
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	258.1 ± 7.2 a	306.5 ± 25.3 a	299.1 ± 36.0 a	85.7 ± 12.8 b	82.7 ± 6.3 b	286.1 ± 72.3 a	270.6 ± 12.8 a	243.9 ± 37.2 a	286.2 ± 63.3 a	169.3 ± 17.8 b
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	694.1 ± 101.9 b	774.6 ± 76.4 b	738.2 ± 46.8 b	1080.1 ± 122.5 a	971.2 ± 39.0 a	745.6 ± 116.0	660.9 ± 41.7	822.7 ± 51.9	881.6 ± 76.8	652.2 ± 91.4

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively. Glc=glucoside

‡ Anthocyanin peak area was integrated by UV-vis spectra at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

Section 3.6 Temperature effects on flavonol accumulation

Flavonol concentration (expressed as μg/g skin FW) was also considerably affected by temperature regimes (**Figure 3.5 A and B**) in Merlot berry skin. The highest flavonol concentrations were observed in low temperature regimes (i.e., 20/10 and 20/15). Differences between the two low temperature regimes and other regimes were observed from 76 DAA to harvest in Experiment 1 and from 93 DAA to harvest in Experiment 2. In Experiment 1, considering the two low temperature regimes, 20/15 regime exhibited higher flavonol concentration than 20/10 regime since 76 DAA. The lowest flavonol concentration was observed in high temperature regimes (i.e., 35/25 and 35/30) in Experiment 1, where the flavonol concentration was reduced by 73.8% (on average) at harvest, in comparison to 20/15 regime which had the highest concentration of flavonols at harvest. In Experiment 2, 25/15, 25/20, and 30/20 regimes had a comparable level of flavonol concentration at harvest (-46.6% compared to the low temperature regimes, i.e., 20/10 and 20/15, on average).

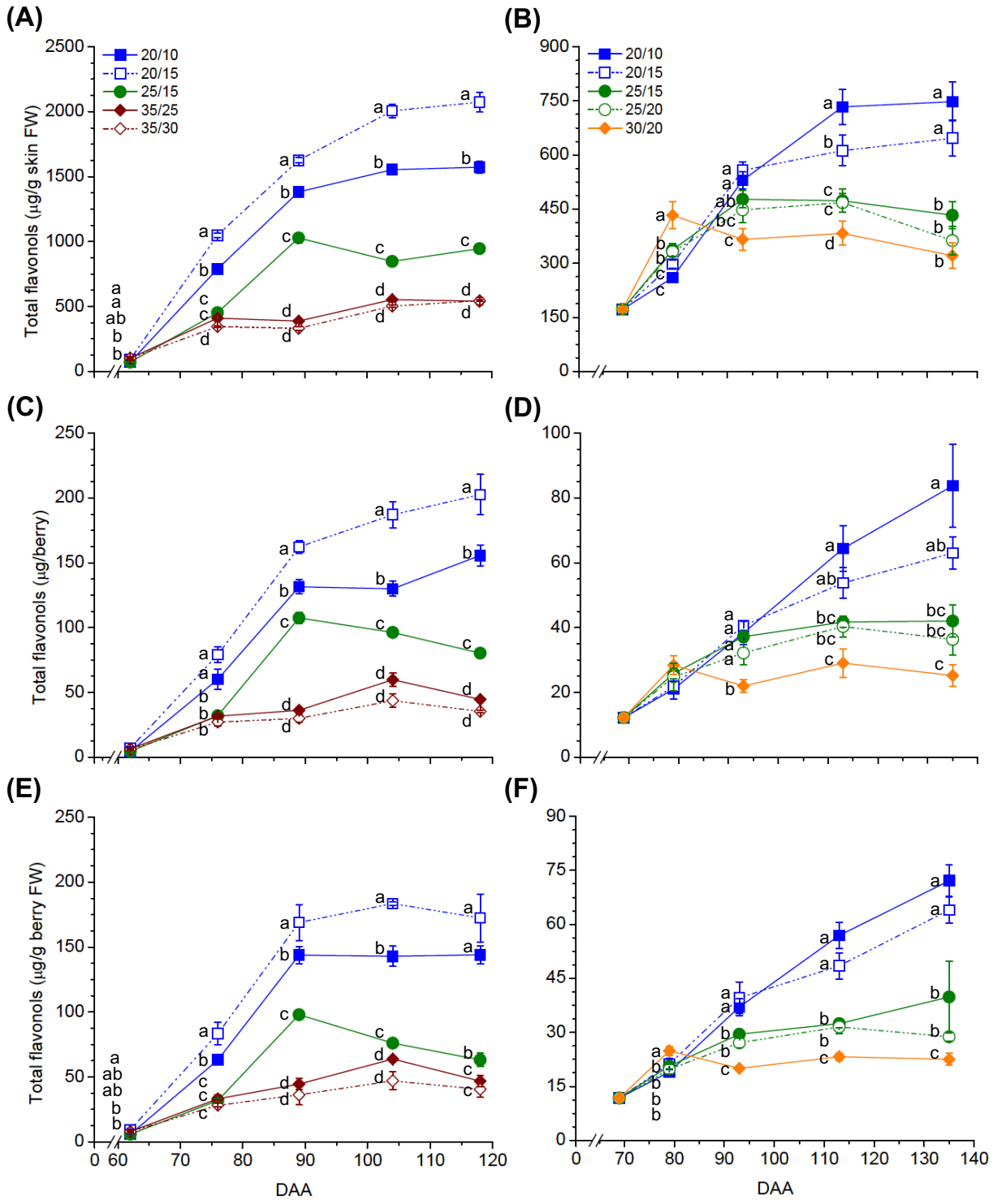


Figure 3.5 Temperature effects on flavonol concentration (µg/g skin FW, A and B; µg/g berry FW, E and F) and content (µg/berry, C and D) in Merlot grapes in Experiment 1 (A, C, and E) and 2 (B, D, and F). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A, C, and E; legends in B indicated the treatments in B, D, and F.

When temperature regimes with the same day temperature but different night temperatures were compared, higher flavonol concentration was observed in the low temperature regime with a smaller difference between day and night temperatures (20/15, ΔT = 5 °C) in comparison to the regime with the same day temperature but a larger difference between day and night temperatures (20/10, ΔT = 10 °C). This was observed throughout the season but only in Experiment 1 (**Figure 3.5 A**). On the other hand, when regimes with the same night temperature but different day temperatures were compared, a smaller difference between day and night temperatures (e.g., 20/15, ΔT = 5 °C versus 25/15, ΔT = 10 °C in Experiment 1 and 2) determined a higher flavonol concentration. Differences between these regimes were remarkable; for example, 20/15 regime had 54.4% higher flavonol concentration than 25/15 regime at harvest in Experiment 1. Similarly, in Experiment 2, 20/15 regime had 33.0% higher flavonol concentration than 25/15 regime at harvest. However, generally no differences were found between the 25/20 and 30/20 regimes in Experiment 2, except for the 103 DAA sampling point when flavonols were lower in 30/20 berries.

Similar effects of the temperature regimes and the difference between day and night temperatures were observed when the data were reported as flavonol content based on per berry weight (μg/berry, **Figure 3.5 C and D**) and flavonol concentration based on per gram of berry FW (μg/g berry FW, **Figure 3.5 E and F**).

Section 3.7 Temperature effects on flavonol profiles

At each sampling point, individual flavonols were quantified (**Appendix 17-23**). Eleven flavonols were profiled in Merlot berry skin (calculated by concentration, μg/g skin FW, **Table 3.4**; calculated by content, μg/berry, **Appendix 15**; calculated by concentration, μg/g berry FW, **Appendix 16**). Quercetin, myricetin, and kaempferol were the major flavonols, while isorhamnetin

and syringetin were accumulated at lower concentrations. In both experiments, quercetin was the most abundant flavonol at harvest (constituting 55.29% and 50.31% of total flavonols, in Experiment 1 and 2, respectively). Four forms of quercetin were detected, namely glucoside, glucuronide, galactoside, and (rhamnosyl)glucoside, with the first two being the majority (**Table 3.4)**.

Temperature regimes strongly affected the relative contribution of flavonol subfamilies, with the proportion of 3'4'5'-substituted and methoxylated flavonols that were increased in high temperature regimes (i.e., 35/25 and 35/30 in Experiment 1, 30/20 in Experiment 2) at harvest (**Figure 3.6**). Consistently, a lower proportion of 3'4'-substituted flavonols was observed in high temperature regimes at harvest, but no differences were observed among temperature regimes for 4'-substituted flavonol proportion. Particularly, the relative concentration of 3'4'5'-substituted flavonols (myricetin and syringetin) at harvest was remarkably higher in berries from 35/25 and 35/30 regimes in Experiment 1 and from 25/15, 25/20, and 30/20 regimes in Experiment 2, than in other temperature regimes considered (i.e., 20/10, 20/15, and 25/15 in Experiment 1, 20/10 and 20/15 in Experiment 2). For example, in Experiment 1, 3'4'5'-substituted relative fraction was 47.5% and 47.3% in 35/25 and 35/30 berries, respectively, while it was 23.8% and 23.0% in 20/10 and 20/15 berries, respectively. In Experiment 2, the relative concentration of 3'4'5'-substituted flavonols accounted for 42.6% of total flavonols in the 30/20 berries and for 28.5% of total flavonols in both 20/10 and 20/15 berries.

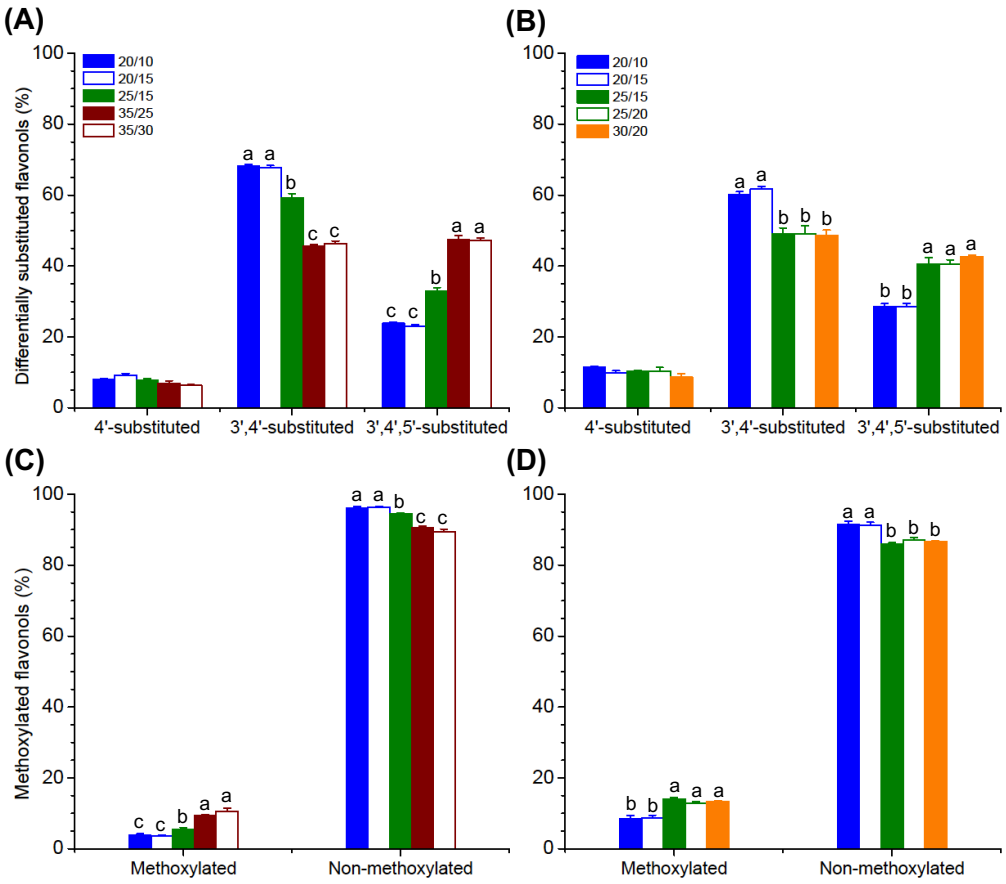


Figure 3.6 Temperature effects on the relative level of substitution and methoxylation of flavonols in Experiment 1 (A and C) and 2 (B and D). Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicate the treatments in A and C; legends in B indicate the treatments in B and D.

Temperature also affected the level of methoxylated flavonols. In Experiment 1, 35/25 and 35/30 berries had the highest proportion of methoxylated flavonols (9.4% and 10.6%, respectively), while 20/10 and 20/15 berries had the lowest (3.8% and 3.6%, respectively). Similar results were found in Experiment 2, but the trend was not as obvious as in Experiment 1. No differences were observed in the proportion of methoxylated flavonols among 25/15, 25/20, and 30/20 regimes, where the proportion of methoxylated flavonols was higher than in 20/10 and 20/15 regimes.

Table 3.4 Temperature effects on flavonol accumulation (concentration, μg/g skin FW) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M\text{-}H^+$)	Mass loss [($M\text{-}H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g skin FW) ‡									
						Experiment 1					Experiment2				
						20/10	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	35.2 ± 4.1 a †	37.9 ± 9.6 a	18.7 ± 3.5 b	13.2 ± 2.2 b	14.2 ± 4.2 b	22.6 ± 3.6 a	16.5 ± 0.5 ab	9.4 ± 1.6 b	9.0 ± 1.1 b	17.2 ± 1.3 ab
2	M-glc	3.9	479	162	317	94.9 ± 13.2 b	129.0 ± 11.8 a	90.0 ± 14.4 b	68.9 ± 10.1 c	66.8 ± 4.4 c	19.6 ± 2.9 a	15.6 ± 1.0 ab	9.4 ± 0.6 b	12.4 ± 1.5 ab	13.4 ± 2.0 ab
3	M-glcU	4.1	493	176	317	218.3 ± 13.5 b	281.5 ± 22.6 a	175.0 ± 18.8 c	131.7 ± 23.8 d	133.0 ± 16.0 d	133.5 ± 8.2 a	118.1 ± 6.2 ab	110.7 ± 9.6 bc	89.7 ± 3.9 c	74.9 ± 4.8 c
4	Q-gal	4.7	463	162	301	96.2 ± 12.1 b	115.0 ± 9.6 a	37.8 ± 5.0 c	27.3 ± 3.5 c	10.7 ± 2.9 d	28.8 ± 2.5 a	25.7 ± 2.79 a	8.0 ± 1.0b	7.1 ± 1.8 b	7.7 ± 0.9 b
5	Q-glcU	4.8	477	176	301	194.4 ± 7.0b	291.7 ± 62.9 a	126.3 ± 6.1 c	59.1 ± 5.0 d	48.4 ± 7.5 d	140.9 ± 8.4 a	124.5 ± 6.9 a	52.5 ± 10.6 b	54.9 ± 4.9 b	29.1 ± 2.4 b
6	Q-glc	4.9	463	162	301	685.2 ± 47.7 b	878.8 ± 76.4 a	333.7 ± 12.4 c	138.8 ± 22.4 d	164.9 ± 12.9 d	215.8 ± 11.9 a	205.1 ± 7.0 a	125.0 ± 20.5 b	95.3 ± 5.3 b	96.8 ± 6.1 b
7	K-(acetyl) glc	5.1	489	204	285	45.2 ± 4.1 b	69.6 ± 1.7 a	47.1 ± 5.0 b	26.2 ± 1.2 c	23.2 ± 2.1 c	57.5 ± 2.6 a	50.6 ± 3.7 a	37.6 ± 4.5 b	30.8 ± 2.5 b	19.5 ± 2.5 c
8	K-glc	5.8	447	162	285	80.5 ± 9.3 b	121.4 ± 11.1 a	27.3 ± 6.0 c	10.9 ± 2.1 d	11.2 ± 2.4 d	27.3 ± 2.2 a	13.1 ± 1.6 b	7.4 ± 1.6 c	6.2 ± 1.1 c	8.6 ± 1.7 bc
9	Q-(rhamnosyl) glc	6.0	609	308	301	62.7 ± 11.0 a	74.2 ± 14.0 a	38.7 ± 6.1 b	14.4 ± 3.1 c	14.9 ± 3.8 c	37.4 ± 5.4 a	20.8 ± 2.5 b	12.8 ± 2.7 bc	10.9 ± 1.8 c	11.0 ± 1.6 c
10	I-glc	6.3	477	162	315	34.3 ± 2.3 b	45.9 ± 2.2 a	22.5 ± 3.1 c	6.9 ± 1.1 e	13.9 ± 2.0 d	26.9 ± 6.2 a	22.4 ± 2.1 a	16.8 ± 2.7 ab	10.4 ± 1.3 b	11.4 ± 1.7 b
11	S-glc	6.5	507	162	345	26.1 ± 3.3 b	29.4 ± 3.8 b	28.8 ± 3.0 b	43.6 ± 2.7 a	43.9 ± 6.9 a	37.8 ± 4.9	34.0 ± 4.1	43.6 ± 3.6	36.0 ± 2.1	31.5 ± 1.0

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively.
‡ Flavonol peaks were identified using the MS fragmentation spectra, but the peak area was integrated by UV-vis spectra at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.
† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

In Experiment 1, 35/25 and 35/30 berries had the highest proportion of 3'4'5'-substituted (**Appendix 32 E**) and methoxylated (**Appendix 33 A**) flavonols from 76 and 62 DAA onwards, respectively. In Experiment 2, a higher proportion of methoxylated flavonols (**Appendix 33 B**) was observed in the berries from the high temperature regime (i.e., 30/20) than other regimes at 113 DAA. At harvest, no differences were observed in the proportion of either 3'4'5'-substituted (**Appendix 32 F**) or methoxylated (**Appendix 33 B**) flavonols between the berries of the high (i.e., 30/20) and intermediate temperature regimes (i.e., 25/15 and 25/20).

Temperature difference between day and night exerted no effect on the relative abundance of 3'4'5'-substituted (**Figure 3.6 A and B**) and methoxylated (**Figure 3.6 C and D**) flavonols when regimes with the same day temperature but different night temperatures were compared (e.g., 20/10 versus 20/15, 25/15 versus 25/20). When regimes with the same night temperature but different day temperatures were considered, a larger difference between day and night temperatures (e.g., 25/15, $\Delta T = 10$ °C) increased the relative concentration of 3'4'5'-substituted and methoxylated flavonols in comparison to the regime with a smaller difference (e.g., 20/15, $\Delta T = 5$ °C).

Section 3.8 Temperature effects on the expression of flavonoid genes

Fourteen genes involved in the flavonoid pathway were selected for assessing the impact of the temperature regimes on the expression of the flavonoid pathway genes at 76 and 89 DAA in Experiment 1 (**Figure 3.7**). No gene expression analysis was conducted for Experiment 2. The effect of temperature regimes on gene expression was tested in samples collected during the day and during the night cycle, in order to evaluate the effect of diurnal and nocturnal conditions on the expression of flavonoid genes.

Out of fourteen genes, three were not affected by the temperature regimes at any of the stages tested: *VviCHI1* (**Figure 3.7 A**), *VviGST4* (**Figure 3.7 L**), and *VviAM3* (**Figure 3.7 M**). The expression of six of the remaining genes tested was not affected by the timing of sampling (day or night). However, the expression of six genes - *VviF3'5'Hf* (**Figure 3.7 D**), *VviFLS5* (**Figure 3.7 G**), *VviUFGT* (**Figure 3.7 I**), *VviAOMT* (**Figure 3.7 J**), *VviAM3* (**Figure 3.7 M**), and *VviMybA* (**Figure 3.7 N**) - was induced at night, for at least one of the developmental stages.

The expression of *VviF3'Ha* (**Figure 3.7 B**) was affected by the temperature regimes at 76 DAA at both day and night, when it generally decreased in the expression level with increasing temperature, with the exception for 25/15 regime that did not differ from 20/15 and 20/10 regimes at night. Similar trends were observed at 89 DAA at day and night, but differences among regimes were not statistically significant.

The impact of temperature regimes on the expression patterns of the two *VviF3'5'H* genes tested (*VviF3'5'Hi* and *VviF3'5'Hf*) was similar (**Figure 3.7 C and D**). Differences among regimes were observed at 76 DAA, when during the day 20/10 regime demonstrated the highest expression level, while during the night the maximum expression level was demonstrated by 25/15 and 35/25 regimes. Interestingly, with both 35 and 20 °C day temperature, a smaller difference between day and night temperatures (i.e., 20/15 and 35/30, $\Delta T = 5$ °C) reduced the expression of both genes in comparison with a larger difference between day and night temperatures (i.e., 20/10 and 35/25, $\Delta T = 10$°C). Neither temperature regimes nor sampling time (day or night) showed significant effects on the expression of these two genes at 89 DAA. Noticeably, the ratio of *VviF3'5'H* (cumulative values of *VviF3'5'Hi* and *VviF3'5'Hf*) to *VviF3'Ha* expression was increased at high temperature regimes (i.e., 35/25 and 35/30), consistent with the increased ratio between 3'4'5'- to 3'4'-substituted anthocyanins observed for the same regimes.

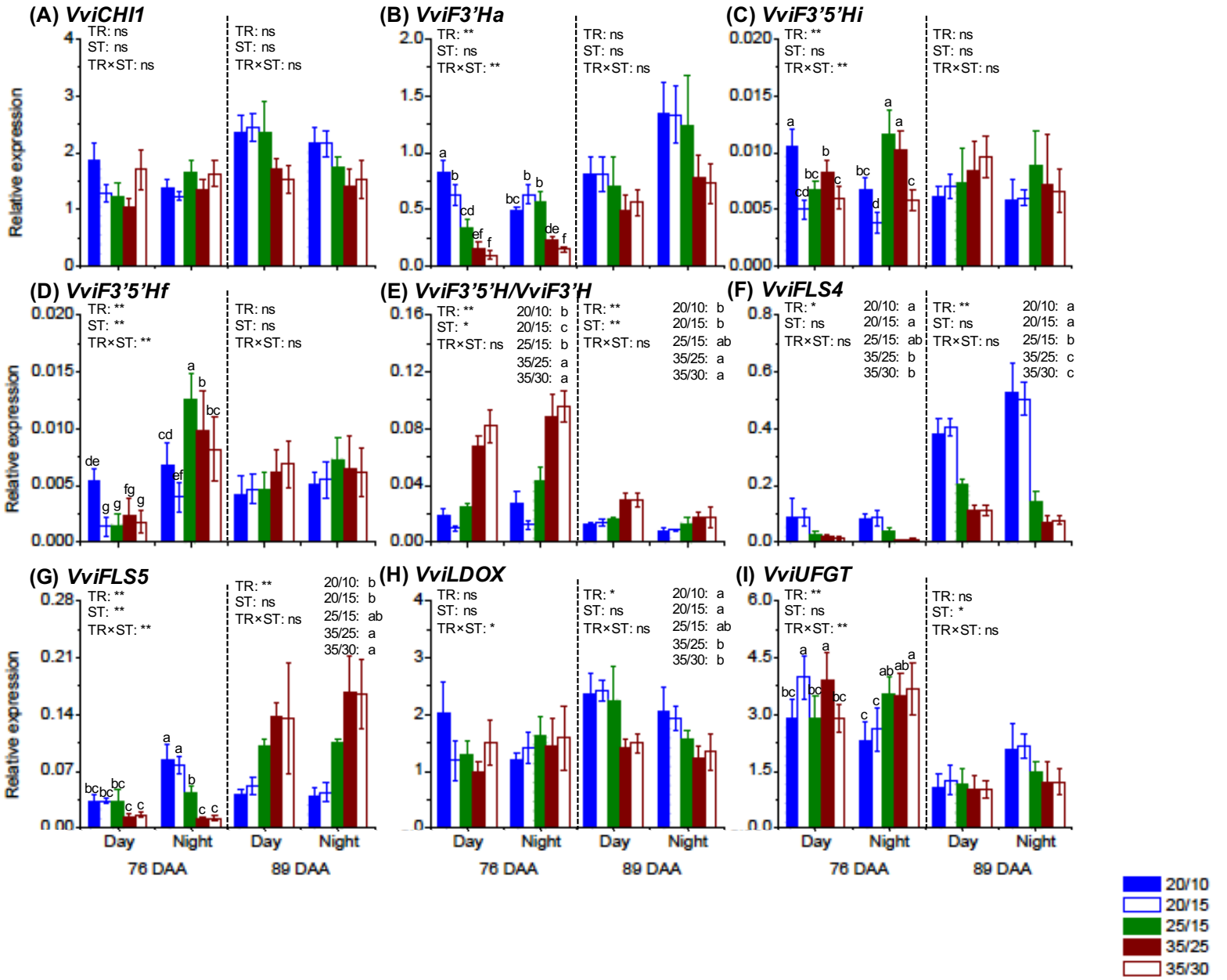
The expression of the two *VviFLS* tested (*VviFLS4* and *VviFLS5*) were differentially regulated by temperature regimes (**Figure 3.7 F and G**). High temperature regimes (i.e., 35/25 and 35/30) down-regulated the expression of *VviFLS4* at day and night, both at 76 and 89 DAA. Similarly, the expression levels of *VviFLS5* were down-regulated by the same regimes at 76 DAA; however, it exhibited an opposite pattern at 89 DAA. The difference between day and night temperatures did not affect the expression of either of the two genes when regimes with the same day temperature but different night temperatures were compared (e.g., 20/10 versus 20/15, 35/25 versus 35/30). On the contrary, when regimes with the same night temperature were considered, the temperature regime with a larger temperature difference between day and night (i.e., 25/15, $\Delta T = 10$ °C) showed lower *VviFLS4* expression than the one with a smaller temperature difference (i.e., 20/15, $\Delta T = 5$ °C).

The expression of *VviLDOX* **(Figure 3.7 H**) was not affected by temperature regimes at 76 DAA, but it was down-regulated in high temperature regimes (i.e., 35/25 and 35/30) at 89 DAA.

VviUFGT was expressed at higher levels in 20/15 and 35/25 berries than in berries from other regimes at 76 DAA during the day, and in 25/15, 35/25, 35/30 berries at night. Despite the fact that there was a trend toward higher expression levels in 20/15 and 20/10 berries than in berries of 35/25 and 35/30 at 89 DAA at night, the differences were not significant.

The expression level of *VviAOMT* (**Figure 3.7 J**) was the highest in 35/25 berries and the lowest in 20/15 berries during the day at 76 DAA. Neither temperature regimes nor sampling time affected the expression at 89 DAA. On the other hand, the expression of *Vvi3AT* (**Figure 3.7 K**) was consistently promoted by high temperature regimes (i.e., 35/25 and 35/30), particularly at 89 DAA.

Two key transcription factors that regulate flavonol and anthocyanin synthesis were tested. The anthocyanin biosynthetic regulator, *VviMybA*, exhibited a consistent expression pattern with *VviUFGT* (**Figure 3.6 N**). Interestingly, despite an interaction effect between temperature regimes × sampling time (day or night) on the expression level being observed at 76 DAA, *VviMybA* was generally expressed at higher levels in low temperature regimes (i.e., 20/10 and 20/15) during the day, and in the intermediate and high temperature regimes (i.e., 25/15, 35/25, and 35/30) at night. At 89 DAA at night, *VviMybA* expression was ~2 fold higher in the berries exposed to low temperature regimes (i.e., 20/10 and 20/15) than in the berries exposed to other regimes. The TF that regulates flavonol biosynthesis, *VviMybF1*, showed a similar expression pattern to *VviFLS4* (that is one of its targets), although the expression levels were much lower than the ones observed for *VviFLS4*, particularly in berries exposed to high temperature regimes (i.e., 35/25 and 35/30) (**Figure 3.6 O**).


(A) VviCHI1
TR: ns
ST: ns
TR×ST: ns
(B) VviF3'Ha
TR: **
ST: ns
TR×ST: **
(C) VviF3'5'Hi
TR: **
ST: ns
TR×ST: **
(D) VviF3'5'Hf
TR: **
ST: **
TR×ST: **
(E) VviF3'5'H/VviF3'H
TR: **
ST: *
TR×ST: ns
20/10: b
20/15: c
25/15: b
35/25: a
35/30: a
(F) VviFLS4
TR: *
ST: ns
TR×ST: ns
20/10: a
20/15: a
25/15: ab
35/25: b
35/30: b
(G) VviFLS5
TR: **
ST: **
TR×ST: **
20/10: b
20/15: b
25/15: ab
35/25: a
35/30: a
(H) VviLDOX
TR: ns
ST: ns
TR×ST: *
20/10: a
20/15: a
25/15: ab
35/25: b
35/30: b
(I) VviUFGT
TR: **
ST: ns
TR×ST: **
TR: ns
ST: *
TR×ST: ns
Relative expression
Day
Night
76 DAA
89 DAA
20/10
20/15
25/15
35/25
35/30

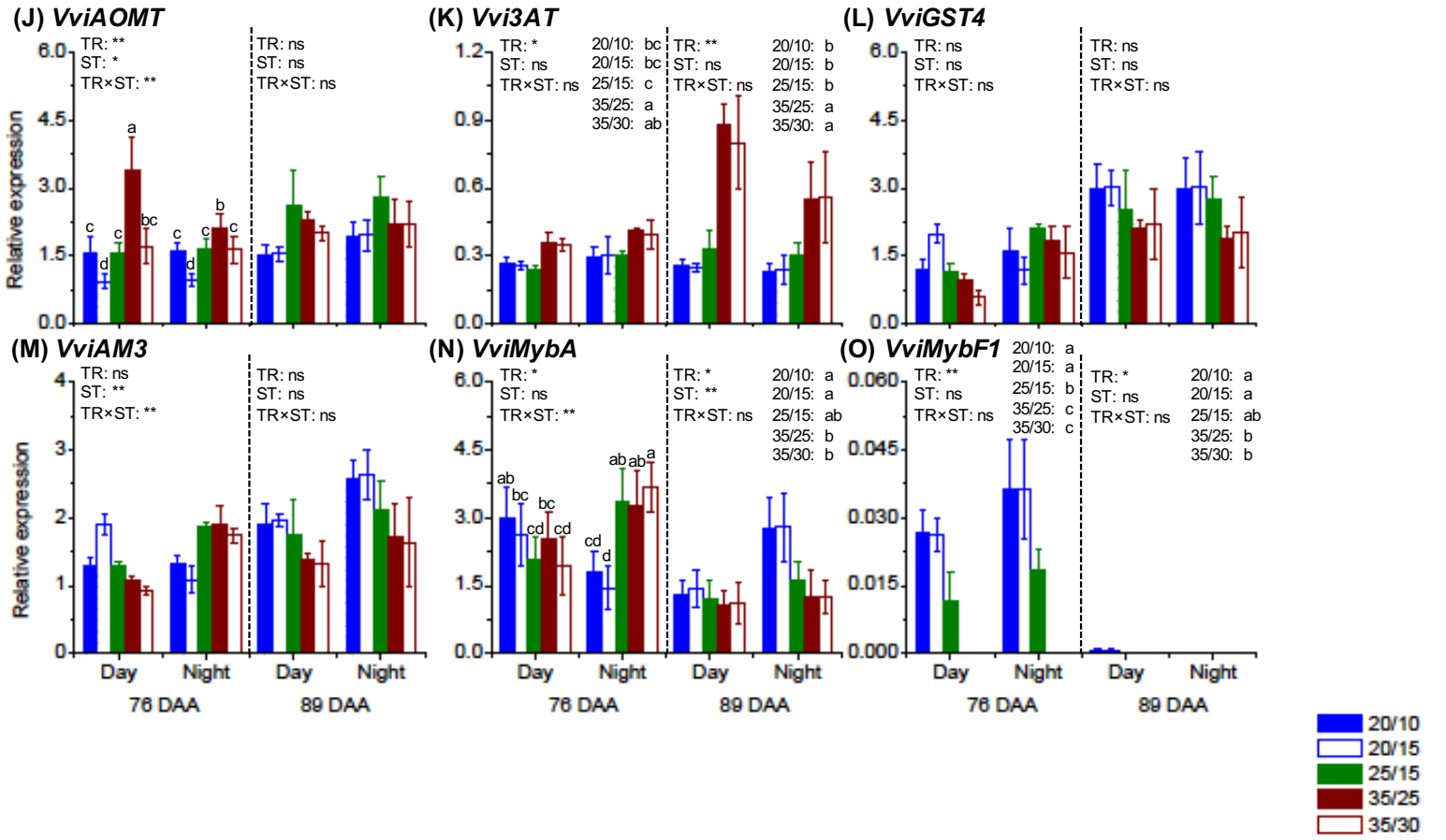


Figure 3.7 Expression analyses of flavonoid genes in Experiment 1. Values were reported as relative expression levels to the expression of the reference gene *VviUbiquitin*. TR and ST indicate temperature regimes and sampling time (between day and night), respectively. Values reported are the mean ± standard error (SE, n = 4). Two-way ANOVA was used to assess the effect of temperature regimes, sampling time, and the interaction effect between temperature regimes and sampling time. *, **, and ns stand for $p \leq 0.05$, $p \leq 0.01$, and not significant, respectively. Different letters indicate significantly different means at each time point according to an LSD test ($p \leq 0.05$).

Chapter 4: Discussion

In the present study, I examined the accumulation of anthocyanins and flavonols and the expression of critical genes involved in the flavonoid pathway in grape berries grown under several temperature regimes (20/10, 20/15, 25/15, 35/25, and 35/30 in Experiment 1; 20/10, 20/15, 25/15, 25/20, 30/20 in Experiment 2). I aimed to investigate how the temperature regimes as well as the difference between day and night temperatures affect anthocyanin and flavonol accumulation in the Merlot berry.

Section 4.1 Growth chamber set up

Two experiments were conducted using temperature-controlled growth chambers during berry ripening stage. The growing conditions of grapevine inside the growth chambers were monitored during berry ripening in both experiments (**Appendix 1 and 2**) to ensure the success of the temperature-controlled experiments. Light intensity around the clusters was similar in both experiments. RH was monitored at 6 h, 24 h, and 30 h after irrigation. The average RH was above 80% in Experiment 1 and 60-70% in Experiment 2, respectively, at 6 h after irrigation. This variation between experiments was possibly due to the higher number of plants in each growth chamber in Experiment 1, which might have resulted in higher amount of gross transpiration, although smaller pots were used. High temperature regimes (i.e., 30/20, 35/25, 35/30) had generally lower RH than the other temperature regimes tested. At 24 h and 30 h after irrigation, RH was 30-40% in high temperature regimes (i.e., 30/20, 35/25, 35/30), while it was 50-70% in the low temperature regimes (i.e., 20/10 and 20/15). Increase in RH have been reported to affect grapevine physiology and development. The increase in RH results in a decrease in water vapor pressure deficit, which might stimulate photosynthesis and plant growth (Bunce et al 1984; Shirke

and Pathre 2004; Ben-Asher et al 2013). However, there are no studies specifically on grapevine and particularly, there are no studies on the effect of RH on flavonoid accumulation hitherto. Shin et al. (2007 and 2008) investigated the effect of post-harvest RH on the level of flavonoid compounds in strawberries during storage, but showed inconsistent effects between the two studies. On the other side, the increase in RH increases the risk of pathogenesis through fungal or bacterial infection (Delp 1954; Thomas et al. 1988), which would induce the accumulation of phenylpropanoid compounds, such as viniferins and stilbene compounds (Emmett et al. 1992 in Downey et al. 2006). However, I did not observe any difference in pest development on the clusters among the temperature regimes, so I speculate that this indirect effect of RH can be ruled out in my experiments. The irrigation schedule and strategy were designed to avoid water stress events as confirmed by the leaf water potential measurement (**Appendix 3**). This measurement was conducted only a few times since it is a destructive measurement that would have reduced the canopies too severely if performed routinely. Although the RH was lower in high temperature regimes at 24 h and 30 h after irrigation, measurement of leaf water potential confirmed that there was no stress during the measurements as indicated by the values of leaf water potential above -1 MPa (Santesteban et al. 2011).

The variation between the temperature set in the growth chamber and the actual air temperature was less than 1% in Experiment 1 and less than 8% in Experiment 2. I speculate that the larger variation observed in Experiment 2 was due to issues with the temperature probe and the location of the temperature probes inside the growth chamber (e.g., under direct radiation or too close to the soil). Testing of the growth chamber temperatures before and after the Experiment 2 confirmed that, as observed in Experiment 1, air temperatures inside the chamber were close to the ones set in each growth chamber. The variation between the berry surface temperature and the

actual air temperature in the growth chamber was less than 2% in Experiment 1. Unfortunately, berry temperature was not tested in Experiment 2, due to the in availability of the infrared thermal gun which is necessary for berry surface temperature measurements. I speculate that, as was observed in Experiment 1, the berry surface temperature and air temperature in the growth chamber were similar in Experiment 2 as well.

Section 4.2 Regulation of total soluble solids and titratable acidity by temperature regimes

Total soluble solid level is a useful indicator of grape ripeness and quality (Du Plessis 1984). Since the sugars accumulated in the berry are synthesized in leaves through photosynthesis, the ratio of grapevine total leaf area to yield (cm^2/g) is considered a critical index for the determination of sugar level (Jackson and Lombard 1993). The ratio below which sugar starts to decline due to excessive crop yield was indicated to be 8-10 cm^2/g (Kliewer and Antcliff 1970; Smart 1985; Jackson and Lombard 1993; Kliewer and Dookoozlian 2005). The data recorded indicates that, in my experiment, this ratio was well above the aforementioned threshold and there should have been no limitation of sugar levels in the berry due to source: sink (leaves: berries) limitations for any of the temperature regimes.

At harvest, TSS was not affected by temperature regimes in Experiment 1 (**Table 3.2, Figure 3.1 A**). This was in agreement with previous studies (Bergqvist et al. 2001; Mori et al. 2005b; Rienth et al. 2016). However, Kliewer (1977b) reported a significantly lower TSS level in Emperor berries grown at 37/32 °C (day/night temperature) than the berries grown at 24/17 °C. The inconsistency with Kliewer (1977b) were probably due to the more extreme temperature conditions (37/32 °C) tested by Kliewer (1977b) than the ones tested in the current study (30/20,

35/25, and 35/30 as high temperature regimes), or possibly also due to the different grape varieties used in the two studies. Nevertheless, the overall trend of TSS during berry ripening in Experiment 1, as well as the difference in TSS observed between the regimes 20/10 and 25/20 at 113 DAA and at harvest in Experiment 2, indicate that low temperature regimes were preferable for sugar accumulation in Merlot berries. Interestingly, in Experiment 2, despite no significant difference among the temperature regimes being observed within each measurement date, a trend toward lower photosynthetic rates in 25/20 and 30/20 vines was observed, consistent with lower sugar levels detected in the berries of the same vines (**Appendix 5**). This indicates that, in growth chamber conditions, the effect of temperature regimes on the vine photosynthesis might play a key role in determining sugar levels in the berry.

The leaf photosynthetic rate was reduced by 35% in Semillon grapevines when they were exposed to a 40 °C day temperature for 4 days (Greer and Weston 2010). In my study, even though a similar trend being observed in Experiment 2, the effect of temperature regimes on photosynthesis was not always consistent among the sampling dates; with no significant effects of the temperature regimes on some dates, and a higher photosynthetic rate at high temperature regimes at 107 DAA in Experiment 1 (**Appendix 4**).

Moreover, the photosynthetic rates measured in my study were lower than the ones normally measured under field conditions (Downton et al. 1987). Similarly, Greer and Weston (2010) reported a maximum photosynthetic rate of 10 μmol CO_2 $m^{-2}s^{-1}$ (mostly at a rate of 3-5 μmol CO_2 $m^{-2}s^{-1}$) in vines grown in temperature-controlled chambers. The limitations of photosynthesis in growth chambers could possibly be attributed to a higher canopy density inside the growth chamber than that in the field conditions, which increased the competition for light

among leaves. Moreover, radiation conditions inside the growth chamber were lower than the ones normally experienced by grapevines in the field.

Despite the harvest date (end of the experiment) being at 118 DAA and 135 DAA in Experiment 1 and Experiment 2, respectively – similar to what has been reported for field experiments that consider Merlot grapes – the TSS at harvest were lower (17.33-20.78 °Brix) than the ones normally observed in field grown Merlot grapes (21-25 °Brix, Tarara et al. 2008; Herrera et al. 2015; Sivilotti et al. 2016). These lower TSS levels could be explained by a lower PAR available to vines in the growth chambers (~500 μmol m^2 s^{-1} PAR measured at the canopy level in the growth chamber versus >1200 μmol m^2 s^{-1} PAR in field conditions on sunny days), which might have limited the photosynthesis and possibly the translocation of sugars into the berry.

On the other hand, TA levels decreased along the ripening process and were similar to the ones observed in field studies (5-7 g/L of tartaric acid equivalents, Herrera et al. 2015; Sivilotti et al. 2016). High temperature regimes (i.e., 30/20, 35/25, and 35/30) enhanced the acid degradation, consistent with what was observed in the previous studies (Kliewer 1977b; Bergqvist et al. 2001; Mori et al. 2005b; Rienth et al. 2016).

Section 4.3 Relationship between sugar and anthocyanin accumulation

Sugars are reported to regulate the accumulation of anthocyanins in grape berry (Agasse et al. 2009) and an increase in the sugar level at the onset of ripening triggers anthocyanin synthesis (Dai et al. 2014). Since there was no difference observed for TSS at harvest in Experiment 1, and only the 25/20 regime was found to have significantly lower TSS than other regimes in Experiment 2, the reduced anthocyanin level observed in high temperature regimes (i.e., 30/20, 35/25, and 35/30) in the current study was not attributed to a delay in sugar accumulation. Linear regression

analysis revealed that there was a decoupling between sugar and anthocyanin levels under high temperature regimes, indicated by the significant difference between the coefficients of the linear regression curves of TSS an total anthocyanin concentration (expressed as µg/g skin FW) under different temperature regimes (**Appendix 28 and 29**). This also reaffirms that while the sugar accumulation was not strongly affected by high temperature regimes, the anthocyanin accumulation was greatly reduced (**Figure 4.1**). Sadras and Moran (2012, 2013) reported the decoupling of anthocyanin and sugar accumulation under elevated temperatures (1-3 °C increase from the control) during early stages of berry development which was attributed to a delayed onset of anthocyanin accumulation under high temperatures rather than to a different rate of anthocyanin accumulation during berry ripening. However, the decoupling observed in the current study may have resulted from a lower rate of anthocyanin accumulation or potentially a higher rate of anthocyanin degradation in high temperature regimes (Mori et al. 2007b), since the temperature regimes were applied at the onset of ripening and anthocyanin levels were actually higher in high temperature regimes (i.e., 30/20, 35/25, and 35/30) just after the application of the temperature regimes or at early stages of ripening (e.g., 62 DAA in Experiment 1 and 79 DAA in Experiment 2, **Figure 3.3**).

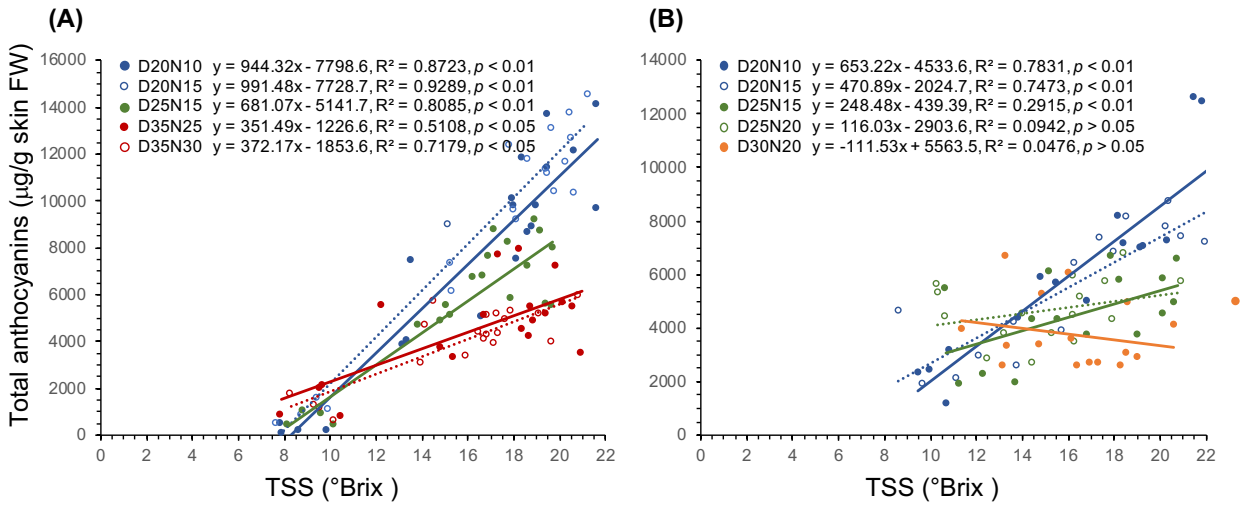


Figure 4.1 Linear regression analysis between sugar and anthocyanin accumulation during berry ripening in Merlot grapes exposed to different temperature regimes in Experiment 1 (A) and 2 (B).

Section 4.4 High temperature reduced anthocyanin accumulation

Anthocyanin levels (as reported by μg/g skin FW, **Figure 3.3 A-B**; mg/berry, **Figure 3.3 C-D**; μg/g berry FW, **Figure 3.3 E-F**) were strongly reduced by high temperature regimes (i.e., 30/20, 35/25, and 35/30), in comparison to low temperature regimes (i.e., 20/10 and 20/15), as well as intermediate temperature regimes (i.e., 25/15 and 25/20) that had intermediate anthocyanin levels. Mori et al. (2005a, 2007a) investigated the effect of increased day and night temperatures on the accumulation of anthocyanins in potted Pinot noir grapes and suggested that 30 °C night temperature (compared to 20 °C) greatly reduced total anthocyanin level in grape skin and shifted anthocyanin profiles (which will be discussed later), while a 35 °C day temperature (compared to 25 °C in control treatment) only shifted anthocyanin profiles. De Rosas et al. (2017) reported a ~40% and 28-41% (depending on the season) loss of total anthocyanins in Bonarda and Malbec berries grown under high temperatures (2.5-3 °C higher than control treatment), respectively. Reduced anthocyanin levels under high temperature regimes have also been reported in different species. Gazula et al. (2005) reported a progressive decrease in anthocyanin levels in lettuce grown under 20/20 °C (day/night temperature), 30/20 °C, and 30/30 °C. Ubi et al. (2006) indicated that storage temperature at 27 °C for 5 days significantly decreased anthocyanin accumulation in different apple varieties, in comparison to a storage temperature at 17 °C. Due to the variation in genetic background, different grape varieties as well as different crop species might have different response to temperature regimes; however, as an overall trend, the negative effect of elevated temperatures on anthocyanin accumulation has been well documented in grapes as well as in other species (Christie et al. 1994; Shvarts et al. 1997; Lo Piero et al. 2005; Tarara et al. 2008). The

current study indicates that the optimal day temperature for anthocyanin accumulation in Merlot grapes is 20 °C, when compared to 25 °C, 30 °C, and 35 °C (**Figure 3.3, Table 3. 3**).

Skin to berry weight ratio plays a major role in determining anthocyanin concentration in the berry (Wong et al. 2016). An increase in the ratio determines an increase in anthocyanin concentration (expressed, for instance, as μg/g berry FW), assuming a similar anthocyanin concentration in the skin. Despite a decrease in skin weight observed in high temperature regimes in both experiments, a decreased skin to berry weight ratio (**Table 3.1**) was observed in the intermediate and high temperature regimes (i.e., 25/15, 25/20, and 30/20 regimes) in Experiment 2. The lower relative amount of skin per berry might have partially contributed to the decreased concentration (μg/g berry FW) of anthocyanins (**Figure 3.3 E and F**). Not consistent with my study, a 0.9-1.1% increase in skin percentage (skin to berry weight ratio) was observed under high temperature regimes (2-3 °C higher than control which was ~25 °C during berry growing season) by Bonada et al. (2015), due to a reduced pulp mass resulting from the inhibition of pericarp cell expansion. The inconsistent results in my study with the previous study (Bonada et al. 2015) could have resulted from the much more extreme temperature regimes imposed in the current study. In the current study, the regimes defined as high temperature regimes (i.e., 30/20, 35/25 and 35/30) were 10-17 °C higher in average daily temperature than the ones defined as low temperature regimes (i.e., 20/10 and 20/15); whereas in Bonada et al. (2015), there was only a 2-3 °C temperature difference between the high temperature treatment and the control. Dokoozlian (2000) reported a decreased expansion of both exocarp and mesocarp cells under a high temperature treatment (~30 °C). Our data indicates that exocarp cells were probably more restricted than mesocarp cells, which resulted in the reduced proportion of skin to berry weight ratio in high temperature regimes (i.e. 30/20, 35/25, and 35/30).

Section 4.5 High temperature affected the expression of anthocyanin genes

To further investigate the molecular mechanism of anthocyanin accumulation under different temperatures, the expression of critical genes involved in the flavonoid pathway was assessed using samples collected at 76 and 89 DAA of Experiment 1. *VviUFGT* codifies for the key enzyme for anthocyanin accumulation (Boss et al. 1996; Kobayashi et al. 2001) and is regulated by *VviMybA* (Goto-Yamamoto et al. 2002). *VviUFGT* expression was higher in 20/15 and 35/25 berries than in other treatments during the day at 76 DAA, and these levels of expression were consistent with the higher anthocyanin levels measured in the same treatments at that specific sampling date. However, at the same developmental stage, the effect of the temperature regimes on the level of expression of the same gene at night was different than the one observed during the day and did not match the anthocyanin accumulation in the berry. Interestingly, at 89 DAA at night, the expression levels of both *VviUFGT* and *VviMybA* genes were lower (although not statistically different for *VviUFGT*) in the berries exposed to the high and intermediate temperature regimes (i.e., 25/15, 35/25, and 35/30) in comparison to low temperature regimes (i.e., 20/10 and 20/15), consistent with the reduced anthocyanin level in high temperature regimes, as described above. Although the anthocyanin accumulation was reduced by high temperatures in the current as well as previous studies, different expression patterns of *VviUFGT* under high temperatures have been documented. Rienth et al. (2014b) indicated that the expression of *VviUFGT* was up-regulated by two-hour heat stress at 37 °C during the day and speculated that the expression of *VviUFGT* escaped from the regulation of *VviMybA* due to the down-regulation of *VviMybA* expression under the same conditions. In the current study, a consistent expression pattern was observed between *VviUFGT* and *VviMybA* among different temperature regimes. Therefore, other mechanisms, such as post-transcriptional regulation, might be involved in determining the inconsistency between

anthocyanin accumulation and *VviUFGT* expression under different temperature regimes. For example, although Mori et al. (2007b) reported that the accumulation of anthocyanins was decreased in 35 °C berries compared to 25 °C berries, the transcript abundance of *VviUFGT* was not affected by the high temperature, while the activity of UFGT enzyme was increased in berries cultured at 35 °C at the same time. This reflected that the lower anthocyanin level under the high temperature condition might result from a higher degradation rate, which would be discussed in the following paragraph.

Recent studies have proposed that anthocyanin degrade under high temperature regimes (Movahed et al. 2016; Niu et al. 2017). It is now believed that anthocyanin accumulation depends on the turnover between biosynthesis and degradation (Niu et al. 2017; Liu et al. 2018). Mori et al. (2007b) have demonstrated by using isotope tracing that *in vitro* grape anthocyanins were more stable at 15 and 25 °C than at 35 °C. Chassy et al. (2015) reported similar results and proposed that the five unidentified compounds with increased concentration under high temperatures were potential degradation products of anthocyanins. Protocatechuic acid, phloroglucinol aldehyde (PHA), and 4-hydroxybenzonic acid have been reported as major degradation products of anthocyanins under thermal conditions in black carrot, strawberry, and elderberries (Sadilova et al. 2006, 2007). In wine, protocatechuic acid, vanillic acid, and syringic acid have been reported as the major degradation products of anthocyanins from human gastrointestinal digestion (Yang et al. 2018). The recent studies by Fang et al. (2015), Zipor et al. (2015), and Luo et al. (2017) identified anthocyanin degradation-related proteins in apples and *Brunfelsia calicina* flowers which shed light on the mechanism of anthocyanin degradation. However, the degradation products of anthocyanins under thermal conditions in grape berries has never been reported with *in vivo* studies. In the current study, potential anthocyanin degradation products, which share the same mass with

protocatechuic acid and PHA, have been detected by LC/MS ananlysis. However, in order to assess if the mass spectrum matches are actually protocatechuic acid or PHA, further analyses using authentic standards are needed. Moreover, whether these compounds are accumulated in the berry from the catabolism of anthocyanins or via other biosynthetic pathways requires further investigation as well.

Section 4.6 High temperature inhibited flavonol accumulation

Flavonol accumulation was strongly inhibited in high temperature regimes (i.e., 30/20, 35/25, and 35/30) (**Figure 3.5 A-F**). The inhibitory effect in Experiment 2 was not as evident as in Experiment 1, probably due to the milder high temperature regimes in Experiment 2. Although temperature was previously shown to have little effect on flavonol accumulation (Spayd et al. 2002; Mori et al. 2005b; Makris et al. 2006; Tarara et al. 2008; Azuma et al. 2012), more recent studies (Degu et al. 2016; Pastore et al. 2017b) reported that flavonol accumulation is strongly inhibited by extremely high temperatures (e.g., 37-40 °C). The results from the more recent studies are in agreement with my study, where the flavonol accumulation was found to decrease in high temperature regimes; however, a reduction of flavonol accumulation was also observed in intermediate temperature regimes (i.e., 25/15 and 25/20) in the current study. The lack of temperature effect on flavonol accumulation in some other studies might be attributed to the application of milder temperature treatments (Spayd et al. 2002), the different grape varieties studied (Mori et al. 2005b), the different scales of the treatment applied (temperature regimes applied at the whole vine level in my study compared to at bunch level in Tarara et al. 2008), or the different study systems adopted (e.g., *in vitro* study, Azuma et al. 2012).

Section 4.7 High temperature affected the expression of flavonol genes

Five *VviFLSs* were reported to be involved in flavonol biosynthesis in *Vitis vinifera* grapes (Owens et al. 2008), among which *VviFLS4* and *VviFLS5* were the two most expressed during ripening (Fujita et al. 2006). The concomitant down-regulation of *VviFLS4* and *VviMybF1* in berries from high temperature regimes (i.e., 35/25 and 35/30) led to a decreased level of flavonols in the same regimes in Experiment 1. Both *VviFLS4* and *VviMybF1* were reported to be strongly or completely inhibited by clusters shading from the sunlight (Downey et al. 2004; Fujita et al. 2006; Azuma et al. 2012), which led to inhibited flavonol accumulation as well. However, to our knowledge, this is the first time the effect of temperature regimes on *VviFLS4* and *VviMybF1* gene expression has been reported *in vivo*. On the contrary, *VviFLS5* showed an opposite expression pattern to the one of *VviMybF1* at 89 DAA. Our data is consistent was in agreement with Czemmel et al. (2017) who reported the negative effect of *VviMybF1* overexpression on the expression of *VviFLS5*, but developmental regulation might also be involved, since the expression of *VviFLS5* showed the same pattern with the expression of *VviMybF1* at 76 DAA.

Section 4.8 Temperature regimes led to dramatic changes in anthocyanin and flavonol profiles

Anthocyanin and flavonol profiles were altered by temperature regimes. The relative concentration of 3'4'5'-substituted anthocyanins and flavonols were induced in the regimes with intermediate and high daily temperature (i.e., 35/25 and 35/30 in Experiment 1; 25/15, 25/20, and 30/20 in Experiment 2) (**Figure 3.4 A and B, Figure 3.6 A and B**). In the case of anthocyanins, Tarara et al. (2008) reported similar results in field grown Merlot grapes. Besides the strongly reduced total anthocyanin accumulation under elevated temperature treatments (~2 °C higher than ambient temperature), the proportions of 3'4'5'-substituted anthocyanins were greatly increased

in the same regimes. Interestingly, Mori et al. (2007a) indicated the opposite results in Pinot noir berries, where a lower proportion of 3’4’5’-substituted anthocyanins was found in the berries exposed to the regime with a higher day temperature (30 °C versus 15 °C in the control regime). Since the increased proportion of 3’4’5’-substituted anthocyanin in high temperature regimes in my study was partially due to the increased concentration of malvidin 3-O-(6"-acetyl)glucoside and 3-O-(6"-*p*-coumaroyl)glucoside, which are absent in Pinot noir grapes, I speculate that the inconsistency of my results compared to the data reported by Mori et al. (2007a) is probably attributed to the different anthocyanin profiles between the two varieties (Merlot versus Pinot noir). The relative concentration of 3’4’5’-substituted anthocyanins was also reported to be induced under deficit irrigation (Castellarin et al. 2006, 2007a) and light exclusion conditions (Downey et al. 2006; Tarara et al. 2008; Guan et al. 2016).

With regards to flavonols, it has been reported that in Cabernet Sauvignon berries grown under heat stress (6 or 9 °C higher than control temperature) which applied three weeks after veraison, kaempferol was no longer detectable, the proportion of quercetin decreased by 80%, and the proportion of myricetin decreased by 65%, resulting in an increased proportion of 3’4’5’-substituted flavonols in high temperature regimes (Pillet et al. 2011, reviewed by Gouot et al. 2018). Similar results have been observed by Pastore et al. (2017a), who reported a dramatic decrease of total flavonols with an increased 3’4’5’-substituted proportion under high temperature regimes. According to Mazza and Brouillard (1987), the substitution of the hydroxyl group at the C5-position on the B-ring increases the stability of flavonoid compounds. Therefore, the altered anthocyanin and flavonol profile under high temperature regimes might be a self-regulatory system in grapevine that promotes the branch of the flavonoid pathway which produces anthocyanins and flavonols that are more stable (e.g., 3’4’5’-substituted anthocyanins and flavonols) and possibly

have a higher resistance to degradation under high temperature regimes. Alternatively or in parallel, the shifted profile toward a higher proportion of 3'4'5'-substituted anthocyanins and flavonols might result from an impaired degradation of those species under high temperature regimes in comparison to 3'4'-substituted anthocyanins and flavonols.

The relative concentrations of methoxylated (peonidin, petunidin, and malvidin based anthocyanins) and acylated anthocyanins [3-O-(6"-acetyl)glucosides and 3-O-(6"-*p*-coumaroyl)glucosides] as well as the relative concentration of methoxylated flavonols were increased in high temperature regimes (i.e., 30/20, 35/25, and 35/30). Mori et al. (2005a) has reported a strong increase of the relative concentration of methoxylated anthocyanins under high night temperature treatment (30 °C versus 15 °C at night) in potted Pinot noir grapes. De Rosas et al. (2017) reported an increased proportion of both methoxylated and acylated anthocyanins in Malbec and Bonarda grapes grown under high temperatures (2.5-3 °C higher than control temperatures). My study revealed consistent effects of high temperatures with these previous studies, although different temperature regimes and grape varieties were considered.

Methoxylation and acylation enhance the chemical and thermal stability of anthocyanins (Jackman and Smith 1996; Mori et al. 2005b; Liu et al. 2018). Since most of the anthocyanin and flavonol compounds decreased in concentration at high temperature regimes (i.e., 30/20, 35/25, and 35/30), the induced (in Experiment 1) or unaffected (in Experiment 2) concentration of malvidin 3-O-(6"-acetyl)glucoside, malvidin 3-O-(6"-*p*-coumaroyl)glucoside, and syringetin 3-O-glucoside at high temperature regimes suggests an induced biosynthesis of these compounds and/or an enhanced stability of these compounds under thermal conditions (**Table 3.3 and 3.4)**. Similar to the 3'4'5'-substitution of anthocyanins and flavonols, enhanced methoxylation and

acylation of flavonoids could be a method for the Merlot grapes to adapt to high temperatures, and/or the result of a lower degradation rate of these species of anthocyanins and flavonols.

Section 4.9 The effect of temperature regimes on anthocyanin and flavonol profiles is partially regulated at the molecular level

The down-regulation of *VviF3'Ha* in 35/25 and 35/30 berries might be responsible for the decreased proportion of 3'4'-substituted anthocyanins and flavonols. Similarly, Azuma et al. (2012) reported a decreased proportion of 3'4'-substituted anthocyanins in berries grown at 35 °C in comparison to berries grown at 15 °C, along with the down-regulation of *VviF3'H* expression. Although the expression of *VviF3'5'Hi* and *VviF3'5'Hf* was not directly related to the increased relative concentration of 3'4'5-substituted anthocyanins and flavonols observed in 35/25 and 35/30 regimes, the ratio of *VviF3'5'H* (cumulative expression levels of *VviF3'5'Hi* and *VviF3'5'Hf*) to *VviF3'Ha* expression level and the ratio of 3'4'5'- to 3'4'-substituted anthocyanin and flavonol accumulation matched. In this regard, *VviF3'Ha*, *VviF3'5'Hi*, and *VviF3'5'Hf* appear to integrate and modulate anthocyanin and flavonol profiles under different temperature regimes.

In the case of flavonols, the increased relative concentration of 3'4'5'-substituted flavonols might also be related to the up-regulation of *VviFLS5* under high temperature regimes (i.e., 35/25 and 35/30). Fujita et al. (2006) has reported that the accumulation of quercetin (3'4'-substituted flavonols) is related to the expression pattern of *VviFLS4* rather than *VviFLS5*, indicating a higher substrate affinity of *VviFLS4* to dihydroquercetin for the production of 3'4'-substituted flavonols (e.g. quercetin and isorhamnetin). Similarly, based on my data, there is a possibility that FLS5 preferably used dihydromyricetin as a substrate for the production of 3'4'5'-substituted flavonols. In this case, the higher proportion of 3'4'-substitued flavonols in low temperature regimes was

partially regulated by the higher expression levels of *VviFLS4* in the same regimes, while the higher proportion of 3'4'5'-substituted flavonols was potentially related to the higher expression levels of *VviFLS5* in high temperature regimes. However, further characterization and functionalization of *VviFLS5* is required in order to confirm the substrate affinity of the codified enzyme.

The expression pattern of *VviAOMT* did not explain much about the increased proportion of methoxylated anthocyanins in the high temperature regimes (i.e., 35/25 and 35/30) of Experiment 1. This indicated that either a regulation at the post-transcriptional level occurs or, as stated above, the higher concentration of methoxylated anthocyanins and flavonols is a result of a higher stability of these compounds. First of all, multiple AOMTs are involved in anthocyanin methoxylation, besides the predominant enzyme AOMT encoded by *VviAOMT* which I tested in the current study (Lücker et al. 2010; Fourier-Level et al. 2011). Other than that, Fourier-Level et al. (2011) suggested that the level of anthocyanin methoxylation was modulated not only at the transcriptional level by *VviAOMTs*, but also by the substrate specificity of their codified enzymes (AOMTs). Moreover, the shifted flavonoid profiles under different temperature regimes could also be attributed to the altered preference at specific temperatures of the responsible enzymes to their specific substrates (Shaked-Sachray et al. 2001). The increased proportion of methoxylated anthocyanins in high temperature regimes in my study, with the increased proportion of malvidin based anthocyanins (3'4'5'-substituted anthocyanins) particularly, could be partially explained by the substrate specificities of the enzymes. It is likely that in high temperature regimes, AOMTs have an increased substrate preference towards delphinidin based anthocyanins, with comparison to that in low temperature regimes, and hence produced a higher proportion of malvidin. Methyltransferases that have a substrate specificity towards flavonols might also be involved.

Flavonol methyltransferase has been reported in arabidopsis (Muzac et al. 2000); however, similar methyltransferases have not been characterized in grapes yet.

With regard to the acylation levels of the anthocyanins, the up-regulation of *Vvi3AT* clearly matches the increased proportion of acylated anthocyanins observed in berries exposed to high temperature regimes (i.e., 35/25 and 35/30) in Experiment 1, especially at 89 DAA.

Section 4.10 Is the difference between day and night temperatures important in the regulation of anthocyanin and flavonol accumulation in grapes?

The experimental design of the current study allowed us to investigate the effect of the difference between day and night temperatures on the accumulation of anthocyanins and flavonols, as well as on their profiles. The difference between day and night temperatures exerted an effect on anthocyanin and flavonol accumulation when the grapevines were subjected to regimes with the same night temperature and different day temperatures. For example, anthocyanin and flavonol levels were higher in 20/15 berries in comparison with 25/15 berries, in both experiments; 3'4'5'-substituted anthocyanins and flavonols had increased their relative concentration in 25/15 berries relative to 20/15 berries.

In contrast to what it is anecdotally believed, when regimes with the same day temperature but different night temperatures were compared, the difference between day and night temperatures had no effect on either anthocyanin and flavonol accumulation or their profiles, except that the accumulation of flavonols in Experiment 1 was higher in 20/15 than in 20/10 berries. Night temperature was suggested to be critical for anthocyanin biosynthesis (Mori et al. 2005a, b) and to modulate the expression of flavonoid genes (Mori et al. 2005b; Rienth et al. 2014a), with anthocyanin biosynthesis being inhibited and gene expression being down-regulated under high

night temperatures (e.g., 30 or 37 °C). Moreover, Gaiotti et al. (2018) reported that low night temperature (10 °C versus 20 °C as the night temperature in control treatment) at veraison hastened anthocyanin accumulation, again suggesting the critical role of night temperature in anthocyanin accumulation. However, my results demonstrated that day temperature plays a more important role in anthocyanin and flavonol accumulation as well as their profiles than night temperature does. Similar results have been reported by Kliewer and Torres (1972) for anthocyanins, with the berry coloration being strongly inhibited by high day temperature treatments (e.g., 35°C) and the effect of night temperature depending on the day temperatures partially. As reviewed by Gouot et al. (2018), the effect of day temperature is much stronger than night temperature with regards to the accumulation of anthocyanins, but an interaction between day and night temperature cannot be excluded. Although my study being arguably the most comprehensive in terms of temperature regimes tested, the different results observed among studies could be attributed to the varieties used, the growing conditions of the plants (growth chamber versus field), and the amplitude of the difference between day and night temperatures.

In my study, the sampling analysis of the gene expression during the day and the night allowed me to assess the effect of night conditions (no light and lower temperature than that during the day) in relation to various temperature regimes. Out of 14 genes tested, the expression of *VviF3'5'Hf*, *VviFLS5*, *VviUFGT*, *VviMybA*, and *VviAOMT* was affected by the sampling time (day versus night), with a higher expression at night, regardless of temperature regimes. Harmer et al. (2000) demonstrated that genes involved in the flavonoid pathway were concomitantly up-regulated at night and peaked before dawn in arabidopsis. However, besides the abovementioned genes, the expression patterns of other genes were similarly affected during day and night in the current study. This was probably related to the tissue specific expression of the genes in different

plant species and/or the development-specific regulated of transcription abundance. Harmer et al. (2000) demonstrated circadian expression of flavonoid pathways genes in the seed of arabidopsis, while in a more recent study, Rienth et al. (2014a) suggested that the expression of flavonoid pathway genes at night was distinct from daytime in grape berries at veraison, but there were few genes consistently regulated by nycthemeral cycle at other developmental stages.

Section 4.11 Limitations of the experiments and future perspectives

Experiments in temperature-controlled chambers allow a better separation of temperature effects from the other environmental factors than field experiments. For this reason, growth chambers have been largely used for experiments including mine here. However, experimental limitations in growth chambers exist including the use of potted grapevines grown in small soil volume, limited growing space for the plants and consequently limited number of biological replicates, lower PAR than field conditions, and different RH, *etc*. These artefacts can affect the grapevine physiology and the berry composition. In addition, temperature fluctuations and photoperiod movement during the growing season are also difficult to reproduce in growth chambers.

In field experiments, the interaction of various environmental factors prevents the dissection of temperature effect from other environmental factors. For example, the effect of temperature is always confounded with the effect of solar radiation, and increased solar radiation always correlates with an increase in temperature. Customized systems (e.g., convective systems) for cooling and warming have been developed and used (Tarara et al. 2008; Cohen et al. 2008, 2012), but are time and space consuming, and might be constrained by the cost as well (Bonada and Sadras 2015). Moreover, these systems generally allow testing only a few treatments at once.

By carefully and comprehensively considering all these factors, I established two experiments conducted in temperature-controlled chambers, with five temperature regimes that had different average daily temperatures and delta temperatures in each experiment. To this end, fruit-bearing cuttings were used as plant material in the current study, which has been demonstrated as a successful model system for vegetative and reproductive growth of field grapevines under controlled environmental conditions (Carbonell-Bejerano et al. 2013, 2014; Martínez-Lüscher et al. 2014). I tried to simulate the field conditions such as light intensity and relative humidity, but temperature fluctuation and movement in the photoperiod during the experiment were not considered due to their complexity and the limitation of the growth chamber programs. The unidentical conditions of light intensity and convection among growth chambers should be considered in further studies as well, by rotating the plants in the chambers regularly.

Statistical analysis of the data generated from the two experiments is another point worth discussing. Due to the limited space inside the growth chambers, the grapevines inside each growth chamber might interact with each other by competing for the light, *etc*. However, in previous growth chamber/phytotron experiments with plant materials, the plant grown in a separated pot were considered as independent and the environmental conditions were assumed as identical among different chambers, except for the variables controlled in the experiments. Rotation of the plants among growth chambers/phytotrons treatment application was conducted as well (Kliewer and Torres 1972), in order to avoid potentially biased conditions in specific chambers. Researchers used ANOVA tests or t-Test to analyze the influence of the applied treatments (Ewart and Kliewer 1977; Coombe 1986; Daudet et al. 2004; Mori et al. 2005a; Wang et al. 2010; Warnock et al. 2010; Shinomiya et al. 2015; Gilardi et al. 2016). In my project the chambers were all of the same model and, as described in **Section 4.1**, environmental conditions inside the growth chambers were

successfully controlled during the two experiments. I did not rotate the plants among chambers within each experiment. However, different chambers were used between the two experiments and the same effect of temperature regimes on anthocyanin and flavonol accumulation and profile was observed. This indicates that the effect of temperature regimes is unrelated to the chamber used. Least significant difference analysis was used for multiple comparison, and a Bonferroni correction was conducted as well, in order to reduce the likelihood of incorrectly rejecting a null hypothesis in multiple comparisons. The results of Bonferroni correction are presented in **Appendix 24 and 25**, and show very strong consistency with the results of the LSD tests, except for the effect of some temperature regimes at certain sampling point (e.g., total anthocyanin concentration, expressed as μg/g skin, did not differ among 20/10, 20/15, and 25/15 regimes in Experiment 1 at 89 DAA after the Bonferroni correction). Moreover, a two-way ANOVA test was conducted to compare the effects of the temperature regimes and the day and night sampling time on the gene expression levels. Since the berry samples used for gene expression analyses were collected from the same grapevines (one plant per replicate), the expression level of one biological replicate at different time points could be considered as a repeated measurement of the same plant and not as independent measurements (as assumed in a two-way ANOVA test). O'Brien and Kaiser (1985) demonstrated that multivariate analysis of variance is suitable for repeated measures designs, and this was supported by Vasey and Thayer (1987) and von Ende (2001). I also conducted a repeated measures ANOVA test in order to confirm the use of two-way ANOVA test in my study was appropriate. The comparison indicated strong consistency between the results of two-way ANOVA test and repeated measures ANOVA test (**Appendix 26 and 27**).

According to the results in the current study, day temperature exerted a larger effect on anthocyanin and flavonol accumulation during grape berry ripening than night temperature or the

difference between day and night temperatures. The impact of the difference between day and night temperatures was not significant when the same day and different night temperatures were considered. However, whether this is due to the complicated interaction between day and night temperatures or simply to the specific difference between day and night temperatures that I tested (ΔT = 5 or 10 °C) remains to be clarified. Further research should explore the effect of larger differences between day and night temperatures on anthocyanin and flavonol accumulation in order to clarify if differences of 15 and 20 °C between day and night temperatures, that are possible in viticultural areas such as the Okanagan Valley (BC), can impact berry quality.

Finally, the limited amounts of berries available in this study, due to the space limitations in the growth chambers that restricted the number of grapevines grown, allowed the analysis of only anthocyanins and flavonols as well as a limited number of genes. In order to better characterize the effect of temperature regimes on grape berry metabolism, further studies should aim at characterizing a larger pool of metabolites, and to analyze the expression of more genes along the biosynthetic pathway as well as the enzymatic activity of key enzymes for flavonoid biosynthesis. Anthocyanin turnover between biosynthesis and degradation should be taken into consideration as well. Future studies would be valuable to characterize the transcripts and proteins responsible for degradation and elucidate the molecular mechanisms of heat induced degradation of anthocyanins.

Chapter 5: Conclusion

Altering grapevine growth conditions by applying different temperature regimes from veraison to harvest strongly affects anthocyanin and flavonol accumulation and shifts their profiles dramatically. Total anthocyanin and flavonol levels are strongly reduced in the berries exposed to temperature regimes with high daily temperatures (i.e. 30/20, 35/25, and 35/30); while higher proportions of 3'4'5'-substituted and methoxylated anthocyanins and flavonols, as well as of acylated anthocyanins, were induced. The difference between day and night temperatures exhibited no effect either on anthocyanin and flavonol accumulation or on their profiles, when regimes with the same day temperature but altered night temperatures were compared. When grapevines were subjected to the same night temperature, a smaller difference between day and night temperatures (e.g., 20/15, ΔT =5 °C) enhanced the level of anthocyanin and flavonol accumulation than a larger difference did (e.g., 25/15, 10 °C). High temperature regimes (e.g., 35/25 and 35/30) and the regimes with the same night temperature but a larger difference between day and night temperatures (e.g., 25/15, ΔT = 10 °C versus 20/15, ΔT = 5 °C) increased the relative concentration of 3'4'5'-substituted and methoxylated anthocyanins. The same results were observed for flavonols. Consequently, day temperature is more important for anthocyanin and flavonol accumulation in grapes, while the effect of night temperature partially depends on day temperature. The ratio of *VviF3'5'H* to *VviF3'H* expression matched the ratio of 3'4'5'- to 3'4'-substituted anthocyanins observed in the berries. The increased expression levels of *Vvi3AT* clearly explained the higher proportion of acylated anthocyanins detected in high temperature regimes (i.e., 35/25 and 35/30). The down-regulation of *VviFLS4* together with *VviMybF1* in the same berries was related to a reduction of flavonol accumulation.

These results provide new insights on grape flavonoid accumulation and support the grape and wine industry in providing useful knowledge for identifying the optimal sites and temperatures for red grape cultivation and wine production.

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Appendices

Appendix 1 Growth chamber conditions in Experiment 1 (during berry ripening)

Temperature regimes (day/night temperature, °C)	Relative humidity (RH, %)			Temperature (°C)				Light intensity (μmol m^{-2} s^{-1} of PAR)
	6 h after irrigation *	24 h after irrigation *	30 h after irrigation *	Air (Day) ‡	Air (Night) ‡	Berry surface (Day) ‡	Berry surface (Night) ‡	Around the cluster †
20/10	88.67 ± 0.26 b	74.16 ± 0.32 b	53.35 ± 0.15 b	20.3 ± 0.1 c	10.1 ± 0.1 d	20.5 ± 0 d	9.7 ± 0.1 e	52.5 ± 9.4
20/15	87.67 ± 0.24 c	77.58 ± 0.29 a	57.60 ± 0.22 a	20.2 ± 0.1 c	14.7 ± 0.1 c	20.2 ± 0.1 d	14.6 ± 0 d	46.7 ± 12.9
25/15	91.06 ± 0.13 a	62.72 ± 0.28 c	48.65 ± 0.24 c	25.1 ± 0.1 b	15.0 ± 0 c	25.1 ± 0.1 c	15.5 ± 0 c	44.2 ± 3.3
35/25	83.96 ± 0.45 d	45.29 ± 0.31 d	41.05 ± 0.06 d	35.0 ± 0 a	25.1 ± 0.1 b	35.1 ± 0.1 a	24.4 ± 0.2 b	53.3 ± 6.5
35/30	79.37 ± 0.47 e	35.56 ± 0.18 e	32.36 ± 0.20 e	34.7 ± 0 a	30.2 ± 0 a	34.4 ± 0.1 b	30.0 ± 0.1 a	51.8 ± 5.3

* Values reported are the mean ± standard error (SE, n = 5). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).
‡ Values reported are the mean ± standard error (SE, n = 10). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).
† Values reported are the mean ± standard error (SE, n = 6). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 2 Growth chamber conditions in Experiment 2 (during berry ripening)

Temperature regimes (day/night temperature, °C)	Relative humidity (RH, %)			Temperature (°C)		Light intensity (µmol m^{-2} s^{-1} of PAR)
	6 h after irrigation *	24 h after irrigation *	30 h after irrigation *	Air (Day) ‡	Air (Night) ‡	Around the cluster †
20/10	64.6 ± 2.15 b	50.85 ± 4.75 b	65.64 ± 1.79 a	22.5 ± 0.2 c	10.4 ± 0.1 e	43.3 ± 15.8
20/15	63.98 ± 1.03 b	60.61 ± 4.45 a	64.64 ± 0.51 a	22.7 ± 0.1 c	14.7 ± 0.1 d	29.4 ± 5.9
25/15	70.22 ± 2.13 a	36.97 ± 1.01 c	68.48 ± 2.34 a	26.3 ± 0.1 b	15.2 ± 0.1 c	26.9 ± 11.4
25/20	73.10 ± 1.64 a	37.93 ± 0.63 c	45.57 ± 1.31 b	26.2 ± 0.1 b	19.8 ± 0.2 b	22.8 ± 8.4
30/20	56.31 ± 3.12 c	32.05 ± 1.41 c	35.25 ± 3.90 c	29.6 ± 0.2 a	20.5 ± 0.1 a	28.7 ± 6.6

* Values reported are the mean ± standard error (SE, n = 5). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).
‡ Values reported are the mean ± standard error (SE, n = 10). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).
† Values reported are the mean ± standard error (SE, n = 6). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 3 Temperature effects on grapevine leaf water potential (Ψ) in Experiment 1 and 2 measured at different times after irrigation

Experiment	Temperature regimes (day/night temperature, °C)	Leaf water potential (Ψ)	
		18 h after irrigation	24 h after irrigation
Experiment 1	20/10	-0.44 ± 0.02	-0.55 ± 0.02 a
	20/15	-0.43 ± 0.04	-0.54 ± 0.02 a
	25/15	-0.37 ± 0.02	-0.51 ± 0.01 a
	35/25	-0.35 ± 0.01	-0.74 ± 0.06 b
	35/30	-0.36 ± 0.02	-0.82 ± 0.05 b
Experiment 2	20/10	-0.72 ± 0.05	-0.81 ± 0.04
	20/15	-0.61 ± 0.05	-0.86 ± 0.02
	25/15	-0.63 ± 0.04	-0.75 ± 0.04
	25/20	-0.64 ± 0.04	-0.81 ± 0.04
	30/20	-0.83 ± 0.07	-0.87 ± 0.02

Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 4 Temperature effects on leaf gas exchange from veraison to harvest (during berry ripening) in Experiment 1

Temperature regimes (day/night temperature, °C)	Photosynthesis rate (A, μmol CO_2 $m^{-2}s^{-1}$)		Stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$)		Transpiration rate (E, mol H_2O $m^{-2}s^{-1}$)	
	78 DAA	107 DAA	78 DAA	107 DAA	78 DAA	107 DAA
20/10	3.42 ± 1.24 a	2.70 ± 0.44 b	0.042 ± 0.013 a	0.147 ± 0.06 b	1.03 ± 0.31 a	1.94 ± 0.20 c
20/15	3.53 ± 0.18 a	3.21 ± 0.26 b	0.067 ± 0.011 a	0.140 ± 0.044 b	1.50 ± 0.18 a	1.96 ± 0.35 c
25/15	3.89 ± 1.19 a	4.66 ± 0.26 a	0.058 ± 0.011 a	0.377 ± 0.113 a	1.18 ± 0.18 a	4.53 ± 0.76 b
35/25	4.85 ± 0.57 a	6.42 ± 1.75 a	0.047 ± 0.022 a	0.261 ± 0.058 a	0.91 ± 0.40 a	4.76 ± 0.80 b
35/30	1.90 ± 0.77 b	4.00 ± 0.67 ab	0.029 ± 0.008 b	0.380 ± 0.018 a	0.55 ± 0.14 b	6.20 ± 0.21 a

Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement date according to an LSD test ($p \leq 0.05$).

Appendix 5 Temperature effects on leaf gas exchange from veraison to harvest (during berry ripening) in Experiment 2

Temperature regimes (day/night temperature, °C)	Photosynthetic rate (A, μmol CO_2 $m^{-2}s^{-1}$)			Stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$)			Transpiration rate (E, mol H_2O $m^{-2}s^{-1}$)		
	83 DAA	104 DAA	118 DAA	83 DAA	104 DAA	118 DAA	83 DAA	104 DAA	118 DAA
20/10	9.07 ± 1.13	7.73 ± 0.94 a	5.30 ± 1.20	0.177 ± 0.007 ab	0.154 ± 0.038	0.095 ± 0.030	3.53 ± 0.12 ab	2.73 ± 0.54	1.76 ± 0.52 b
20/15	9.57 ± 1.56	6.78 ± 0.68 a	4.33 ± 0.94	0.120 ± 0.038 bc	0.129 ± 0.023	0.074 ± 0.007	2.96 ± 0.75 abc	2.56 ± 0.38	1.45 ± 0.13 b
25/15	9.11 ± 0.75	5.95 ± 0.85 ab	4.50 ± 0.52	0.239 ± 0.016 a	0.116 ± 0.037	0.149 ± 0.009	4.62 ± 0.21 a	2.38 ± 0.71	2.73 ± 0.14 a
25/20	6.54 ± 1.40	5.29 ± 1.19 ab	5.00 ± 0.92	0.103 ± 0.041 c	0.176 ± 0.057	0.121 ± 0.007	2.71 ± 0.83 bc	3.41 ± 0.91	2.40 ± 0.12 a
30/20	6.85 ± 1.09	5.28 ± 0.72 b	4.15 ± 2.05	0.090 ± 0.022 c	0.101 ± 0.009	0.102 ± 0.021	1.98 ± 0.43 c	2.31 ± 0.19	2.13 ± 0.39 ab

Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement date according to an LSD test ($p \leq 0.05$).

Appendix 6 Temperature effects on anthocyanin accumulation (content, μg/berry) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin content (μg/berry FW) ‡									
						Experiment 1					Experiment 2				
						20/10†	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	89.5 ± 4.5 b	122.3 ± 4.2 a	46.3 ± 11.2 c	10.3 ± 0.8 d	7.5 ± 1.3 d	66.3 ± 15.9 a	35.6 ± 2.7 ab	18.2 ± 3.0 b	27.3 ± 4.4 b	7.0 ± 1.3 b
2	Cy 3-O-glc	2.9	449	162	287	96.0 ± 3.8 a	93.5 ± 14.4 a	33.1 ± 8.2 b	3.6 ± 0.4 c	3.3 ± 0.2 c	47.7 ± 13.0 a	42.9 ± 4.3 a	10.3 ± 2.0 b	15.6 ± 3.0 b	8.5 ± 2.1 b
3	Pt 3-O-glc	3.4	479	162	317	94.7 ± 6.9 a	82.7 ± 8.6 a	37.9 ± 2.7 b	12.5 ± 1.2 c	9.8 ± 0.9 c	52.9 ± 16.9 a	44.2 ± 4.8 ab	22.1 ± 4.1 bc	20.9 ± 4.1 bc	5.6 ± 2.3 c
4	Pe 3-O- glc	3.9	463	162	301	193.0 ± 14.3 a	200.2 ± 9.7 a	83.0 ± 4.8 b	22.0 ± 1.9 c	21.9 ± 2.5 c	136.1 ± 27.5 a	119.2 ± 9.8 ab	61.0 ± 3.8 bc	56.3 ± 8.8 bc	17.2 ± 6.1 c
5	Mv 3-O- glc	4.4	493	162	331	269.9 ± 10.6 a	296.8 ± 17.7 a	146.6 ± 12.4 b	82.1 ± 6.6 c	77.5 ± 2.7 c	276.7 ± 59.0 a	242.2 ± 19.2 a	142.2 ± 13.7 b	128.4 ± 26.1 b	66.0 ± 10.9 b
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	32.0 ± 3.5 a	29.1 ± 1.9 a	20.1 ± 6.8 a	2.7 ± 0.3 b	1.3 ± 0.1 b	33.1 ± 13.2 a	20.2 ± 1.4 ab	5.3 ± 1.2 b	3.6 ± 1.0 b	1.7 ± 0.7 b
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	13.9 ± 0.8 a	14.3 ± 2.2 a	8.1 ± 2.9 ab	2.3 ± 0.3 b	1.2 ± 0 b	9.6 ± 3.8 a	5.5 ± 0.4 ab	2.4 ± 0.5 b	2.1 ± 0.3 b	1.3 ± 0.4 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	25.3 ± 3.5 a	17.2 ± 2.2 b	12.4 ± 2.1 b	3.8 ± 0.4 c	1.9 ± 0.2 c	21.9 ± 9.1 a	9.8 ± 0.8 ab	5.6 ± 1.0 b	3.7 ± 1.0 b	1.9 ± 0.6 b
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	30.0 ± 0.8 a	32.3 ± 2.1 a	20.9 ± 3.7 b	5.8 ± 0.9 c	4.2 ± 0.4 c	44.2 ± 9.3 a	31.5 ± 2.9 ab	17.6 ± 0.7 bc	12.8 ± 3.6 bc	1.7 ± 0.4 c
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	99.6 ± 8.1 a	101.8 ± 8.2 a	81.5 ± 5.9 ab	98.6 ± 2.9 a	70.2 ± 3.9 b	119.7 ± 24.9 a	97.9 ± 9.8 ab	99.5 ± 4.7 ab	97.1 ± 17.0 ab	62.5 ± 8.2 b
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	11.1 ± 1.7 a	10.1 ± 1.1 a	7.4 ± 1.6 ab	3.9 ± 0.7 b	2.1 ± 0.2 b	31.3 ± 6.0 a	35.9 ± 4.5 a	24.3 ± 1.8 ab	18.1 ± 2.9 ab	8.5 ± 2.5 b
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	9.2 ± 0.7 a	11.6 ± 1.6 a	6.0 ± 1.3 b	1.2 ± 0.2 c	0.6 ± 0.1 c	5.4 ± 1.7	4.9 ± 0.8	3.0 ± 0.6	3.8 ± 1.1	1.2 ± 0.4
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	9.5 ± 1.5 a	8.6 ± 0.8 ab	4.9 ± 0.5 bc	7.4 ± 0.9 abc	3.9 ± 0.3 c	8.4 ± 2.2 a	5.3 ± 0.5 ab	4.2 ± 0.6 ab	3.6 ± 1.1 ab	2.4 ± 0.6 b
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	25.5 ± 1.1 a	29.7 ± 2.0 a	25.4 ± 3.2 a	7.0 ± 0.7 b	5.4 ± 0.4 b	33.4 ± 11.4	26.2 ± 1.4	23.6 ± 3.0	28.7 ± 8.1	13.4 ± 2.5
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	67.6 ± 8.0	74.9 ± 6.9	62.5 ± 3.9	88.4 ± 6.0	63.1 ± 3.5	85.2 ± 20.7	64.5 ± 6.3	80.0 ± 3.6	89.2 ± 16.9	52.7 ± 12.6

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/berry based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

Appendix 7 Temperature effects on anthocyanin accumulation (concentration, μg/g berry FW) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g berry FW) ‡									
						Experiment 1					Experiment 2				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	83.6 ± 7.3 b	103.6 ± 6.5 a	36.2 ± 8.7 c	10.8 ± 1.2 d	9.1 ± 2.7 d	55.6 ± 6.4 a	36.2 ± 2.0 b	17.5 ± 5.2 cd	21.5 ± 1.1 c	6.2 ± 0.6 d
2	Cy 3-O-glc	2.9	449	162	287	89.5 ± 6.7 a	78.1 ± 9.7 a	25.8 ± 6.5 b	3.8 ± 0.3 c	3.9 ± 0.9 c	39.7 ± 5.9 a	43.5 ± 3.4 a	10.0 ± 3.3 b	12.2 ± 1.1 b	7.4 ± 1.2 b
3	Pt 3-O-glc	3.4	479	162	317	88.8 ± 10.1 a	69.4 ± 5.9 b	29.8 ± 2.9 c	12.9 ± 0.6 c	11.7 ± 3.1 c	43.4 ± 8.4 a	44.8 ± 3.9 a	21.4 ± 6.9 b	16.3 ± 1.2 bc	4.7 ± 1.6 c
4	Pe 3-O- glc	3.9	463	162	301	177.6 ± 6.4 a	169.0 ± 8.3 a	65.0 ± 5.1 b	22.9 ± 1.3 c	26.6 ± 7.9 c	115.8 ± 11.4 a	120.8 ± 5.9 a	56.8 ± 11.1 b	44.5 ± 3 b	14.7 ± 4.0 c
5	Mv 3-O- glc	4.4	493	162	331	250.4 ± 12.7 a	251.7 ± 20.4 a	115.0 ± 11.8 b	85.3 ± 4.5 b	88.5 ± 13.5 b	234.4 ± 24.1 a	246.5 ± 16.7 a	134.2 ± 31.3 b	99.7 ± 6.6 bc	58.0 ± 4.6 c
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	30.3 ± 4.8 a	24.8 ± 2.3 ab	15.7 ± 5.5 b	2.8 ± 0.4 c	1.5 ± 0.2 c	26.5 ± 6.9 a	20.5 ± 0.7 a	5.2 ± 1.8 b	2.8 ± 0.7 b	1.5 ± 0.5 b
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	13.0 ± 1.2 a	11.9 ± 1.5 a	6.3 ± 2.3 b	2.4 ± 0.3 bc	1.4 ± 0.3 c	7.7 ± 2.0 a	5.7 ± 0.6 a	2.3 ± 0.7 b	1.7 ± 0.3 b	1.1 ± 0.3 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	24.1 ± 4.4 a	14.4 ± 1.5 b	9.7 ± 1.7 bc	4.0 ± 0.6 c	2.3 ± 0.6 c	17.5 ± 4.9 a	9.9 ± 0.5 b	5.4 ± 1.6 bc	2.9 ± 0.7 bc	1.6 ± 0.4 c
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	27.8 ± 1.0 a	27.2 ± 1.1 a	16.3 ± 3.0 b	6.0 ± 0.5 c	5.1 ± 1.4 c	37.6 ± 4.1 a	31.9 ± 1.6 a	16.2 ± 2.7 b	10.0 ± 2.0 b	1.4 ± 0.2 c
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	94.0 ± 12.6 ab	85.7 ± 6.5 ab	63.8 ± 5.5 b	103.1 ± 5.6 a	80.5 ± 12.9 ab	101.6 ± 10.2 a	99.0 ± 6.4 a	92.2 ± 16.4 a	76.5 ± 6.5 ab	55.6 ± 3.1 b
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	10.7 ± 2.2 a	8.4 ± 0.5 ab	5.8 ± 1.3 bc	3.9 ± 0.4 c	2.5 ± 0.5 c	26.7 ± 2.7 b	36.2 ± 3.1 a	22.6 ± 4.3 bc	14.4 ± 1.4 cd	7.3 ± 1.6 d
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	8.6 ± 1.0 a	9.7 ± 0.9 a	4.7 ± 1.1 b	1.3 ± 0.3 c	0.8 ± 0.2 c	4.5 ± 0.8 a	4.9 ± 0.5 a	2.9 ± 1.0 ab	3.0 ± 0.7 ab	1.0 ± 0.3 b
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	9.1 ± 1.9 a	7.3 ± 0.7 ab	3.8 ± 0.4 c	7.6 ± 0.7 ab	4.4 ± 0.6 bc	7.0 ± 0.9 a	5.4 ± 0.4 ab	4.0 ± 1.0 bc	2.8 ± 0.8 c	2.0 ± 0.4 c
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	23.7 ± 1.2 a	24.9 ± 1.0 a	19.9 ± 2.8 a	7.2 ± 0.4 b	6.4 ± 1.6 b	27.2 ± 5.8 a	26.7 ± 0.9 a	22.5 ± 6.1 ab	22.7 ± 5.1 ab	12.0 ± 1.6 b
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	64.2 ± 10.9 b	62.6 ± 2.6 b	49.3 ± 5.0 b	91.8 ± 0.6 a	72.6 ± 12.4 ab	71.7 ± 9.6 ab	65.2 ± 4.2 ab	74.2 ± 13.3 a	69.9 ± 6.3 ab	45.7 ± 7.0 b

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g berry FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

Appendix 8 Temperature effects on anthocyanin accumulation at 62 DAA in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (µg/g skin FW) ‡				
						62 DAA				
						20/10 †	20/15	25/15	35/25	35/30
1	Dp 3-O-glc	2.2	465	162	303	38.4 ± 21.1 b	172.3 ± 48.0 a	113.5 ± 31.7 ab	140.2 ± 37.9 ab	143.2 ± 52.5 ab
2	Cy 3-O-glc	2.9	449	162	287	44.5 ± 12.6 b	202.3 ± 29.5 a	63.0 ± 11.4 b	64.3 ± 19.1 b	52.5 ± 19.9 b
3	Pt 3-O-glc	3.4	479	162	317	14.1 ± 5.5 b	87.7 ± 26.5 ab	65.6 ± 18.5 ab	106.5 ± 32.5 a	94.1 ± 24.4 a
4	Pe 3-O- glc	3.9	463	162	301	38.4 ± 11.7 b	157.3 ± 30.8 a	84.6 ± 13.4 ab	139.1 ± 31.9 a	100.5 ± 21.6 ab
5	Mv 3-O- glc	4.4	493	162	331	32.8 ± 10.2 b	106.2 ± 25.5 b	157.4 ± 40.8 b	444.2 ± 109.9 a	370.6 ± 54.9 a
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	10.1 ± 6.1	52.6 ± 22.6	28.3 ± 7.7	36.7 ± 12.1	30.8 ± 10.7
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	8.6 ± 3.4 b	39.1 ± 6.0 a	14.1 ± 1.9 b	14.6 ± 4.7 b	10.1 ± 3.2 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	7.1 ± 3.7	31.1 ± 12.7	20.2 ± 5.3	33.6 ± 10.2	27.6 ± 7.7
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	8.6 ± 3.4 b	37.6 ± 6.4 a	20.7 ± 3.4 ab	38.7 ± 9.7 a	26.3 ± 5.8 ab
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	10.6 ± 4.5 b	41.1 ± 17.3 b	47.9 ± 10.9 b	187.0 ± 42.4 a	144.0 ± 19.2 a
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	3.5 ± 1.0 c	8.0 ± 2.2 bc	8.1 ± 2.5 bc	17.6 ± 4.9 ab	19.3 ± 3.1 a
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	5.1 ± 1.3 b	16.0 ± 2.8 a	6.6 ± 1.5 b	9.5 ± 2.6 ab	9.4 ± 2.9 ab
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	1.0 ± 1.0 b	6 ± 2.2 b	6.6 ± 1.5 b	21.1 ± 4.0 a	22.3 ± 4.3 a
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	4.5 ± 1.3 c	15.5 ± 4.1 abc	12.6 ± 2.3 bc	29.1 ± 7.6 a	24.9 ± 4.3 ab
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	3.5 ± 1.5 b	9.0 ± 2.7 b	20.2 ± 5.4 b	132.7 ± 28.3 a	127.9 ± 19.5 a

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.
‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as µg/g skin FW based on malvidin 3-O-glucoside equivalents.
† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means between columns according to an LSD test ($p \leq 0.05$).

Appendix 9 Temperature effects on anthocyanin accumulation at 76 DAA at day and night in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡									
						76 DAA (day)					76 DAA (night)				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	35/25	35/30
1	Dp 3-O-glc	2.2	465	162	303	742.5 ± 143.2 b	1336.4 ± 164.5 a	481.9 ± 28.0 bc	402.9 ± 26.6 cd	151.5 ± 26.1 d	759.0 ± 117.7 b	1386.2 ± 160.1 a	524.7 ± 30.3 bc	454.4 ± 58.8 c	152.9 ± 24.8 d
2	Cy 3-O-glc	2.9	449	162	287	591.6 ± 120.3 b	1138.1 ± 164.6 a	267.5 ± 15.9 c	80.3 ± 14.4 d	28.5 ± 7.2 d	681.0 ± 120.3 b	1158.6 ± 170.9 a	264.4 ± 32.1 c	96.2 ± 25.9 d	34.4 ± 10.5 d
3	Pt 3-O-glc	3.4	479	162	317	508.6 ± 124.1 b	718.9 ± 68.9 a	384.2 ± 34.9 bc	366.2 ± 40.2 bc	172.4 ± 27.6 c	515.8 ± 132.4 ab	679.5 ± 89.5 a	394.6 ± 7.1 bc	413.6 ± 72.3 b	162.8 ± 17.0 c
4	Pe 3-O- glc	3.9	463	162	301	903.7 ± 113.9 b	1366.2 ± 81.7 a	612.7 ± 56.3 c	466.8 ± 31.1 c	134.5 ± 24.7 d	929.6 ± 135.1 b	1333.8 ± 79.4 a	563.0 ± 71.1 c	474.9 ± 18.0 c	125.5 ± 16.8 d
5	Mv 3-O- glc	4.4	493	162	331	1121.7 ± 141.4 b	1515.9 ± 102.0 ab	1647.9 ± 122.2 a	1640.9 ± 179.1 a	1464.2 ± 158.1 ab	1155.5 ± 193.0 b	1534.2 ± 91.8 ab	1678.6 ± 82.2 a	1568.7 ± 149.0 ab	1414.6 ± 171.3 ab
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	153.1 ± 34.3 b	312.2 ± 52.0 a	146.2 ± 22.2 b	110.2 ± 22.1 b	54.0 ± 7.7 b	176.2 ± 41.2 b	283.4 ± 40.4 a	143.6 ± 15.3 bc	132.3 ± 31.0 bc	57.9 ± 9.8 c
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	114.8 ± 44.0 b	184.7 ± 24.3 a	48.6 ± 1.7 bc	27.0 ± 8.1 c	5.0 ± 1.7 c	128.9 ± 43.6 a	175.6 ± 14.0 a	60.7 ± 10.2 b	30.6 ± 9.9 b	7.5 ± 1.7 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	115.5 ± 10.1	156.2 ± 11.6	115.9 ± 17.4	118.9 ± 23.2	62.4 ± 6.8	82.2 ± 10.9 bc	155.7 ± 11.1 a	98.6 ± 14.6 bc	130.7 ± 31.1 ab	68.4 ± 11.2 c
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	153.0 ± 20.0 b	261.6 ± 31.7 a	157.4 ± 4.1 b	166.0 ± 29.4 b	85.0 ± 8.8 c	111.0 ± 43.7 b	272.0 ± 20.8 a	115.2 ± 32.8 b	150.3 ± 23.1 b	61.3 ± 12.6 b
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	235.7 ± 16.0 b	315.4 ± 27.9 b	554.7 ± 47.0 b	1316.0 ± 206.4 a	1005.9 ± 147.0 a	216.3 ± 33.2 b	312.1 ± 40.9 b	538.4 ± 49.6 b	1295.6 ± 194.6 a	1043.1 ± 164.6 a
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	58.0 ± 8.1 b	87.3 ± 9.0 ab	59.2 ± 7.2 b	102.9 ± 20.6 a	60.5 ± 7.6 b	105.1 ± 16.3 ab	91.2 ± 9.8 ab	104.1 ± 12.2 ab	126.1 ± 26.0 a	68.4 ± 10.0 b
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	63.6 ± 15.2 b	118.9 ± 20.6 a	36.7 ± 1.8 bc	31.1 ± 6.7 bc	12.0 ± 0.9 c	49.5 ± 5.6 b	86.8 ± 16.7 a	41.1 ± 8.8 c	28.1 ± 6.0 cd	10.5 ± 3.8 d
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	43.6 ± 15.0 b	49.4 ± 6.0 b	52.7 ± 4.8 b	127.1 ± 26.5 a	82.0 ± 10.3 ab	33.1 ± 9.8 b	51.9 ± 6.5 b	59.2 ± 4.7 b	151.6 ± 38.1 a	86.8 ± 16.2 b
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	109.6 ± 21.7 ab	154.8 ± 11.4 a	111.2 ± 3.3 ab	155.7 ± 32.8 a	92.5 ± 10.7 b	96.1 ± 12.8	129.9 ± 17.5	107.2 ± 10.1	166.2 ± 46.2	93.8 ± 15.1
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	168.1 ± 26.3 b	195.4 ± 12.1 b	366.2 ± 24.7 b	1457.3 ± 142.3 a	1234.1 ± 141.0 a	163.0 ± 45.5 b	196.9 ± 28.6 b	343.1 ± 36.7 b	1433.9 ± 144.2 a	1280.5 ± 107.2 a

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 10 Temperature effects on anthocyanin accumulation at 89 DAA at day and night in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡									
						89 DAA (day)					89 DAA (night)				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	35/25	35/30
1	Dp 3-O-glc	2.2	465	162	303	1454.1 ± 66.9 a	1442.8 ± 130.4 a	725.6 ± 121.7 b	219.8 ± 28.0 c	165.4 ± 17.5 c	1444.7 ± 64.4 a	1447.2 ± 136.4 a	746.4 ± 102.5 b	216.2 ± 34.1 c	144.5 ± 22.7 c
2	Cy 3-O-glc	2.9	449	162	287	934.2 ± 86.7 b	1366.3 ± 228.6 a	370.4 ± 83.5 c	69.6 ± 10.8 d	51.3 ± 6.3 d	964.0 ± 98.5 b	1372.0 ± 250.3 a	393.2 ± 52.2 c	47.5 ± 24.3 d	29.5 ± 9.1 d
3	Pt 3-O-glc	3.4	479	162	317	747.2 ± 59.6 a	841.8 ± 68.5 a	548.9 ± 79.2 b	215.3 ± 29.7 c	170.9 ± 18.6 c	752.0 ± 99.4 ab	820.2 ± 32 a	602.4 ± 49.0 b	182.4 ± 45.8 c	157.5 ± 16.9 c
4	Pe 3-O- glc	3.9	463	162	301	1662.1 ± 84.5 b	2175.8 ± 71.7 a	853.6 ± 26.1 c	270.2 ± 73.3 d	249.2 ± 23.7 d	1627.8 ± 94.9 b	2121.4 ± 125.3 a	1044.8 ± 128.1 c	254.0 ± 89.7 d	330.9 ± 106.2 d
5	Mv 3-O- glc	4.4	493	162	331	1878.3 ± 79.4 b	2391.8 ± 51.1 a	2421.3 ± 83.0 a	1588.1 ± 219.0 bc	1506.1 ± 52.7 c	1935.9 ± 75.8 ab	2272.3 ± 129.3 a	2375.6 ± 202.6 a	1668.9 ± 229.4 b	1470.4 ± 76.4 b
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	240.6 ± 20.4 b	294.4 ± 10.5 a	204.5 ± 27.4 b	65.6 ± 6.1 c	56.3 ± 7.6 c	272.2 ± 50.2 ab	335.5 ± 31 a	234.8 ± 29.1 b	44.5 ± 11.5 c	45.9 ± 7.8 c
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	151.1 ± 16.8 b	196.8 ± 25.2 a	56.9 ± 6.9 c	21.4 ± 3.8 c	20.9 ± 2.9 c	174.4 ± 16.4 a	192.2 ± 29.7 a	81.6 ± 9.6 b	6.5 ± 2.1 c	6.0 ± 0.8 c
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	159.3 ± 23.0 a	185.3 ± 7.0 a	161.1 ± 12.0 a	64.2 ± 11.8 b	55.8 ± 9.6 b	308.6 ± 26 a	220.6 ± 10.8 b	187.4 ± 24.1 b	59.5 ± 13.3 c	51.3 ± 8.7 c
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	274.9 ± 16.0 b	356.6 ± 31.4 a	251.8 ± 30.5 b	147.7 ± 13.9 c	108.6 ± 11.8 c	159.9 ± 22.7 b	295.9 ± 27.9 a	104.6 ± 9.2 c	73.4 ± 13.7 c	53.3 ± 4.2 c
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	468.1 ± 42.2 b	536.9 ± 54.1 b	958.0 ± 36.9 a	1145.8 ± 146.0 a	1198.5 ± 63.3 a	448.5 ± 55.6 b	646.2 ± 161.1 b	1047.3 ± 146.2 a	1231.7 ± 129.1 a	1150.5 ± 67.6 a
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	87.9 ± 12.4 ab	104.4 ± 6.9 a	84.3 ± 10.4 ab	58.7 ± 11.5 bc	51.3 ± 7.0 c	307.7 ± 18.5 a	124.4 ± 9.0 b	278.8 ± 20.8 a	101.4 ± 24.5 b	96.6 ± 28.1 b
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	101.7 ± 15.9 a	127.4 ± 15.9 a	48.4 ± 8.0 b	17.4 ± 3.3 bc	14.0 ± 1.6 c	135.1 ± 21.1 a	132.9 ± 10.9 a	69.1 ± 10.4 b	18.0 ± 3.0 c	11.0 ± 2.7 c
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	63.9 ± 6.8	74.8 ± 4.7	83.3 ± 8.6	93.5 ± 17.7	85.8 ± 8.0	74.7 ± 13.9	97.6 ± 18.9	93.0 ± 9.4	101.9 ± 18.6	72.7 ± 7.3
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	178.1 ± 8.0 b	262.2 ± 16.9 a	201.0 ± 19.1 b	175.0 ± 20.9 b	113.2 ± 11.7 c	247.1 ± 15.7 b	367.4 ± 56.3 a	192.6 ± 11.3 b	188.4 ± 37.6 b	141.7 ± 45.6 b
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	270.6 ± 18.7 c	376.1 ± 47.5 c	717.8 ± 20.9 b	1284.6 ± 152.4 a	1087.3 ± 75.1 a	321.7 ± 44.2 c	378.8 ± 64.6 c	717.6 ± 37.1 b	1278.0 ± 182.8 a	1050.7 ± 96.4 a

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 11 Temperature effects on anthocyanin accumulation at 104 DAA in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$) -MS^2]	MS^2	Anthocyanin concentration (µg/g skin FW) ‡				
						104 DAA				
						20/10 †	20/15	25/15	35/25	35/30
1	Dp 3-O-glc	2.2	465	162	303	1688.5 ± 204.5 a	1632.6 ± 62.2 a	562.9 ± 82.6 b	134.7 ± 25.0 c	99.8 ± 8.6 c
2	Cy 3-O-glc	2.9	449	162	287	1286.1 ± 123.7 a	1357.8 ± 82.3 a	317.4 ± 74.2 b	22.0 ± 4.1 c	25.2 ± 7.4 c
3	Pt 3-O-glc	3.4	479	162	317	1058.4 ± 89.7 a	1013.2 ± 71.4 a	422.3 ± 55.5 b	144.2 ± 22.1 c	115.4 ± 8.0 c
4	Pe 3-O- glc	3.9	463	162	301	2110.7 ± 170.3 b	2711.4 ± 136.0 a	1076.9 ± 74.7 c	176.3 ± 32.6 d	293.0 ± 27.3 d
5	Mv 3-O- glc	4.4	493	162	331	2905.8 ± 224.3 a	3183.5 ± 108.4 a	2158.6 ± 177.1 b	1356.4 ± 139.0 c	1244.0 ± 127 c
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	385.1 ± 50.7 a	350.5 ± 22.1 a	153.8 ± 31.0 b	36.5 ± 6.3 c	25.7 ± 3.4 c
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	234.6 ± 26.4 a	226.7 ± 14.9 a	44.7 ± 9.5 b	21.0 ± 3.3 b	23.1 ± 4.8 b
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	243.0 ± 28.2 a	234.1 ± 8.3 a	128.1 ± 23.9 b	49.1 ± 6.7 c	38.3 ± 3.8 c
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	398.9 ± 32.0 a	432.7 ± 16.4 a	240.0 ± 19.8 b	100.6 ± 13.9 c	98.7 ± 23.9 c
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	720.9 ± 60.6 b	766.5 ± 44.2 b	893.1 ± 85.7 ab	1016.1 ± 125.0 a	1171.4 ± 129.4 a
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	143.2 ± 16.9 a	139.4 ± 7.8 a	69.8 ± 9.7 b	48.6 ± 9.7 b	41.8 ± 9.2 b
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	157.2 ± 17.8 a	154.0 ± 14.4 a	54.7 ± 9.7 b	11.0 ± 2.4 c	12.6 ± 2.8 c
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	102.4 ± 5.9 a	98.3 ± 5.2 ab	66.3 ± 8.2 c	74.6 ± 8.5 bc	72.0 ± 13.5 bc
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	318.3 ± 33.2 ab	377.5 ± 12.5 a	271.1 ± 28.7 b	126.8 ± 8.9 c	124.3 ± 29.0 c
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	590.6 ± 36.1 c	630.3 ± 45.1 c	837.8 ± 73.3 bc	1274.6 ± 118.7 a	1147.3 ± 190.0 ab

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as µg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means among columns according to an LSD test ($p \leq 0.05$).

Appendix 12 Temperature effects on anthocyanin accumulation at 79 DAA in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡				
						79 DAA				
						20/10 †	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	234.7 ± 35.7	169.6 ± 54.8	202.3 ± 125.3	338.2 ± 36.4	240.5 ± 97.4
2	Cy 3-O-glc	2.9	449	162	287	133.7 ± 27.4	109.2 ± 23.1	118.1 ± 79.2	137.6 ± 23.2	90.3 ± 34.4
3	Pt 3-O-glc	3.4	479	162	317	150.4 ± 41.3	180.1 ± 70.0	169.7 ± 100.2	266.2 ± 29.3	325.6 ± 38.7
4	Pe 3-O- glc	3.9	463	162	301	299.3 ± 74.8	420 ± 55.7	360 ± 128.9	559.3 ± 38.7	430.1 ± 88.4
5	Mv 3-O- glc	4.4	493	162	331	813.2 ± 105.8 b	818.4 ± 122.6 b	854.1 ± 214.1 b	1809.5 ± 87.0 a	1920.9 ± 132.6 a
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	49.6 ± 13.6 ab	53.7 ± 21.6 ab	37.8 ± 19.8 b	95.8 ± 11.6 a	65.3 ± 12.0 ab
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	22.5 ± 6.4	17.6 ± 4.4	16.4 ± 9.7	27 .0± 4.5	16.6 ± 5.6
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	40.1 ± 10.6	48.4 ± 17.8	36.4 ± 18.4	75.6 ± 9.8	53.9 ± 19.5
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	19.0 ± 4.2	29.4 ± 10.2	32.6 ± 14.6	47.2 ± 6.3	40.6 ± 13.6
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	225.8 ± 47.7 c	469.0 ± 111.7 abc	406.2 ± 45.3 bc	644.5 ± 68.8 ab	708.4 ± 132.8 a
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	59.1 ± 13.6 b	96.9 ± 15.8 ab	77.5 ± 25.5 ab	128.0 ± 10.1 a	118.1 ± 8.5 a
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	13.0 ± 3.9	15.2 ± 3.0	18.0 ± 8.6	24.6 ± 3.7	21.0 ± 6.0
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	17.6 ± 5.1 b	37.7 ± 9.9 ab	39.1 ± 12.4 ab	51.1 ± 5.6 ab	61.9 ± 18.2 a
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	38.6 ± 7.6 c	69.0 ± 14.3 bc	95.9 ± 22.3 ab	124.5 ± 11.1 a	125.7 ± 15.8 a
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	153.5 ± 16.9 c	358.5 ± 94.5 b	429.8 ± 36.2 b	620.2 ± 22.2 a	643.0 ± 83.1 a

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means among columns according to an LSD test ($p \leq 0.05$).

Appendix 13 Temperature effects on anthocyanin accumulation at 93 DAA at day and night in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡									
						93 DAA (day)					93 DAA (night)				
						20/10 †	20/15	25/15	25/20	30/20	20/10	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	599.9 ± 95.1 a	282.8 ± 69.4 b	217.0 ± 74.6 b	178.1 ± 66.5 b	219.3 ± 63.0 b	633.6 ± 80.1 a	351.4 ± 54.9 b	178.1 ± 52.5 b	175.8 ± 63.5 b	237.2 ± 91.2 b
2	Cy 3-O-glc	2.9	449	162	287	248.6 ± 54.2 a	127.4 ± 28.1 b	95.4 ± 14.2 b	88.9 ± 29.3 b	114.3 ± 28.7 b	259.5 ± 60.7 a	130.5 ± 31.1 ab	94.0 ± 28.5 b	70.5 ± 25.2 b	137.6 ± 56.9 ab
3	Pt 3-O-glc	3.4	479	162	317	511.4 ± 49 a	202.3 ± 53.4 b	204.0 ± 65.7 b	154.7 ± 60.1 b	217.7 ± 24.8 b	448.9 ± 90.4 a	256.6 ± 51.8 ab	164.5 ± 42.9 b	174.0 ± 58.8 b	131.2 ± 91.8 b
4	Pe 3-O- glc	3.9	463	162	301	628.1 ± 111.6	408.9 ± 89.5	446.0 ± 63.6	320 ± 113.6	393.3 ± 93.7	567.1 ± 101.0	472.4 ± 68.3	455.5 ± 81.2	388.4 ± 65.4	405.3 ± 167.2
5	Mv 3-O- glc	4.4	493	162	331	2026 ± 251.4 a	1870.2 ± 236 ab	1954.7 ± 169.3 b	1237.4 ± 157.8 bc	1022.0 ± 256.4 c	2111.4 ± 280.3	1773.2 ± 126.7	1880.9 ± 86.1	1159.8 ± 192.1	1047.9 ± 241.5
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	167.9 ± 26.9 a	63.0 ± 21.6 b	62.5 ± 22.2 b	42.0 ± 15.6 b	66.5 ± 13.9 b	166.6 ± 35.5 a	80.4 ± 20.6 b	46.0 ± 14.4 b	45.5 ± 16.2 b	56.7 ± 16.1 b
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	41.7 ± 11.3 a	27.6 ± 5.7 ab	20.0 ± 3.9 ab	15.2 ± 5.3 b	29.2 ± 6.1 ab	35.7 ± 15.9	27.2 ± 6.2	18.6 ± 5.0	14.3 ± 4.7	26.4 ± 7.3
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	135.8 ± 21.8 a	55.5 ± 18.7 b	60.9 ± 20.2 b	41.6 ± 14.5 b	42.5 ± 16.9 b	136.3 ± 24.5 a	71.8 ± 17.1 b	50.4 ± 14.2 b	46.8 ± 18.0 b	29.9 ± 17.8 b
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	59.5 ± 13.2	30.8 ± 11.4	42.2 ± 12.1	31.3 ± 13.4	36.2 ± 15.0	45.9 ± 16.7	41.1 ± 9.3	35.8 ± 8.6	37.4 ± 11.9	37.3 ± 9.6
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	702.5 ± 89.2 b	353.1 ± 99.4 c	989.1 ± 93.8 a	516.1 ± 94.5 bc	651.1 ± 64.4 b	689.8 ± 58.4 b	409.7 ± 35.3 d	971.3 ± 89.5 a	467.0 ± 92 cd	668.7 ± 45.1 bc
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	155.9 ± 27	83.0 ± 21.0	146.2 ± 28.4	83.8 ± 27.8	98.7 ± 18.0	143.0 ± 26.1	123.3 ± 18.6	120.9 ± 21.5	108.4 ± 12.2	87.1 ± 27.4
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	32.6 ± 7.3	17.8 ± 4.2	21.1 ± 5.3	15.2 ± 5.3	17.1 ± 8.3	26.4 ± 8.8	23.9 ± 5.6	18.1 ± 4.4	16.8 ± 4.0	16.0 ± 9.1
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	64.8 ± 9.4	31.7 ± 10.2	58.7 ± 15.6	37.7 ± 15.2	48.2 ± 14.8	48.7 ± 11.5	40.2 ± 9.0	41.2 ± 9.4	44.1 ± 13.3	33.3 ± 9.3
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	117.6 ± 12.9 ab	58.3 ± 14.7 c	142.3 ± 17.2 a	73.0 ± 18.9 bc	86.6 ± 23.6 abc	100.8 ± 13.7	116.5 ± 13.2	132.2 ± 20.3	134.3 ± 15.6	100.4 ± 33.2
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	538 ± 41 c	665.7 ± 118.6 bc	876.4 ± 19.8 ab	981.6 ± 122.6 a	781.4 ± 123.8 abc	479.9 ± 73.9 b	516.6 ± 26.2 b	845.7 ± 21.0 a	878.5 ± 84.9 a	708.1 ± 37.6 a

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 14 Temperature effects on anthocyanin accumulation at 113 DAA at day and night in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M+H^+$)	Mass loss [($M+H^+$)-MS^2]	MS^2	Anthocyanin concentration (μg/g skin FW) ‡									
						113 DAA (day)					113 DAA (night)				
						20/10 †	20/15	25/15	25/20	30/20	20/10	20/15	25/15	25/20	30/20
1	Dp 3-O-glc	2.2	465	162	303	539.5 ± 160.8 a	411.6 ± 24.9 ab	200.2 ± 45.3 bc	164.3 ± 41.2 bc	71.6 ± 37.7 c	524.1 ± 181.7 a	390.9 ± 40.3 ab	189.5 ± 53.9 b	146.1 ± 42.5 b	93.3 ± 72.8 b
2	Cy 3-O-glc	2.9	449	162	287	240.5 ± 118.3	195.9 ± 19.5	97.5 ± 10.2	87.2 ± 10.1	55.0 ± 36.4	223 ± 90.7 a	152.5 ± 47.2 ab	92.0 ± 19.7 ab	71.5 ± 12.1 ab	47.4 ± 35.0 b
3	Pt 3-O-glc	3.4	479	162	317	467.9 ± 168.7 a	323.1 ± 38.9 ab	203.7 ± 40.9 ab	207.2 ± 26.3 ab	85.5 ± 41.2 b	444.2 ± 146.5 a	272.5 ± 44.1 ab	182.8 ± 44.9 b	148.8 ± 37.0 b	87.9 ± 56.3 b
4	Pe 3-O- glc	3.9	463	162	301	928.3 ± 266.2 a	846.5 ± 40.9 ab	498.2 ± 33.0 b	552.8 ± 95.9 ab	432.1 ± 61.6 b	836.8 ± 225.9	747.1 ± 35.2	569.3 ± 97.4	595.6 ± 91.2	466.6 ± 56.0
5	Mv 3-O- glc	4.4	493	162	331	2706.4 ± 332.3 a	2526.0 ± 27.2 a	1288.2 ± 123.5 b	1313.8 ± 201.9 b	760.5 ± 103.1 b	2852.8 ± 464.5 a	2412.1 ± 33.7 a	1415.2 ± 182.2 b	1358.2 ± 204.5 b	834.1 ± 107.8 b
6	Dp 3-O-(6"-acetyl) glc	4.6	507	204	303	140.4 ± 42.4 a	129.9 ± 9.3 ab	75.6 ± 18.5 abc	58.8 ± 15.7 bc	23.9 ± 13.0 c	186.4 ± 81.5 a	95.6 ± 18.1 ab	56.3 ± 18.7 b	36.6 ± 13.2 b	17.1 ± 11.9 b
7	Cy 3-O-(6"-acetyl) glc	5.2	491	204	287	61.4 ± 31.8	44.0 ± 2.9	22.0 ± 5.1	15.4 ± 2.6	30.8 ± 12.1	58.7 ± 28.2	33.5 ± 9.9	18.1 ± 4.0	34.5 ± 13.4	12.6 ± 4.6
8	Pt 3-O-(6"-acetyl) glc	5.6	521	204	317	118.1 ± 36.1 a	93.7 ± 11.6 ab	66.5 ± 17.6 abc	40.3 ± 12.1 bc	24.8 ± 9.3 c	146.1 ± 57.4 a	83.7 ± 14.0 ab	54.7 ± 14.8 b	40.7 ± 13.4 b	19.0 ± 8.4 b
9	Pe 3-O-(6"-acetyl) glc	6.3	505	204	301	79.1 ± 31.5 a	63.7 ± 5.2 ab	47.7 ± 11.0 ab	36.7 ± 6.7 ab	23.7 ± 6.4 b	79.9 ± 29.7 a	50.6 ± 6.0 ab	36.8 ± 8.1 ab	35.6 ± 10.5 ab	18.3 ± 5.9 b
10	Mv 3-O-(6"-acetyl) glc	6.5	535	204	331	1221.8 ± 217.4 a	1219.0 ± 51.2 a	900.2 ± 117.7 ab	932.4 ± 156.4 ab	664.9 ± 119.5 b	1134.9 ± 206.9	1111.1 ± 52.4	928.3 ± 141.7	948.7 ± 168.0	699.2 ± 96.5
11	Dp 3-O-(6"-*p*-coumaroyl) glc	6.2	611	308	303	267.0 ± 75.0 a	217.0 ± 10.4 ab	216.1 ± 38.9 ab	223 ± 43.2 ab	115.9 ± 16.9 b	232.2 ± 64.8 a	185.0 ± 18.8 ab	216.0 ± 48.7 a	139.3 ± 25.9 ab	76.5 ± 22.0 b
12	Cy 3-O-(6"-*p*-coumaroyl) glc	6.8	595	308	287	45.3 ± 18.6 a	46.5 ± 2.5 a	24.6 ± 3.9 ab	23.3 ± 2.3 ab	14.3 ± 4.2 b	44.2 ± 14.5 a	34.0 ± 9.6 ab	27.2 ± 7.1 ab	22.9 ± 2.3 ab	11.2 ± 4.1 b
13	Pt 3-O-(6"-*p*-coumaroyl) glc	7.0	625	308	317	77.7 ± 25.1	62.2 ± 3.2	52.7 ± 10.8	42.7 ± 9.5	37.9 ± 7.7	70.3 ± 16.1 a	51.6 ± 5.4 ab	45.3 ± 11.2 ab	35.6 ± 8.7 b	23.8 ± 4.5 b
14	Pe 3-O-(6"-*p*-coumaroyl) glc	7.4	609	308	301	200.1 ± 46.8	231.2 ± 11	205.3 ± 29.2	237.3 ± 49.9	132.0 ± 13.1	203.6 ± 40.6 ab	234.6 ± 9.5 a	226.3 ± 36.9 a	229.6 ± 49.9 a	110.3 ± 22.2 b
15	Mv 3-O-(6"-*p*-coumaroyl) glc	7.5	639	308	331	808.1 ± 85.7	926.2 ± 14.3	863.4 ± 103.6	840.5 ± 100	741.0 ± 92.5	787.7 ± 84.0	851.3 ± 33.7	857.2 ± 103.6	886.4 ± 194.2	754.2 ± 69.0

* Dp, Cy, Pt, Pe, Mv indicate cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin, respectively; glc=glucoside.

‡ Anthocyanins were identified using their mass spectra, and anthocyanin peak areas were integrated using UV-vis absorbance at 520 nm; anthocyanin concentration was calculated and expressed as μg/g skin FW based on malvidin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 15 Temperature effects on flavonol accumulation (content, μg/berry) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol content (μg/berry FW) ‡									
						Experiment 1					Experiment2				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	3.5 ± 0.3 a	3.7 ± 0.6 a	1.6 ± 0.2 b	1.1 ± 0.1 b	0.9 ± 0.1 b	2.6 ± 0.6 a	1.6 ± 0.1 ab	0.9 ± 0.1 b	0.9 ± 0.2 b	1.4 ± 0.2 ab
2	M-glc	3.9	479	162	317	9.4 ± 0.9 b	12.6 ± 0.9 a	7.6 ± 0.4 bc	5.7 ± 0.1 cd	4.3 ± 0.2 d	2.2 ± 0.5 a	1.5 ± 0.2 a	0.9 ± 0.1 a	1.3 ± 0.3 a	1.0 ± 0.2 a
3	M-glcU	4.1	493	176	317	21.6 ± 1.1 b	27.4 ± 2.0 a	14.9 ± 1.0 c	10.8 ± 0.6 cd	8.6 ± 0.7 d	14.9 ± 2.1 a	11.4 ± 0.7 ab	10.8 ± 0.7 ab	9.1 ± 1.5 b	5.9 ± 0.8 b
4	Q-gal	4.7	463	162	301	9.5 ± 0.8 a	11.2 ± 0.5 a	3.2 ± 0.2 b	2.3 ± 0.2 b	0.7 ± 0.1 c	3.2 ± 0.5 a	2.5 ± 0.4 a	0.8 ± 0.1 b	0.7 ± 0.1 b	0.6 ± 0.1 b
5	Q-glcU	4.8	477	176	301	19.2 ± 0.7 b	28.6 ± 4.1 a	10.7 ± 0.4 bc	4.9 ± 0.4 c	3.1 ± 0.2 c	15.7 ± 2.1 a	12.2 ± 1.4 a	5.1 ± 0.9 b	5.4 ± 0.5 b	2.2 ± 0.1 b
6	Q-glc	4.9	463	162	301	67.8 ± 3.6 b	86.0 ± 7.4 a	28.4 ± 1.2 c	11.4 ± 0.3 cd	10.7 ± 0.7 d	24.0 ± 3.0 a	20.0 ± 1.8 a	12.1 ± 1.7 b	9.5 ± 1.3 b	7.7 ± 1.2 b
7	K-(acetyl) glc	5.1	489	204	285	4.5 ± 0.1 b	6.8 ± 0.4 a	4.0 ± 0.3 b	2.2 ± 0.2 c	1.5 ± 0.1 c	6.4 ± 0.7 a	5.0 ± 0.6 ab	3.7 ± 0.4 bc	3.2 ± 0.6 bc	1.5 ± 0.3 c
8	K-glc	5.8	447	162	285	8.0 ± 0.6 b	11.8 ± 0.3 a	2.3 ± 0.2 c	0.9 ± 0.2 c	0.7 ± 0.1 c	3.1 ± 0.5 a	1.3 ± 0.2 b	0.7 ± 0.1 b	0.7 ± 0.2 b	0.7 ± 0.1 b
9	Q-(rhamnosyl) glc	6.0	609	308	301	6.2 ± 0.7 a	7.2 ± 0.8 a	3.3 ± 0.3 b	1.2 ± 0.2 b	1.0 ± 0.1 b	4.3 ± 1.0 a	2.0 ± 0.1 b	1.2 ± 0.2 b	1.1 ± 0.1 b	0.9 ± 0.2 b
10	I-glc	6.3	477	162	315	3.4 ± 0.1 b	4.5 ± 0.1 a	1.9 ± 0 c	0.6 ± 0.1 d	0.9 ± 0 d	3.1 ± 1.0 a	2.2 ± 0.2 ab	1.6 ± 0.2 ab	1.0 ± 0 b	0.9 ± 0.2 b
11	S-glc	6.5	507	162	345	2.6 ± 0.2 b	2.8 ± 0.1 ab	2.5 ± 0.2 b	3.6 ± 0.2 a	2.8 ± 0.2 ab	4.3 ± 0.9	3.3 ± 0.3	4.2 ± 0.3	3.6 ± 0.5	2.5 ± 0.3

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/berry based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

Appendix 16 Temperature effects on flavonol accumulation (concentration, μg/g berry FW) at harvest in Experiment 1 and 2

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g berry FW) ‡									
						Experiment 1					Experiment2				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	3.2 ± 0.2 a	3.2 ± 0.6 a	1.3 ± 0.2 b	1.1 ± 0.1 b	1.1 ± 0.2 b	2.2 ± 0.3 a	1.7 ± 0.1 ab	0.9 ± 0.2 c	0.7 ± 0.1 c	1.2 ± 0.1 bc
2	M-glc	3.9	479	162	317	8.7 ± 0.7 a	10.6 ± 0.9 a	5.9 ± 0.4 b	5.9 ± 0.5 b	4.9 ± 0.7 b	1.9 ± 0.3 a	1.5 ± 0.1 a	0.8 ± 0.1 b	1.0 ± 0.1 b	0.9 ± 0.2 b
3	M-glcU	4.1	493	176	317	20.0 ± 0.7 a	23.4 ± 2.5 a	11.7 ± 1.2 b	11.4 ± 1.3 b	9.7 ± 1.2 b	12.9 ± 0.9 a	11.6 ± 0.4 a	10.0 ± 2.0 a	7.1 ± 0.4 b	5.3 ± 0.5 b
4	Q-gal	4.7	463	162	301	8.8 ± 0.7 a	9.5 ± 0.7 a	2.5 ± 0.1 b	2.4 ± 0.2 b	0.8 ± 0.2 c	2.8 ± 0.2 a	2.6 ± 0.4 a	0.7 ± 0.1 b	0.6 ± 0.2 b	0.5 ± 0.1 b
5	Q-glcU	4.8	477	176	301	17.8 ± 0.8 a	24.6 ± 4.4 b	8.5 ± 0.8 c	5.2 ± 0.8 c	3.7 ± 0.8 c	13.6 ± 0.6 a	12.4 ± 1.1 a	4.9 ± 1.6 b	4.4 ± 0.5 bc	2.0 ± 0.2 c
6	Q-glc	4.9	463	162	301	62.8 ± 3.6 a	73.1 ± 8.2 a	22.3 ± 2 b	11.9 ± 0.9 b	12.2 ± 1.9 b	20.8 ± 0.8 a	20.3 ± 1.4 a	11.6 ± 3.3 b	7.6 ± 0.6 b	6.8 ± 0.5 b
7	K-(acetyl) glc	5.1	489	204	285	4.2 ± 0.3 b	5.8 ± 0.5 a	3.1 ± 0.3 c	2.3 ± 0.3 cd	1.7 ± 0.2 d	5.5 ± 0.2 a	5.0 ± 0.4 a	3.4 ± 0.8 b	2.4 ± 0.2 bc	1.4 ± 0.2 c
8	K-glc	5.8	447	162	285	7.4 ± 0.5 b	10.0 ± 0.8 a	1.8 ± 0.2 c	1.0 ± 0.2 c	0.8 ± 0.1 c	2.6 ± 0.2 a	1.3 ± 0.2 b	0.7 ± 0.2 c	0.5 ± 0.1 c	0.6 ± 0.1 c
9	Q-(rhamnosyl) glc	6.0	609	308	301	5.7 ± 0.5 a	6.1 ± 0.6 a	2.6 ± 0.3 b	1.3 ± 0.2 bc	1.2 ± 0.3 c	3.6 ± 0.4 a	2.1 ± 0.2 b	1.2 ± 0.4 bc	0.9 ± 0.2 c	0.8 ± 0.1 c
10	I-glc	6.3	477	162	315	3.1 ± 0.2 b	3.8 ± 0.3 a	1.5 ± 0.1 c	0.6 ± 0.1 d	1.0 ± 0.2 cd	2.6 ± 0.5 a	2.2 ± 0.1 ab	1.6 ± 0.4 bc	0.8 ± 0.1 c	0.8 ± 0.1 c
11	S-glc	6.5	507	162	345	2.4 ± 0.2 bc	2.4 ± 0.2 bc	1.9 ± 0.2 c	3.8 ± 0.4 a	3.3 ± 0.8 ab	3.7 ± 0.5 a	3.3 ± 0.3 ab	4.0 ± 0.8 a	2.9 ± 0.2 ab	2.2 ± 0.1 b

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g berry FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each experiment according to an LSD test ($p \leq 0.05$).

Appendix 17 Temperature effects on flavonol accumulation at 62 DAA in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M\text{-}H^+$)	Mass loss [$(M\text{-}H^+)\text{-}MS^2$]	MS^2	Flavonol concentration (µg/g skin FW) ‡				
						62 DAA				
						20/10 †	20/15	25/15	35/25	35/30
1	M-gal	3.8	479	162	317	1.0 ± 1.0	2.0 ± 1.1	0 ± 0	2.0 ± 1.1	1.0 ± 1.0
2	M-glc	3.9	479	162	317	4.0 ± 0	3.9 ± 0	4.0 ± 0	4.0 ± 0	4.5 ± 0.5
3	M-glcU	4.1	493	176	317	3.0 ± 0 b	3.7 ± 0.7 b	3.0 ± 0 b	4.5 ± 0.9 b	6.7 ± 0.8 a
4	Q-gal	4.7	463	162	301	4.0 ± 0	3.9 ± 0	2.0 ± 1.2	2.0 ± 1.1	2.0 ± 1.1
5	Q-glcU	4.8	477	176	301	28.3 ± 4.1 b	37.0 ± 1.7 ab	30.7 ± 1.7 b	43.5 ± 5.1 a	34.6 ± 3.1 ab
6	Q-glc	4.9	463	162	301	29.8 ± 3.9 b	38.9 ± 1.3 ab	28.7 ± 2.5 b	39.6 ± 5.8 ab	46.8 ± 3.5 a
7	K-(acetyl) glc	5.1	489	204	285	1.0 ± 0 b	1.2 ± 0.2 b	1.0 ± 0 b	1.5 ± 0.3 b	2.5 ± 0.3 a
8	K-glc	5.8	447	162	285	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5	0 ± 0	0 ± 0
9	Q-(rhamnosyl) glc	6.0	609	308	301	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5	0 ± 0	0 ± 0
10	I-glc	6.3	477	162	315	0.2 ± 0.2 c	1.0 ± 0.1 ab	0.8 ± 0 bc	1.4 ± 0.4 ab	1.5 ± 0.1 a
11	S-glc	6.5	507	162	345	0 ± 0 b	0.4 ± 0.2 b	0.8 ± 0.2 b	3.3 ± 0.9 a	2.8 ± 0.4 a

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as µg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means among columns according to an LSD test ($p \leq 0.05$).

Appendix 18 Temperature effects on flavonol accumulation at 76 DAA at day and night in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g skin FW) ‡									
						76 DAA (day)					76 DAA (night)				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	35/25	35/30
1	M-gal	3.8	479	162	317	10.8 ± 1.0 b	27.6 ± 3.4 a	15.2 ± 1.3 b	14.3 ± 1.5 b	9.8 ± 1.4 b	13.6 ± 1.3 b	32.5 ± 3.1 a	18.3 ± 1.2 b	15.3 ± 1.4 b	9.8 ± 1.4 b
2	M-glc	3.9	479	162	317	18.7 ± 1.3 b	63.9 ± 3.0 a	19.1 ± 2.0 b	19.3 ± 6.2 b	13.2 ± 3.7 b	16.7 ± 1.3 b	74.8 ± 2.8 a	19.2 ± 2.1 b	22.1 ± 6 b	20.3 ± 3.8 b
3	M-glcU	4.1	493	176	317	52.8 ± 1.3 c	131.3 ± 4.3 a	65.9 ± 3.9 c	89.2 ± 6.6 b	64.5 ± 3.1 c	57.0 ± 1.3 d	127.1 ± 4.6 a	76.4 ± 3.6 c	83.9 ± 6.2 b	62.3 ± 2.2 cd
4	Q-gal	4.7	463	162	301	46.2 ± 2.0 a	41.8 ± 2.7 a	11.2 ± 0.9 b	11.3 ± 1.0 b	10.8 ± 1.3 b	48.7 ± 2.0 a	44.5 ± 2.6 a	15.3 ± 1.0 b	13.3 ± 0.9 b	12.8 ± 1.3 b
5	Q-glcU	4.8	477	176	301	154.8 ± 2.3 b	214.0 ± 7.9 a	60.5 ± 2.6 c	45.1 ± 3.3 d	39.0 ± 2.0 d	155.2 ± 2.5 b	210.6 ± 6.7 a	64.0 ± 2.7 c	42.0 ± 3.2 d	43.1 ± 2.3 d
6	Q-glc	4.9	463	162	301	407.0 ± 7.4 b	484.5 ± 12.7 a	197.0 ± 10.9 c	130.9 ± 9.9 d	131.2 ± 4.2 d	408.0 ± 7.0 b	483.8 ± 13.4 a	202.6 ± 10.0 c	125.4 ± 9.2 d	136.4 ± 3.6 d
7	K-(acetyl) glc	5.1	489	204	285	22.7 ± 1.2 ab	25.8 ± 3.7 ab	22.0 ± 1.8 ab	29.4 ± 2.4 a	20.5 ± 1.8 b	24.8 ± 1.2	25.2 ± 3.9	23.2 ± 1.7	22.4 ± 2.6	20.5 ± 1.7
8	K-glc	5.8	447	162	285	34.9 ± 1.8 a	17.2 ± 2.5 bc	19.1 ± 2.7 b	13.8 ± 1.9 bc	10.8 ± 1.3 c	33.6 ± 1.8 a	17.2 ± 2.4 ab	19.2 ± 2.6 ab	11.2 ± 1.9 b	8.2 ± 0.8 b
9	Q-(rhamnosyl) glc	6.0	609	308	301	16.2 ± 1.2 a	16.2 ± 1.5 a	10.8 ± 1.3 b	6.9 ± 0.6 c	10.3 ± 1.0 bc	17.3 ± 1.2 a	16.8 ± 1.5 a	12.8 ± 1.2 b	6.0 ± 0.4 c	10.3 ± 0.9 bc
10	I-glc	6.3	477	162	315	13.8 ± 1.4 ab	17.7 ± 1.7 a	11.2 ± 2.0 bc	8.9 ± 0.8 c	3.9 ± 0.4 d	13.4 ± 1.4 ab	16.3 ± 1.8 a	12.3 ± 2.2 bc	9.8 ± 0.6 c	3.2 ± 0.5 d
11	S-glc	6.5	507	162	345	10.8 ± 0.4 d	9.3 ± 1.3 d	21.0 ± 1.2 c	41.4 ± 3.2 a	31.2 ± 2.2 b	11.6 ± 0.4 d	10.7 ± 1.5 d	24.2 ± 1.9 c	44.3 ± 3.9 a	34.3 ± 2.7 b

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 19 Temperature effects on flavonol accumulation at 89 DAA at day and night in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g skin FW) ‡									
						89 DAA (day)					89 DAA (night)				
						20/10 †	20/15	25/15	35/25	35/30	20/10	20/15	25/15	35/25	35/30
1	M-gal	3.8	479	162	317	33.9 ± 2.5 a	30.2 ± 1.6 a	22.1 ± 2.2 b	14.7 ± 1.3 c	14.7 ± 1.2 c	34.1 ± 2.4 a	32.1 ± 1.5 ab	27.1 ± 2.2 bc	13.9 ± 1.3 c	16.2 ± 1.3 c
2	M-glc	3.9	479	162	317	64.8 ± 4.2 a	72.7 ± 3.5 a	70.6 ± 3.7 a	39.1 ± 3.0 b	35.4 ± 3.1 b	67.4 ± 4.1 b	79.5 ± 3.7 a	73.9 ± 3.8 ab	32.3 ± 3.2 c	37.4 ± 3.2 c
3	M-glcU	4.1	493	176	317	129.2 ± 7.0 b	157.7 ± 3.4 a	116.8 ± 4.5 b	67.7 ± 2.0 c	52.2 ± 5.0 d	123.2 ± 6.7 b	169.4 ± 4.2 a	122.1 ± 4.7 b	62.0 ± 1.9 c	59.2 ± 5.4 c
4	Q-gal	4.7	463	162	301	57.5 ± 4.5 a	68.2 ± 6.4 a	34.3 ± 2.3 b	9.3 ± 1.7 c	14.7 ± 1.3 c	52.9 ± 4.4 a	63.0 ± 6.3 a	31.4 ± 2.3 b	12.3 ± 1.7 c	11.0 ± 1.3 c
5	Q-glcU	4.8	477	176	301	203.7 ± 9.1 a	195.7 ± 12.3 a	112.4 ± 7.5 b	51.1 ± 3.3 c	47.9 ± 12.1 c	205.4 ± 8.5 a	201.4 ± 12.9 a	132.6 ± 6.9 b	57.3 ± 3.2 c	52.9 ± 12.3 c
6	Q-glc	4.9	463	162	301	700.0 ± 15.4 b	874.2 ± 22.5 a	470.1 ± 10.9 c	97.8 ± 4.9 d	59.4 ± 7.0 d	706.1 ± 17.3 b	852.0 ± 19.5 a	491.4 ± 9.2 c	108.2 ± 5.3 d	63.3 ± 7.0 d
7	K-(acetyl) glc	5.1	489	204	285	42.7 ± 3.0 b	52.4 ± 3.1 a	40.7 ± 2.4 b	22.4 ± 1.5 c	25.8 ± 3.4 c	48.1 ± 2.9 a	48.3 ± 3.1 a	35.9 ± 2.5 ab	23.5 ± 1.6 b	24.7 ± 3.2 b
8	K-glc	5.8	447	162	285	36.3 ± 4.3 a	40.0 ± 2.8 a	19.7 ± 1.9 b	9.8 ± 1.4 c	12.3 ± 1.7 bc	39.6 ± 4.2 b	45.9 ± 2.8 a	22.7 ± 1.4 c	11.4 ± 1.4 d	16.3 ± 2.1 cd
9	Q-(rhamnosyl) glc	6.0	609	308	301	49.5 ± 5.8 a	60.3 ± 2.2 a	49.1 ± 4.3 a	11.7 ± 2.1 b	16.7 ± 2.6 b	59.9 ± 5.8 a	67.2 ± 2.3 a	53.2 ± 4.1 a	16.8 ± 2.1 b	14.9 ± 2.9 b
10	I-glc	6.3	477	162	315	32.9 ± 2.1 b	43.6 ± 0.8 a	36.9 ± 3.1 ab	19.6 ± 3.1 c	10.8 ± 1.2 d	35.2 ± 3.4 a	34.5 ± 1.1 a	43.0 ± 3.7 a	25.6 ± 3.1 b	14.8 ± 1.3 b
11	S-glc	6.5	507	162	345	31.7 ± 2.8 c	30.6 ± 1.6 c	55.4 ± 2.5 a	44.5 ± 1.3 b	42.0 ± 3.6 b	32.0 ± 2.3 bc	28.5 ± 1.7 c	63.5 ± 2.1 a	51.7 ± 1.5 ab	45.0 ± 3.7 abc

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 20 Temperature effects on flavonol accumulation at 104 DAA in Experiment 1

No.	Compound *	Retention time (min)	*m/z* ($M\text{-}H^+$)	Mass loss [$(M\text{-}H^+)\text{-}MS^2$]	MS^2	Flavonol concentration (μg/g skin FW) ‡				
						104 DAA				
						20/10 †	20/15	25/15	35/25	35/30
1	M-gal	3.8	479	162	317	24.6 ± 1.2 b	26.1 ± 2.4 b	22.7 ± 2.1 b	24.2 ± 2.5 b	33.7 ± 1.8 a
2	M-glc	3.9	479	162	317	70.3 ± 2.6 b	93.2 ± 4.1 a	42.0 ± 4.7 c	40.5 ± 3.5 c	46.1 ± 2.4 c
3	M-glcU	4.1	493	176	317	176.4 ± 4.8 ab	169.2 ± 7.2 b	197.6 ± 6.7 a	98.6 ± 6.0 c	101.1 ± 9.8 c
4	Q-gal	4.7	463	162	301	91.9 ± 2.7 a	93.2 ± 6.1 a	52.8 ± 5.7 b	21.2 ± 1.7 c	6.9 ± 1.3 d
5	Q-glcU	4.8	477	176	301	215.7 ± 3.1 b	340.9 ± 21.7 a	91.9 ± 5.1 c	85.3 ± 4.8 c	52.0 ± 4.4 d
6	Q-glc	4.9	463	162	301	799.8 ± 21.2 b	1029.0 ± 26.8 a	282.6 ± 17.9 c	185.9 ± 6.3 d	151.6 ± 5.2 d
7	K-(acetyl) glc	5.1	489	204	285	33.9 ± 2.1 c	70.1 ± 4.6 a	53.4 ± 3.8 b	30.4 ± 1.7 c	29.4 ± 2.9 c
8	K-glc	5.8	447	162	285	34.4 ± 3.3 b	53.8 ± 3.5 a	16.3 ± 1.7 cd	8.9 ± 1.3 d	17.8 ± 2.9 c
9	Q-(rhamnosyl) glc	6.0	609	308	301	59.5 ± 4.7 a	57.7 ± 4.0 a	31.1 ± 2.1 b	12.3 ± 1.3 c	18.3 ± 1.7 c
10	I-glc	6.3	477	162	315	24.1 ± 0.9 c	55.3 ± 2.2 a	30.6 ± 2.8 b	12.3 ± 1.7 d	11.4 ± 1.7 d
11	S-glc	6.5	507	162	345	23.4 ± 1.8 bc	17.7 ± 1.3 c	25.9 ± 1.8 b	33.5 ± 1.1 a	34.6 ± 4.0 a

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means among columns according to an LSD test ($p \leq 0.05$).

Appendix 21 Temperature effects on flavonol accumulation at 79 DAA in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M\text{-}H^+$)	Mass loss [$(M\text{-}H^+)\text{-}MS^2$]	MS^2	Flavonol concentration (μg/g skin FW) ‡				
						79 DAA				
						20/10 †	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	5.9 ± 0.8 c	7.2 ± 1.7 bc	12.2 ± 1.3 a	10.7 ± 1.3 ab	4.7 ± 1.2 c
2	M-glc	3.9	479	162	317	5.9 ± 0.8	4.8 ± 1.2	4.9 ± 1.3	4.8 ± 0.5	4.3 ± 1.0
3	M-glcU	4.1	493	176	317	12.0 ± 0.8 c	22.2 ± 1.9 b	27.3 ± 1.3 b	29.2 ± 2.4 b	46.8 ± 5.5 a
4	Q-gal	4.7	463	162	301	9.3 ± 0.9 a	3.9 ± 0.8 bc	6.4 ± 1.0 b	4.3 ± 0.9 bc	2.4 ± 0.5 c
5	Q-glcU	4.8	477	176	301	146.9 ± 7.6 ab	143.2 ± 8.9 ab	133.6 ± 8.8 b	133.0 ± 10.5 b	167.9 ± 8.5 a
6	Q-glc	4.9	463	162	301	51.7 ± 5.9 c	53.6 ± 2.7 c	92.5 ± 7.7 b	91.0 ± 5.5 b	133.8 ± 5.1 a
7	K-(acetyl) glc	5.1	489	204	285	5.9 ± 0.8 c	11.1 ± 1.6 b	12.2 ± 1.6 ab	14.1 ± 1.0 ab	16.3 ± 2.0 a
8	K-glc	5.8	447	162	285	12.8 ± 1.3 c	30.4 ± 2.3 a	20.6 ± 2.2 b	10.2 ± 1.1 c	11.4 ± 1.5 c
9	Q-(rhamnosyl) glc	6.0	609	308	301	0 ± 0 d	0 ± 0 d	4.9 ± 1.3 c	11.7 ± 1.8 b	15.8 ± 1.7 a
10	I-glc	6.3	477	162	315	3.2 ± 0.5 c	4.8 ± 0.5 bc	7.9 ± 0.5 a	5.3 ± 0.5 b	8.2 ± 1.0 a
11	S-glc	6.5	507	162	345	6.4 ± 1.0 c	16.4 ± 2.0 ab	14.7 ± 1.5 b	17.5 ± 1.6 ab	21.5 ± 2.8 a

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means among columns according to an LSD test ($p \leq 0.05$).

Appendix 22 Temperature effects on flavonol accumulation at 93 DAA at day and night in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g skin FW) ‡									
						93 DAA (day)					93 DAA (night)				
						20/10 †	20/15	25/15	25/20	30/20	20/10	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	24.1 ± 3.2 b	33.1 ± 3.9 a	18.2 ± 2.5 b	15.5 ± 1.4 b	19.8 ± 2.9 b	27.4 ± 3.4 a	31.8 ± 4.3 a	19.9 ± 2.1 b	14.3 ± 1.5 b	18.6 ± 3.1 b
2	M-glc	3.9	479	162	317	7.6 ± 1.7	10.7 ± 1.4	10.8 ± 1.3	11.6 ± 1.5	11.4 ± 1.8	9.7 ± 1.8 c	10.5 ± 1.3 bc	10.7 ± 1.2 ab	11.5 ± 1.7 a	11.1 ± 1.5 ab
3	M-glcU	4.1	493	176	317	89.3 ± 7.8 a	72.8 ± 5.9 ab	88.4 ± 8.1 a	81.1 ± 5.4 a	59.6 ± 3.4 b	90.4 ± 8.2 a	70.1 ± 6.8 ab	87.3 ± 5.9 a	80.3 ± 6.0 ab	58.5 ± 2.5 b
4	Q-gal	4.7	463	162	301	1.4 ± 1.4 b	9.2 ± 1.2 a	1.4 ± 1.4 b	1.0 ± 1.0 b	7.1 ± 2.4 a	1.5 ± 1.5 b	9.0 ± 1.1 a	1.5 ± 1.5 b	0.9 ± 0.9 b	8.1 ± 2.4 ab
5	Q-glcU	4.8	477	176	301	180.9 ± 7.2	197.0 ± 1.7	189.2 ± 8.8	194.9 ± 9.3	143.3 ± 6.0	183.3 ± 8.6 bc	199.7 ± 5.7 a	186.1 ± 9.8 ab	189.3 ± 6.4 ab	131.1 ± 7.4 c
6	Q-glc	4.9	463	162	301	138.9 ± 6.9 a	130.9 ± 6.2 a	70.1 ± 5.9 b	77.7 ± 5.6 b	58.5 ± 6.4 b	140.6 ± 7.3 a	129.6 ± 8.2 a	69.4 ± 5.0 b	77.0 ± 6.3 b	58.1 ± 7.8 b
7	K-(acetyl) glc	5.1	489	204	285	27.4 ± 2.2 ab	32.5 ± 3.3 a	21.7 ± 3.9 bc	15.9 ± 3.1 c	19.9 ± 1.3 bc	24.7 ± 2.2 ab	37.3 ± 3.8 a	26.4 ± 3.5 ab	12.9 ± 3.4 b	13.5 ± 1.1 b
8	K-glc	5.8	447	162	285	19.3 ± 3.5 b	27.7 ± 2.5 a	11.2 ± 2.3 c	8.7 ± 1.2 c	5.7 ± 0.8 c	19.6 ± 3.7 a	29.4 ± 2.7 a	13.7 ± 2.5 b	6.6 ± 1.3 b	5.6 ± 0.8 b
9	Q-(rhamnosyl) glc	6.0	609	308	301	4.7 ± 1.2 b	4.4 ± 1.2 b	13.7 ± 2.2 a	4.8 ± 2.8 b	9.5 ± 3.9 ab	4.8 ± 1.2 b	6.1 ± 1.3 b	11.6 ± 2.2 a	5.9 ± 2.8 b	8.3 ± 3.7 b
10	I-glc	6.3	477	162	315	10.4 ± 2.4 ab	5.8 ± 1.7 b	14.2 ± 1.6 a	5.8 ± 2.0 b	6.1 ± 1.4 b	10.5 ± 2.3 ab	5.7 ± 1.6 b	14.1 ± 1.5 a	5.8 ± 2.2 b	6.1 ± 1.5 b
11	S-glc	6.5	507	162	345	25.5 ± 2.4 b	33.4 ± 3.7 ab	38.4 ± 3 a	31.0 ± 1.9 ab	25.1 ± 1.7 b	25.8 ± 2.6	33.1 ± 4.1	38.4 ± 4.0	30.7 ± 2.2	24.6 ± 1.3

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 23 Temperature effects on flavonol accumulation at 113 DAA at day and night in Experiment 2

No.	Compound *	Retention time (min)	*m/z* ($M-H^+$)	Mass loss [($M-H^+$)-MS^2]	MS^2	Flavonol concentration (μg/g skin FW) ‡									
						113 DAA (day)					113 DAA (night)				
						20/10 †	20/15	25/15	25/20	30/20	20/10	20/15	25/15	25/20	30/20
1	M-gal	3.8	479	162	317	19.4 ± 3.1 a	13.9 ± 0.8 ab	13.9 ± 0.9 ab	11.7 ± 1.6 b	13.5 ± 1.3 b	22.7 ± 1.3 a	13.2 ± 0.9 b	12.8 ± 0.9 b	12.0 ± 1.7 b	13.1 ± 1.4 b
2	M-glc	3.9	479	162	317	32.4 ± 2.7 a	23.8 ± 2.9 b	17.5 ± 2.8 b	20.9 ± 2.2 b	24.6 ± 2.5 ab	42.9 ± 3.7 a	22.5 ± 2.8 b	15.2 ± 2.5 c	21.5 ± 2.1 bc	25.2 ± 2.1 bc
3	M-glcU	4.1	493	176	317	101.3 ± 5.4 a	101.1 ± 6.0 a	96.0 ± 8.6 a	87.3 ± 11.6 a	54.8 ± 3.6 b	97.2 ± 3.6 a	95.2 ± 5.1 ab	95.7 ± 8.0 a	89.6 ± 10.4 ab	56.2 ± 2.7 b
4	Q-gal	4.7	463	162	301	18.0 ± 3.3 a	9.0 ± 1.3 b	5.0 ± 1.3 b	4.9 ± 1.3 b	7.7 ± 0.7 b	21.1 ± 4.2 a	8.5 ± 1.3 ab	4.7 ± 1.3 c	5.0 ± 1.3 c	7.9 ± 0.7 bc
5	Q-glcU	4.8	477	176	301	164.8 ± 9.6 a	121.8 ± 15.0 b	123.3 ± 6.3 ab	126.8 ± 21.0 ab	124.3 ± 5.7 ab	161.8 ± 10.9 a	114.8 ± 13.7 b	120.0 ± 6.3 b	129.9 ± 20.0 a	127.9 ± 6.2 b
6	Q-glc	4.9	463	162	301	217.9 ± 10.9 a	166.8 ± 10.4 b	92.4 ± 6.1 cd	97.6 ± 6.5 c	69.2 ± 8.6 d	221.4 ± 12.1 a	163.9 ± 10.2 b	87.9 ± 4.7 c	100.6 ± 6.9 c	70.7 ± 7.8 c
7	K-(acetyl) glc	5.1	489	204	285	46.5 ± 4.5 a	51.3 ± 3.6 a	31.8 ± 3.3 b	29.3 ± 4.2 b	18.3 ± 1.2 c	58.8 ± 5.5 a	48.3 ± 3.2 a	30.5 ± 2.8 b	30.0 ± 3.8 b	18.7 ± 0.9 b
8	K-glc	5.8	447	162	285	25.0 ± 1.6 a	25.9 ± 1.9 a	10.9 ± 1.2 b	10.2 ± 1.7 b	6.7 ± 1.2 b	33.9 ± 3.2 a	24.4 ± 1.6 b	12.5 ± 1.4 c	9.5 ± 1.7 c	6.9.0 ± 1.2 c
9	Q-(rhamnosyl) glc	6.0	609	308	301	21.6 ± 4.1 b	32.3 ± 0.8 a	28.2 ± 1.2 ab	26.6 ± 1.8 ab	8.7 ± 1.2 c	24.6 ± 7.1 a	30.5 ± 0.9 a	29.3 ± 1.7 a	27.6 ± 2.4 a	9.0 ± 1.4 b
10	I-glc	6.3	477	162	315	24.9 ± 2.5 a	15.0 ± 1.4 b	11.8 ± 2.0 b	10.7 ± 1.4 bc	5.8 ± 0.8 c	23.7 ± 2.4 a	14.1 ± 1.4 b	13.8 ± 1.9 b	11.0 ± 1.3 b	5.9 ± 0.8 b
11	S-glc	6.5	507	162	345	61.4 ± 3 a	51.3 ± 3.5 b	42.5 ± 2.4 b	41.9 ± 3.6 b	49.1 ± 2.4 b	53.6 ± 3.9 a	48.4 ± 3.4 ab	42.5 ± 3.1 ab	43.0 ± 3.1 b	50.6 ± 3.2 ab

* M, Q, K, I, S indicate myricetin, quercetin, kaempferol, isorhamnetin, and syringetin, respectively; glc, gal, and glcU indicate glucoside, galactoside, and glucuronide, respectively.

‡ Flavonols were identified using their mass spectra, and flavonol peak areas were integrated using UV-vis absorbance at 353 nm; flavonol concentration was calculated and expressed as μg/g skin FW based on quercetin 3-O-glucoside equivalents.

† Values reported are the mean ± standard error (SE, n = 4). Different letters indicate significantly different means within each measurement according to an LSD test ($p \leq 0.05$).

Appendix 24 Temperature effects on the evolution of anthocyanin and flavonol concentration (μg/g skin FW) by one-way ANOVA test in Experiment 1

Temperature regimes (day/night temperature, °C)	Temperature effects on anthocyanin concentration (μg/g skin FW)					Temperature effects on flavonol concentration (μg/g skin FW)				
	62 DAA	76 DAA	89 DAA	104 DAA	118 DAA	62 DAA	76 DAA	89 DAA	104 DAA	118 DAA
20/10	b *	b	**ab** ‡	a	a	b	b	b	b	b
20/15	a	a	a	a	a	ab	a	a	a	a
25/15	b	b	**bc**	b	b	b	c	c	c	c
35/25	a	ab	c	c	c	a	d	d	d	d
35/30	a	b	c	c	c	a	d	d	d	d

* Different letters indicate significantly different means within each measurement by a Bonferroni correction ($p \leq 0.05$).
‡ Bold font indicates different results between LSD test and Bonferroni correction.

Appendix 25 Temperature effects on the evolution of anthocyanin and flavonol concentration (μg/g skin FW) by one-way ANOVA test in Experiment 2

Temperature regimes (day/night temperature, °C)	Temperature effects on anthocyanin concentration (μg/g skin FW)				Temperature effects on flavonol concentration (μg/g skin FW)			
	79 DAA	93 DAA	113 DAA	135 DAA	79 DAA	93 DAA	113 DAA	135 DAA
20/10	b *	a	a	a	c	**ab**	a	a
20/15	b	a	a	**ab ‡**	**bc**	a	b	a
25/15	b	a	b	**abc**	b	ab	c	b
25/20	a	a	b	**bc**	b	bc	c	b
30/20	a	a	c	c	a	c	**c**	b

* Different letters indicate significantly different means within each measurement by a Bonferroni correction ($p \leq 0.05$).
‡ Bold font indicates different results between LSD test and Bonferroni correction.

Appendix 26 Effects of temperature regimes and sampling time on flavonoid gene expression level according to repeated measures ANOVA and two-way ANOVA tests in Experiment 1 at 76 DAA

Method of statistical analysis	Sources	*p* value														
		VviCHI 1	*VviF3' Ha*	*VviF3'5 'Hi*	*VviF3'5 'Hf*	*VviF3'5 'H/VviF 3'H*	*VviFLS 4*	*VviFLS 5*	*VviLD OX*	*VviUF GT*	*VviAO MT*	*Vvi3AT*	*VviGST 4*	*VviAM3*	*VviMyb A*	*VviMyb F1*
Repeated measures analysis of variance	TR *	0.266	< 0.001	< 0.001	0.001	< 0.001	0.100	0.001	0.165	0.012 ‡	< 0.001	0.038	0.631	0.257	0.035	0.001
	ST	0.851	0.934	0.320	< 0.001	0.022	0.914	< 0.001	0.689	0.198	0.020	0.129	0.070	0.014	0.116	0.185
	TR×ST	0.276	0.006	< 0.001	< 0.001	0.624	0.978	0.001	0.047	0.001	0.001	0.997	**0.049**	0.001	< 0.001	0.686
two-way ANOVA test	TR	0.137	< 0.001	< 0.001	< 0.001	< 0.001	0.017	< 0.001	0.219	0.005	< 0.001	0.037	0.421	0.277	0.036	< 0.001
	ST	0.680	0.952	0.453	< 0.001	0.024	0.966	0.001	0.443	0.239	0.017	0.108	0.060	0.008	0.186	0.234
	TR×ST	0.151	0.001	< 0.001	< 0.001	0.653	0.992	0.007	0.010	0.001	< 0.001	0.986	**0.059**	< 0.001	< 0.001	0.876

* TR and ST represent for temperature regimes and sampling time (between day and night), respectively.
‡ Bold font indicates inconsistent (in term of significance or lack of significance) results between repeated measures ANOVA and two-way ANOVA test. *p* value ≤ 0.01, ≤ 0.05, and > 0.05 were indicated as **, *, and ns in the gene expression figure, respectively.

Appendix 27 Effects of temperature regimes and sampling time on flavonoid gene expression level according to repeated measures ANOVA and two-way ANOVA tests in Experiment 1 at 89 DAA

Method of statistical analysis	Sources	*p* value														
		VviCHI 1	*VviF3' Ha*	*VviF3'5' Hi*	*VviF3'5' Hf*	*VviF3'5' H/VviF 3'H*	*VviFLS 4*	*VviFLS 5*	*VviLD OX*	*VviUF GT*	*VviAO MT*	*Vvi3AT*	*VviGST 4*	*VviAM3*	*VviMyb A*	*VviMyb F1*
Repeated measures analysis of variance	TR	0.082	0.245	0.669	0.626	0.020	0.043	< 0.001	**0.056**	0.232	0.185	0.001	0.111	0.163	0.006	0.018
	ST	0.311	0.055	0.768	0.431	0.009	0.439	0.439	0.113	0.034	0.293	0.231	0.913	0.120	0.010	0.613
	TR×ST	0.970	0.919	0.834	0.885	0.092	0.218	0.218	0.932	0.805	0.952	0.715	0.987	0.991	0.317	0.068
two-way ANOVA test	TR	0.120	0.406	0.827	0.717	0.003	< 0.001	0.003	**0.032**	0.439	0.191	< 0.001	0.406	0.189	0.019	0.039
	ST	0.255	0.056	0.554	0.465	0.007	0.472	0.595	0.110	0.040	0.430	0.123	0.923	0.090	0.003	0.450
	TR×ST	0.954	0.967	0.909	0.899	0.079	0.180	0.961	0.938	0.669	0.981	0.613	0.998	0.986	0.150	0.054

* TR and ST represent for temperature regimes and sampling time (between day and night), respectively.
‡ Bold font indicates inconsistent (in term of significance or lack of significance) results between repeated measures ANOVA and two-way ANOVA test. *p* value ≤ 0.01, ≤ 0.05, and > 0.05 were indicated as **, *, and ns in the gene expression figure, respectively.

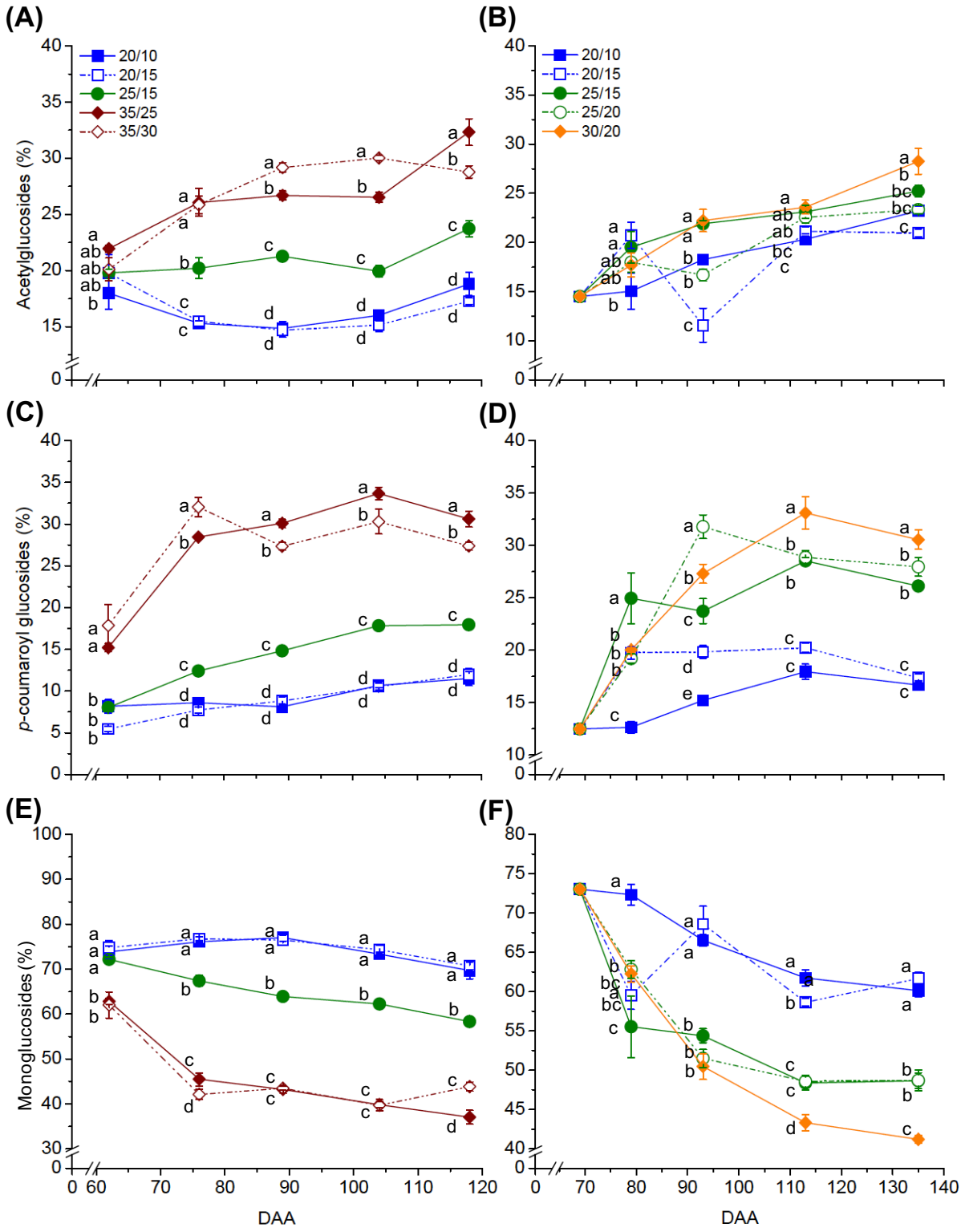
Appendix 28 *p* values resulting from an ANCOVA analysis of linear regression curves between TSS and total anthocyanin concentration (expressed as μg/g skin FW) under different temperature regimes in Experiment 1

Temperature regimes	20/10	20/15	25/15	35/25	35/30
20/10	N/A	0.714	0.037	< 0.001	< 0.001
20/15	0.714	N/A	0.004	< 0.001	< 0.001
25/15	0.037	0.004	N/A	0.007	0.003
35/25	< 0.001	< 0.001	0.007	N/A	0.842
35/30	< 0.001	< 0.001	0.003	0.842	N/A

Appendix 29 *p* values resulting from an ANCOVA analysis of linear regression curves between TSS and total anthocyanin concentration (expressed as μg/g skin FW) under different temperature regimes in Experiment 2

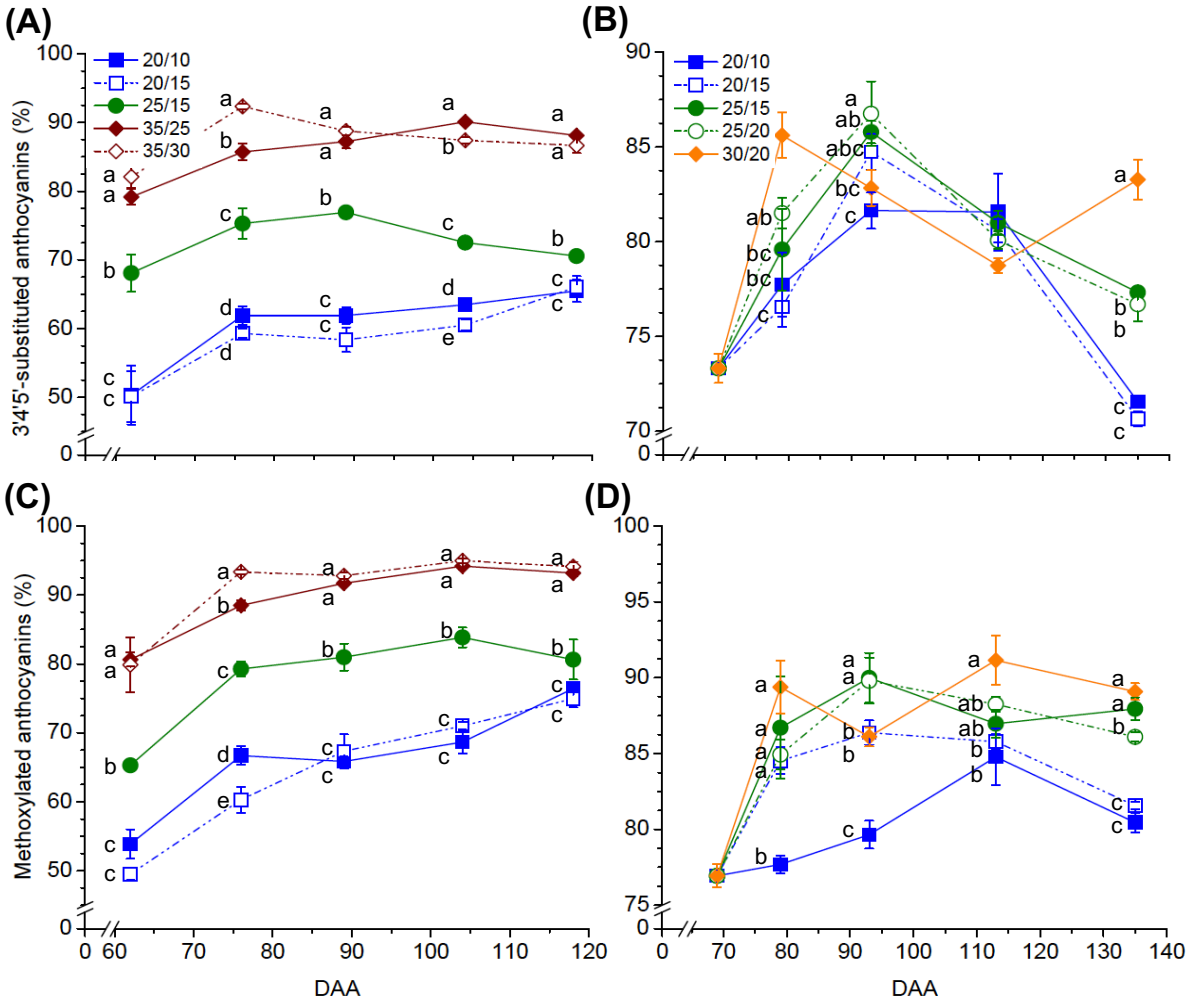
Temperature regimes	20/10	20/15	25/15	25/20	30/20
20/10	N/A	0.132	0.009	0.001	< 0.001
20/15	0.132	N/A	0.039	0.007	0.001
25/15	0.009	0.039	N/A	0.367	0.027
25/20	0.001	0.007	0.367	N/A	0.046
30/20	< 0.001	0.001	0.027	0.046	N/A

Appendix 30 Temperature effect on the evolution of the relative concentration of acylated anthocyanins during berry ripening in Experiment 1 (A, C, and E) and 2 (B, D, and F). Acetyl glucosides (A and B), *p*-coumaroyl glucosides (C and D), and non-acylated anthocyanins (monoglucosides, E and F).



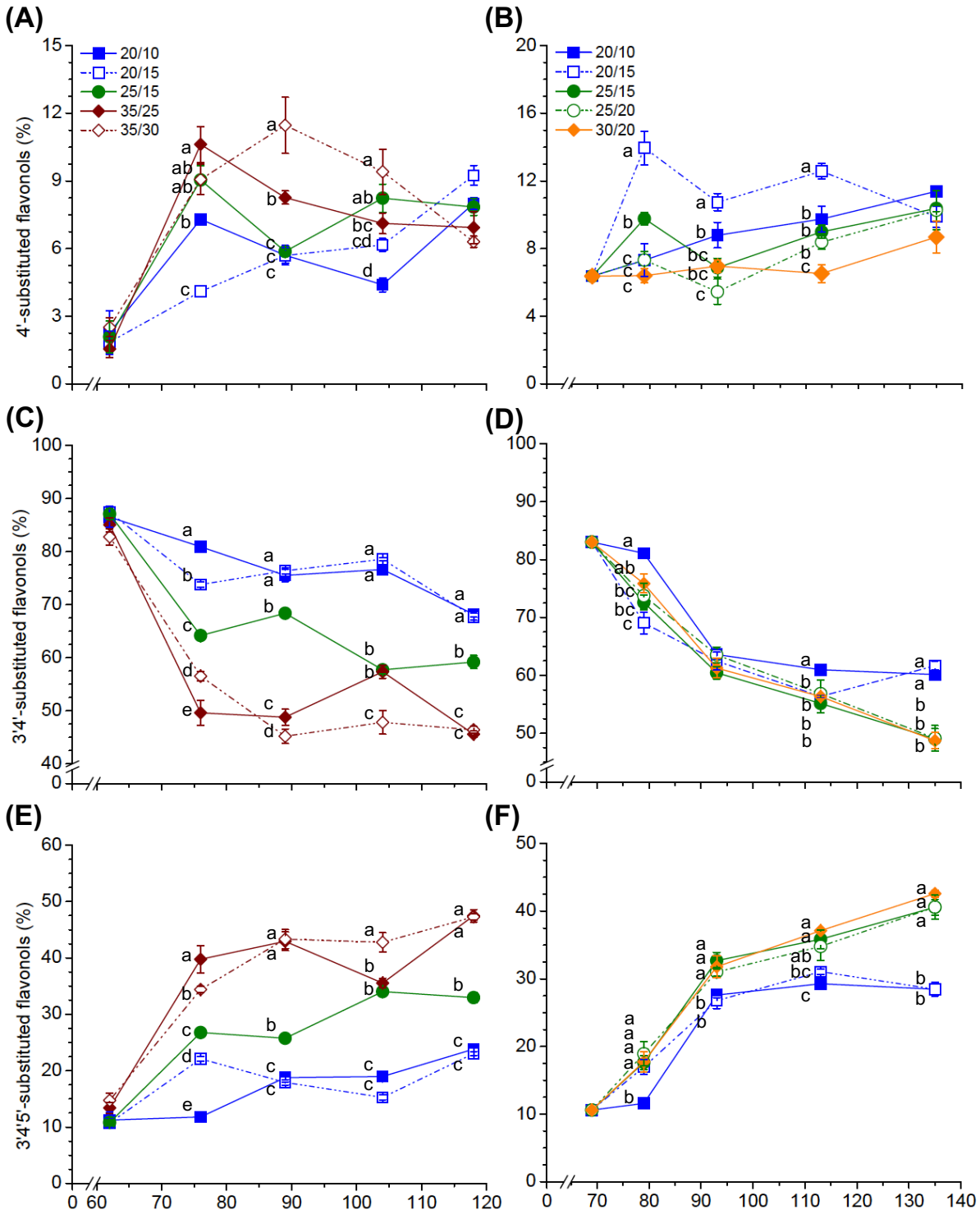
Values represent the mean ± standard error (SE, n = 4). Different letters indicate significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A, C, and E; legends in B indicated the treatments in B, D, and F.

Appendix 31 Temperature effect on the evolution of the relative concentration of 3'4'5'-substituted (A and B) and methoxylated (C and D) anthocyanins during berry ripening in Experiment 1 (A and C) and 2 (B and D).



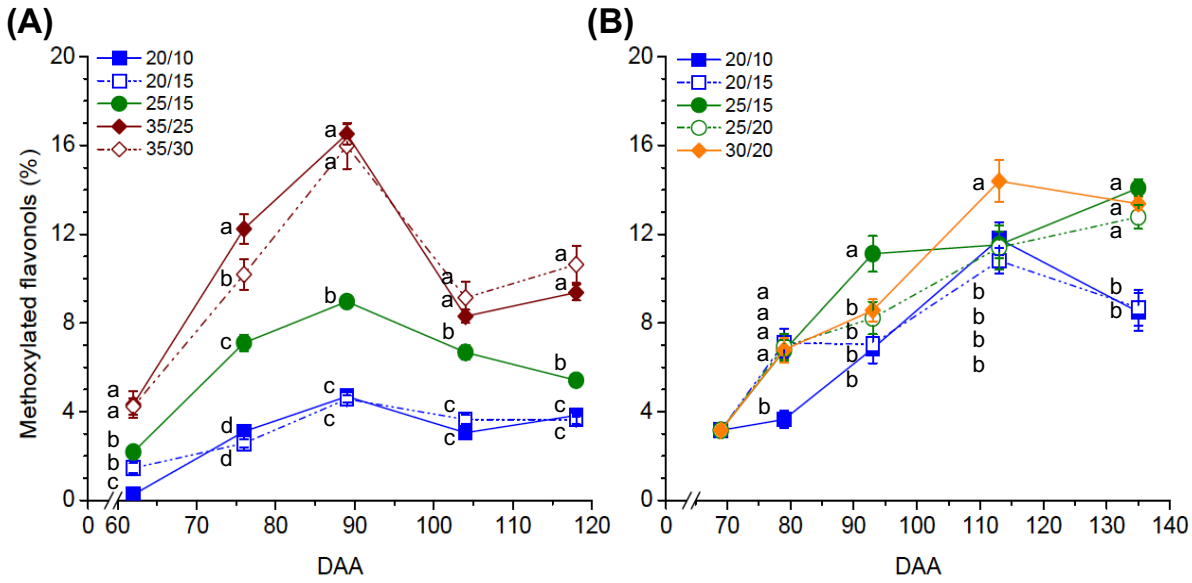
Values represent the mean ± standard error (SE, n = 4). Different letters represent significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A and C; legends in B indicated the treatments in B and D.

Appendix 32 Temperature effect on the evolution of the relative concentration of differentially substituted flavonols during berry ripening in Experiment 1 (A, C, and E) and 2 (B, D, and F).



Values represent the mean ± standard error (SE, n = 4). Different letters represent significantly different means according to an LSD test ($p \leq 0.05$). Legends in A indicated the treatments in A, C, and E; legends in B indicated the treatments in B, D, and F.

Appendix 33 Temperature effect on the evolution of the relative concentration of methoxylated flavonols during berry ripening in Experiment 1 (A) and 2 (B).



Values represent the mean ± standard error (SE, n = 4). Different letters represent significantly different means according to an LSD test ($p \leq 0.05$).