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Impact of short temperature exposure of *Vitis vinifera* L. cv. Shiraz grapevine bunches on berry development, primary metabolism and tannin accumulation

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ABSTRACT

Heatwaves are expected to become more frequent, reach higher maximum temperatures, last longer and occur earlier during the grape growing season. Such heat events during berry development are already known to dramatically reduce berry growth and quality by affecting berry size and primary metabolites (e.g. sugars, organic acids, and amino acids). In this study, the effect of three days of exposure to extreme high temperature after fruit-set was investigated with pot-grown fruiting grapevines (*Vitis vinifera* L. cv. Shiraz). Using a factorial design, heat treatments were applied for three days (+8 °C) and/or three nights (+6 °C), 20 days after the start of flowering, when tannin biosynthesis is at its maximum. Berry growth responses were recorded, and detailed flavan-3-ol and tannin composition determined for individual berry tissues to elucidate the sensitivity of these compounds to high temperature.

Locally heated bunches reached a maximum close to 45 °C on the first day of the heating treatment and berry growth and compositional parameters were immediately and substantially affected by high day temperature. In particular, a significant increase in galloylated skin flavan-3-ol subunits and consequently, percentage of galloylation of skin tannins, were observed two weeks after the end of the treatment. However, total skin tannins and size were not affected and no differences in composition were found closer to véraison. A considerable decrease in total seed tannins and changes in other seed compositional parameters were associated with disruption of berry and seed development upon day heating. In the pulp, various compounds (from the *myo*-inositol pathway, amino acids) were significantly increased under high day temperature while high night temperature reduced malic acid accumulation. Berries heated day and night exhibited the most differences but this was mainly driven by high day temperature.

1. Introduction

The record high temperatures observed around the world in the last two decades (e.g. summer 2003 and 2018 in the northern hemisphere, spring and summer 2008/09 and 2018/19 in Australia) have been directly attributed to climate change and global warming (Kornhuber et al., 2018; Rahmstorf and Coumou, 2011). Under those conditions, the grape growing season is shifted with key phenological stages advancing by many weeks in some locations, and average harvest dates moving forward (Petrie and Sadras, 2008; Sadras and Petrie, 2011). Heatwaves, which are expected to increase in severity (Perkins-Kirkpatrick and Pitman, 2018; Perkins-Kirkpatrick et al., 2016), can be defined by a range of indices including a period of at least three consecutive days with maximum temperatures above the 90th percentile for

Abbreviations: ANR, anthocyanidin reductase; C, (+)-catechin; CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating; dT, temperature difference; DW, dry weight; EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; EGC, (-)-epigallocatechin; FW, fresh weight; F+, days after flowering; GC, (+)-gallocatechin; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; mDP, mean degree of polymerisation; N, nitrogen; PVC, polyvinyl chloride; RH, relative humidity; SD, sampling date; T°C, temperature; TT_{seed}, total seed tannin; TT_{skin}, total skin tannin; TSS, total soluble solids; VPD, vapour pressure deficit; UV, ultra-violet; X_{term}, X terminal subunits; X_{up}, X upper subunits; %gall, percentage of galloylation

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a specific location and period of the year (Perkins and Alexander, 2013).

In Australia, Shiraz is the most planted red varieties in locations where maximum day temperatures can reach above 40 °C, including the mid-spring to early summer period when flowering and early stages of berry development occur. Occasionally, temperature can also peak around 45 °C, with for example, 45.5 °C recorded in Mildura in the Murray Valley on November 29, 2012 or 44.0 °C on December 31 in Griffith in the Riverina (Bureau of Meteorology, 2019). Without heat discharge, night temperatures may also remain above 30 °C. High temperature around flowering has been found to significantly affect the reproductive performance of several grape varieties (Buttrose and Hale, 1973; Dunn and Martin, 2000; Kliewer, 1977; Pagay and Collins, 2017). In addition, previous studies investigating high temperature effects on early berry development found that temperature is an important factor regulating berry growth and metabolism (Buttrose et al., 1971; Greer and Weston, 2010; Lecourieux et al., 2017; Matsui et al., 1986; Rienth et al., 2014a, Rienth et al., 2014b; Rienth et al., 2016; Sweetman et al., 2014). In these studies, short-term treatments varied in duration from several hours (2 to 48 h) to several days (4 to 14 days), and maximum temperatures reported for heating treatments spaned 30 to 43 °C. Longer treatments were also applied varying from several weeks/months or lasting the whole grape growing season but intensities were often milder (Gouot et al., 2019b). An important parameter identified during experiments conducted on Shiraz grapevines (*Vitis vinifera*) was the effect of day versus night temperatures, both impacting differently on grape primary metabolism and gene regulation (Sweetman et al., 2014). Understanding the impacts of heat events on key metabolites accumulated during early berry development will therefore be a key part of adapting viticulture and winemaking techniques to a warmer climate.

Organic acids, for example, are produced and accumulated in the pulp until véraison, and malic acid has been found to increase under warm night temperature (Sweetman et al., 2014). Other compounds such as the secondary metabolites are also synthetised early on to protect plants against stresses. In particular, synthesis of flavan-3-ols and tannins, also known as proanthocyanidins, starts in the inflorescence prior to flowering. Key enzymes involved in the phenylpropanoid pathway, such as leucoanthocyanidin/anthocyanidin reductase (LAR/ANR) and leucoanthocyanidin dioxygenase (LDOX) play an important role in tannin biosynthesis with maximum gene expression (*VvLDOX, VvANR*, *VvLAR1* and *VvLAR2*) within two weeks from flowering (Bogs et al., 2005). About two weeks after flowering, berry skin and seeds can be analysed separately and display different patterns of tannin biosynthesis (Gouot et al., 2019b; Rousserie et al., 2019). However, for both berry tissues, expression of known genes involved in tannin biosynthesis has been recorded at their maximum two-three weeks after flowering and then decline, except for *VvLDOX* in skin and *VvLAR2* in seeds (Bogs et al., 2005). To date, only one set of studies has investigated tannin accumulation and biosynthesis in detail. Heating and cooling of individual Merlot bunches was conducted for six weeks in the field pre- or post-véraison with tannin composition and gene expression slightly affected by temperature (Cohen et al., 2012a, 2008, 2012b). However, the effect of short spells of more extreme high temperature (> 35/40 °C) on these compounds remains unknown as studies investigating this aspect on flavonoids have mostly focussed on véraison, berry ripening and anthocyanins (Gouot et al., 2019b).

Grape berries, like other plant tissues exposed to extreme heat stress, would be expected to activate heat-tolerance mechanisms within the first hours to protect DNA and other cellular components from oxidation (Wahid et al., 2007). Under such stress, reactive oxygen species are produced and could contribute to visible damage such as browning and necrosis or compositional changes in the berry tissues. Flavonoids, as antioxidants, could be either directly affected by changes in biosynthesis, or chemically and enzymatically degraded, with their concentrations thus expected to decrease. The main hypothesis tested in this study is whether tannins and flavan-3-ols have a thermotolerance role by moderating the intracellular oxidation. If this is the case, then changes in tannin concentrations should be observed immediately after heat stress.

The study described here focussed on the impact of high temperature on flavan-3-ol and tannin biosynthesis during berry development and short-term responses of berry metabolism during and after heat stress. The experiment was conducted on potted Shiraz grapevines in a UV-transparent glasshouse, using a 2*2 factorial design with day and night temperatures as parameters. Bunches were individually heated for three days (+ 8 °C) and/or three nights (+ 6 °C) and skin and pulp flavan-3-ols and skin and seed tannins were measured during berry development. A wide range of metabolites (primary and secondary) were also analysed to assess treatment efficacy by monitoring the accumulation of known heat stress markers in grape berries. The system employed in this study heated bunches without major stress on the canopy. Common canopy manipulation practice of early leaf removal are used to decrease berry size or bunch compactness (Hickey and Wolf, 2018; Intrieri et al., 2008; Tardaguila et al., 2010), but as a consequence expose bunches to more sunlight and higher surface temperature (Hickey et al., 2018). In these experiments, changes in berry composition have been attributed to several parameters such as higher sink:source ratio, sunlight and/or temperature while in the present study, the sole effect of high temperature was studied.

2. Materials and methods

2.1. Plant material and experimental conditions

The experiment was carried out at Charles Sturt University, Wagga Wagga (35 °S, 147 °E) from October 2016 until December 2016 with six-year-old own-rooted Shiraz grapevines (V. *vinifera* L., clone EVOVS12) planted in 30 L pots and filled with premium commercial potting mix. Three weeks before flowering (October 24), vines were moved from an outdoor bird-proof enclosure into a UV-transmitting glasshouse (PLEXIGLAS® Alltop SDP 16/980 (/1053, /1200) – 64). The bay was orientated north-north-east and was fully illuminated from sunset to zenith. Air temperature, relative humidity (RH) and light were monitored at canopy height at two points in the centre of each bench. These consisted of a Tinytag Plus 2 TGP-4500 dual channel dataloggers (Gemini dataloggers, West Sussex, UK) and a SQ-110 quantum sensor (Apogee Instruments Inc, Logan, Utah, USA). The glasshouse temperature was regulated by evaporative air conditioning and gas heating, with daily maxima averaging 32 ± 3 °C, daily minima 17 ± 2 °C, and the average glasshouse RH varying between 36 and 74%. The photosynthetically active radiation varied from 0 at night to 2100 $W/m^2/s$ on the sunniest day. The Plexiglas allowed up to 70% of UV A (365 nm) and B (312 nm) to reach the vines at 2 p.m. on a sunny day, as measured with a portable UV sensor (VLX 3 W, Cole Parmer) and compared with outside. The glasshouse CO_2 concentration was measured with a LCA-4 portable infrared gas analyser (ADC Bioscientific Ltd., Hoddesdon, Hertfordshire, UK) and averaged 425 ± 9 ppm.

2.2. Treatment application

Before flowering, vines were pruned to six shoots, trained vertically with bamboo stakes and crop-thinned to four inflorescences per vine. Flowering (E-L 19) was defined as the average date of the first cap fall for each inflorescence (Coombe, 1995). Heating treatments started 21 days after flowering, at the end of berry set (E-L 31; berry pea size). Day heating ran from 7 a.m. to 7 p.m., and night heating from 8 p.m. to 6 a.m. for three days and three nights over a period of four days. Treatments were applied to individual bunches using a heater system adapted from Tarara et al. (2000). Polyvinyl chloride (PVC) delivery tubes with a 9 cm diameter and length of 30 cm were positioned 10 cm away from bunches at a 45° angle. Hot or ambient air was blown

(around 1 m^3/min) with axial fans installed at the lower end of the delivery tube. Hot air was produced with commercial heaters (2000 W) blowing at 10 m^3/min into an insulated box (1 m^3) and delivered through flexible ducts to the fans and PVC tubes. To minimise any interactions between the heat treatment and plant water status, irrigation was scheduled to maintain soil water near saturation. This was checked by regular soil moisture measurements using a ML2x ThetaProbe soil moisture sensor connected to a HH2 moisture meter (Delta-T Devices Ltd, Cambridge, UK) with an average volumetric water content of 30% for the duration of the study.

The temperature of the air delivered to each bunch by the heating system was measured by bare fine-wire thermocouples (0.13 mm diameter; Type T) positioned in front of the bunch, about 10 cm from the exit of the PVC tubes. Sensor signals were scanned and recorded every 10 s by two data acquisition systems (AM-25T and CR-1000, Campbell Scientific, Logan, UT, USA) from flowering until the end of the treatment application. Berry surface temperature was also recorded using a thermal imaging camera (FLIR One for Android, FLIR systems, Wilsonville, OR, USA) at several times of the day and night during the experiment and images were processed using the FLIR tool software (Version 5.13.17214.2001, FLIR systems, Wilsonville, OR, USA).

2.3. Experimental design

A total of 16 vines (3 replicates per treatment and 4 buffers) were arranged on two steel mesh benches in a randomised block design generated using DiGGer (Coombes, 2009). A complete factorial design was applied to the effect of temperature on berry composition. The effects of two independent factors, day and night temperatures (Day TºC and Night TºC), were investigated. The temperatures tested varied between two levels: high (+) where bunches were heated, and low (-) where only the fan was used to blow ambient air (slight cooling effect). The design generated four treatments: control blower (CB; day and night fan only), day heating (DH; with night fan), night heating (NH; with day fan), and day and night heating (DNH). Untreated vines were used as buffers at the extremity of each bench.

2.4. Berry sampling

Berries were sampled at four key stages: Sampling Date 0 (SD0), two days before treatment application; SD1, one day after the end of the treatment; SD2, 2 weeks later; and SD3, 3 weeks later (onset of véraison - softening and colouration - determined for the most advanced bunches). Each sampling date is referenced to the number of days after flowering (F+) 19, 25, 40 and 47, respectively. At each SD, two to four berries were collected per bunch and pooled together per vine. Samples were snap-frozen in liquid nitrogen (N) after determining berry fresh weight (FW) and stored at -80 °C. At processing, berries were slightly thawed and quickly separated into skin, pulp and seeds on ice. Pulp and juice were homogenised and immediately ground into a fine powder using mortar and pestle under liquid N. Total soluble solids (TSS) and sugar content were determined for the last two SDs as follows: ground powder (around 1 g) was transferred to an Eppendorf tube, thawed for 5 min at 20 °C, vortexed and then centrifuged at 14,000 rpm for 2 min at 4 °C (Eppendorf microcentrifuge 5430R, Hamburg, Germany); TSS (in ºBrix) was determined from the supernatant with an Atago refractometer (PR-101, Atago, Tokyo, Japan). The remaining pulp/juice powder was kept frozen and then freeze-dried until constant weight (Gamma 1–16 LSC, Christ, Osterode am Harz, Germany). Skins were carefully peeled from the berries with tweezers and then blotted dry. Skin and seed fresh mass was determined before snap-freezing in liquid N and storage at -80 °C. Before analysis, skins and seeds were ground with a mortar and pestle under liquid N and freeze-dried until constant weight.

2.5. Chemical analyses

2.5.1. Primary metabolites

Pulp samples (20 mg of freeze-dried powder) were extracted, stored at -80 °C, and derivatised as detailed in Gouot et al. (2019a). GC–MS analyses were performed as per Rossouw et al. (2019) on an Agilent system consisting of a 7890A gas chromatograph and 5975C mass spectrometer with an electron impact ionisation source and a quadrupole analyser (all from Agilent Technologies, Agilent, Santa Clara, CA, USA). Compound identification was conducted after spectral deconvolution as previously described (Gouot et al., 2019a; Rossouw et al., 2019). Compounds were semi-quantified after data normalisation to internal standard and tissue dry weight (DW).

2.5.2. Secondary metabolites

The extraction protocol was adapted from Pinasseau et al. (2017). Freeze-dried skin samples of 35 mg were used for extraction, matching the optimised solid:liquid ratio used by Pinasseau et al. (2017) with 100 mg of fresh skin and average skin moisture of 65% determined as per Section 2.4 above. First, 500 µL of methanol were added, followed by 3.5 mL of 0.05% (v/v) trifluoroacetic acid in acetone/water (70/30, v/v) and 100 µL of an internal standard solution containing corticosterone and ampicillin at 0.5 g/L each in 50% (v/v) aqueous methanol. Seed samples (10 mg of freeze-dried powder) were similarly extracted with volumes of solvent reduced by 2.5 (200 µL of methanol, 1.4 mL of acetone/water and 40 µL of internal standard). Samples were first sonicated in ice for 10 min and then, shaken in a cool dark room using a Ratek rotary mixer for 20 min before centrifugation (5 min, 4000 rpm, 4 °C). An aliquot of 500 µL was dried under constant flow of N_2 gas for polyphenol analysis. Additional aliquots of 500 µL were transferred into separate tubes and dried with Genevac (EZ-2 Plus, SP Scientific, Ipswich, England) for tannin analysis.

Skin and seed samples for tannin analysis were prepared by phloroglucinolysis and both tannin and polyphenol analyses were conducted on an Agilent LC-QQQ system exactly as described in Gouot et al. (2019a). Tannin terminal (X_{term}) and upper (X_{up}), also called extension, subunits were quantified as per Gouot et al. (2019a) using (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC) standards (Extrasynthese, Genay, France). Polyphenols were semi-quantified using the multiple reaction monitoring mode. Both data sets were normalised to tissue DW.

2.6. Statistical analyses

Samples at F + 19 were very small (less than 0.3 g per berry) and, except for berry weight measurements, were combined to give one replicate per treatment, hence no error bars appear on graphs for the first SD. From F + 25 onwards, for each variable at a single sampling date, factors (Day TºC and Night TºC) were analysed by 2-way ANOVA and treatments (CB, DH, NH and DNH) by one-way ANOVA followed by post-hoc Tukey HSD test for mean comparison (R software, version 3.5.1).

3. Results

3.1. Temperature

Air (thermocouples) and bunch surface (thermal images) temperatures were measured during the experiment (Fig. 1). The average temperature difference between high and low levels (dT) over the four-day treatment period was calculated from the air temperature recordings in front of the bunches (Fig. 1A). During the day, $dT_{(DH)}$ and $dT_{(DNH)}$ were maintained at +7.8 ± 2.2 °C and +8.1 ± 2.1 °C, respectively, and at night, $dT_{(NH)}$ = +5.6 ± 0.4 °C and $dT_{(DNH)}$ = +5.9 ± 0.4 °C. Night heating was stable from one bunch to another. However, day heating was also dependent on external sunlight

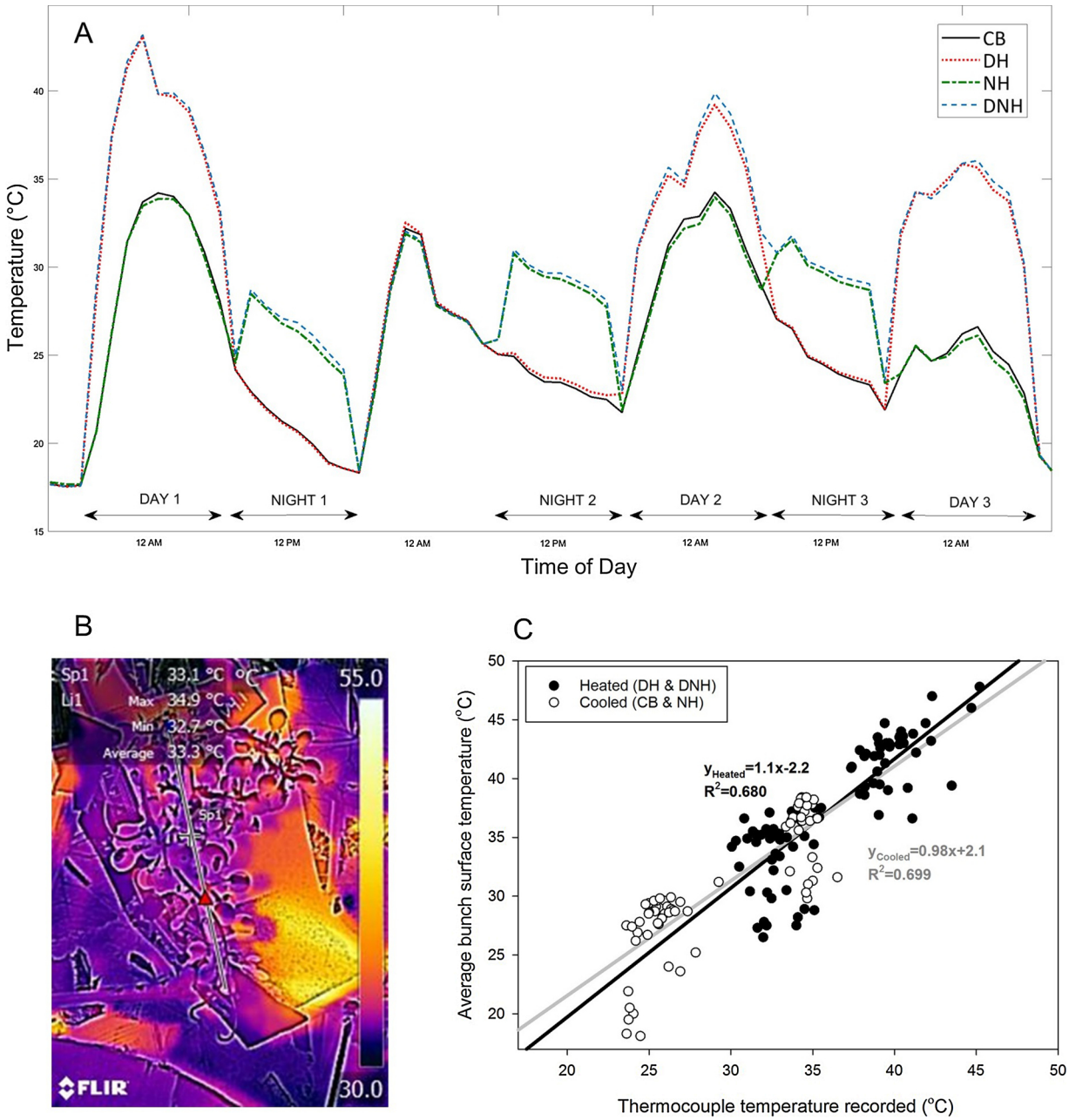

Fig. 1. Temperature records during the experiment with (A) mean air temperature (90 min intervals) averaged per treatment (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating), (B) example of thermal pictures with temperature (recorded at the red triangle position - Sp1) and maximum (Max), minimum (Min) and average (FLIR tool software) and (C) relationship between berry surface and air temperatures during the day. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

exposure and more variations occurred. Thermal images (Fig. 1B) were taken at different times of day during heating to span a wide range of temperatures and compared with data obtained from the thermocouples (Fig. 1C). In general, air and average bunch surface temperatures were well correlated (R^2 = 0.699 with fans only, and R^2 = 0.680 when heated).

Day and night temperatures followed a typical diurnal pattern. During daylight hours, on the first day of treatment for example, the temperature quickly increased from 20.0 °C at 7 a.m. to 44.8 °C at 2 p.m. The next two days of treatment were less intense with maxima recorded at 41.5 °C (day 2) and 37.5 °C (day 3) (Supplementary – Table A.1). At night, treatments reached their maxima early on (28.9–32.8 °C) before temperatures slowly decreased until their minimum just before sunrise.

RH was recorded during treatment application using two sensors in the glasshouse. Vapour pressure deficit (VPD) was estimated, assuming RH was uniform in the glasshouse and values recorded at canopy height were representative of the bunch microclimate. VPD is a function between temperature and RH, hence VPD was automatically higher for heated bunches, reaching a maximum of 8 kPa on the first treatment day (Supplementary – Table A.1).

3.2. Berry physiology

Bunch damage appeared within four hours of heating with browning of the bunch stem, pedicel and skin of green berries. This was followed by rapid berry desiccation the following day (Supplementary – Fig. A.1). Damage was localised to berries exposed to temperatures

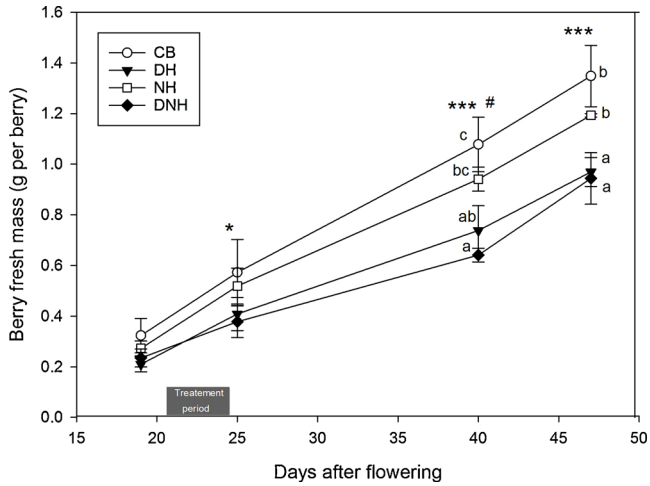

Fig. 2. Effect of treatments (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating) and heat factors (day and night temperature) on berry fresh weight (n = 3, mean ± stdev). Significant effects tested by 2-way ANOVA are indicated with * (day), # (night) and X (interactions) for each sampling date ($p < 0.05$, 0.01, 0.001). Significant differences between treatments for each sampling date are indicated with lower case letters ($p < 0.05$).

higher than the average. For example, the bottom of the bunch of DNH was higher in temperature than average with 48.1 versus 42.9 °C (Supplementary – Fig. A.1). Only berries without visible damage were collected for physiology and metabolite analyses. Berry weight was regularly measured throughout the experiment and averaged 261 ± 64 mg on SD0 (F + 19; all treatments combined) with some pre-existing differences between treatments (Fig. 2). Berry weight appeared to be immediately significantly affected by Day T°C with DH and DNH berries both smaller than CB from F + 25 onward. While Night T°C did not have any immediate effect on berry weight, berries heated overnight were smaller than their counterpart at F + 40 (NH vs CB; DNH vs DH).

In addition, parameters such as the proportion of pulp and skin, and their percentage of moisture, were most affected by Day T°C with minimal impact from Night T°C and interactions (Table 1). The number of seeds per berry were recorded to check sample homogeneity with no differences in treatments. Only at F + 25, Day T°C had an effect leading to treated berries with a slightly higher number of seeds ($p = 0.048$). Seed fresh mass and percentage of moisture were the most affected parameters by both Day T°C and Night T°C. TSS, recorded at the last sampling date, was not affected, however, sugar content, calculated from TSS and berry weight, was significantly lower under high Day T°C.

3.3. Pulp primary metabolites

About a third of the 85 compounds detected by GC–MS in the pulp was affected by day and/or night heating (Fig. 3). The carbohydrate metabolism was significantly affected with some minor sugars such as tagatose, rhamnose, dulcitol and fucose increased by high Day T°C. However, the accumulation of sucrose, glucose and fructose, central to the carbohydrate metabolism, was only slightly impacted by temperature with a small increase of fructose under high Day T°C. The *myo*-inositol metabolism was the most affected pathway with a rapid increase of *myo*-inositol, galactinol, raffinose and melibiose under high Day T°C, just after treatment. Metabolite abundance then returned to that of CB and NH. *Myo*-inositol and raffinose were also slightly increased by high Night T°C at F + 25 and 40, respectively. Glycerate, a key compound at the intersection of several pathways, was slightly increased under high Day T°C at F + 25. Several amino acids such as methionine, asparagine, valine, leucine and isoleucine were also immediately increased under high Day T°C. In addition, some of the main organic acids involved in the tricarboxylic acid cycle were significantly affected with a slight decrease in malic and maleic acid under high Night T°C at F + 47, and succinic and oxoglutaric acid at F + 25 under high Day T°C. Despite a significant increase in shikimate as well as tryptophan and phenylalanine under high Day T°C, no effect was observed on the phenylpropanoid pathway in the pulp.

3.4. Skin and seed secondary metabolites

3.4.1. Skin tannins

Total skin tannin (TT_{skin}) content increased from 3.6 to 6.6 mg/berry during development without any differences between treatments (Supplementary - Fig. A.2A). When expressed as concentration (Supplementary - Fig. A.2B), TT_{skin} followed the opposite trend with a slow decline from about 14.2 to 6.1 mg/g berry. Day T°C impacted TT_{skin} at every sampling date with a significantly higher concentration observed for DNH berries at F + 25 and F + 40 compared to CB (Supplementary - Fig. A.2B). Night T°C also impacted on TT_{skin} at F + 40 with slightly higher concentration in treated berries. However, TT_{skin} concentration expressed per gram of skin oscillated between 60 and 100 mg/g skin without differences between treatments (Supplementary - Fig. A.2C).

To study the metabolic response of tannin accumulation, concentrations on a DW basis were however preferred (Fig. 4) as mass and moisture were affected by the treatments. TT_{skin} concentrations varied around 360 mg/g skin DW at F + 19 before treatment application, and slightly decreased during berry development to around 330 mg/g skin DW (Fig. 4A). Tannin size, characterised by the mean degree of polymerisation (mDP) (Kennedy and Jones, 2001), slightly increased during berry development, from around 28 at F + 19 to about 33 at F + 47 but without any effect from treatments or factors (data not shown). After polymer cleavage by phloroglucinolysis, nine compounds were detected with (-)-epicatechin upper subunit (EC_{up}) the most abundant (around 65%), spanning 170–245 mg/g skin DW and (-)-epigallocatechin upper subunit (EGC_{up}) following (about 20%), spanning 62–86 mg/g skin DW. Other upper and terminal subunits of ECG, C, GC and EC were also detected in lower concentrations (from 10 to 0.01%). The percentage of dihydroxylated (C, EC, ECG) and trihydroxylated (GC, EGC) flavan-3-ols was relatively stable throughout berry development, spanning 64.7–67.0% and 33.0–35.3%, respectively, with no effect of treatments and factors on di/trihydroxylated proportions and ratio (data not shown). Day T°C had a small effect on tannin composition by affecting both EGC_{up} and EGC_{term} at F + 25 (Fig. 4B–C). Both DH and DNH had lower EGC concentrations, however no significant differences between treatments were found.

The percentage of galloylation (%gall) was calculated as per Le Bourvellec et al. (2005) and decreased over time from 7% at F + 19 to 5% at F + 47. It was also significantly affected by Day T°C at F + 40, leading to a significantly higher %gall for DNH (6.0%) than CB (4.2%) (Fig. 4D). This effect was concomitant with significantly higher concentrations in ECG_{up} and ECG_{term} (Fig. 4E–F).

3.4.2. Seed tannins

The total seed tannin (TT_{seed}) content increased from 1.7 to 7 mg/berry while TT_{seed}, expressed in mg/g berry FW, slightly increased from F + 19 to F + 25 before returning to pre-treatment concentrations around 6–7 mg (Fig. 5A–B). Significant differences were found between treatments at F + 40 and 47 for tannin content with Day T°C significantly decreasing TT_{seed}, and both DH and DNH significantly reduced compared to CB. The greatest treatment effect was recorded for the tannin content on a seed basis with Day T°C significantly decreasing TT_{seed} at every SD as well as an effect of Night T°C and interactions at F + 40 and 47 (Fig. 5C). TT_{seed} concentrations (mg/g seed) marginally increased during berry development with significant interaction at F + 47 (Fig. 5D). When expressed on a DW basis, TT_{seed} (mg/g seed)

Table 1
Berry parameters for each treatment (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating) and heat factors: day and night temperatures (Day T°C and Night T°C) tested by 2-way ANOVA (n = 3, mean ± stdev). Significant differences between treatments for a given sampling date are indicated with lower case letters ($p < 0.05$). Significant effects of Day T°C and Night T°C as well as interactions for each sampling date are indicated with asterisks (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS; not significant).

Parameters	Date	Treatment				Factors		
		CB	DH	NH	DNH	Day T°C	Night T°C	Int[a]
Proportion of pulp per berry (% fresh mass)	F+25	75 ± 2 ab	73 ± 1 a	77 ± 1 b	75 ± 1 ab	*	*	NS
	F+40	83.2 ± 0.4	82.6 ± 0.5	84.2 ± 0.4	83 ± 2	NS	NS	NS
	F+47	85.2 ± 0.2	85 ± 1	85.6 ± 0.3	85 ± 1	NS	NS	NS
Pulp moisture (%)	F+25	90 ± 3	88 ± 2	91.4 ± 0.8	88.1 ± 0.7	*	NS	NS
	F+40	92.5 ± 0.6	90.6 ± 0.9	92 ± 1	91 ± 1	NS	NS	NS
	F+47	91 ± 1	91.1 ± 0.6	91.7 ± 0.3	89.7 ± 0.4	NS	NS	*
Proportion of skin per berry (% fresh mass)	F+25	13 ± 1 a	16 ± 1 b	12.7 ± 0.1 a	14.9 ± 0.4 ab	**	NS	NS
	F+40	8.9 ± 0.5	10 ± 1	8.6 ± 0.4	10 ± 1	*	NS	NS
	F+47	7.5 ± 0.6 a	8.1 ± 0.1 ab	7.9 ± 0.3 ab	9 ± 1 b	**	*	NS
Skin moisture (%)	F+25	78 ± 2	79 ± 1	78 ± 2	77 ± 2	NS	NS	NS
	F+40	73 ± 2	73 ± 3	70 ± 3	69 ± 3	*	NS	NS
	F+47	72 ± 5	70 ± 3	75.7 ± 0.2	70 ± 1	NS	NS	NS
Proportion of seed per berry (% fresh mass)	F+25	12 ± 2	11.6 ± 0.8	10 ± 1	10 ± 1	NS	*	NS
	F+40	7.9 ± 0.5	7.4 ± 0.6	7.2 ± 0.6	6.4 ± 0.9	NS	NS	NS
	F+47	7.4 ± 0.3	6.9 ± 1	6.5 ± 0.4	6.1 ± 0.7	NS	NS	NS
Number of seed per berry	F+25	2.6 ± 0.4	3.1 ± 0.2	2.5 ± 0.4	2.9 ± 0.2	*	NS	NS
	F+40	2.2 ± 0.2	2.3 ± 0.2	2.6 ± 0.3	2.3 ± 0.3	NS	NS	NS
	F+47	2.4 ± 0.3	2.5 ± 0.5	2.7 ± 0.1	2.3 ± 0.5	NS	NS	NS
Seed fresh mass (mg)	F+25	25 ± 6 b	15 ± 2 ab	22 ± 4 ab	13 ± 3 a	**	NS	NS
	F+40	38 ± 3 c	24 ± 3 b	26 ± 1 b	17.5 ± 0.6 a	***	***	NS
	F+47	41 ± 6 b	26 ± 1 a	29 ± 2 a	24.9 ± 0.1 a	**	**	*
Seed moisture (%)	F+25	72 ± 9	81 ± 2	77 ± 8	83 ± 1	NS	NS	NS
	F+40	43 ± 4 a	61 ± 4 bc	52 ± 3 ab	65 ± 5 c	***	*	NS
	F+47	43 ± 1 a	50 ± 2 b	43 ± 2 a	50 ± 2 b	***	NS	NS
TSS[b] (°Brix)	F+47	5 ± 1	3.67 ± 0.06	4.2 ± 0.4	4.1 ± 0.6	NS	NS	NS
Sugar content(mg per berry)	F+47	61 ± 18	35 ± 1	49 ± 4	38 ± 8	*	NS	NS

[a] Interactions between Day T°C and Night T°C.
[b] Total soluble solids, F+: days after flowering.

was however impacted by Day T°C for all three SDs after treatment application (data not shown).

The nine upper and terminal subunits found in skin were also detected in seeds after polymer cleavage but in different proportions with trihydroxylated compounds, for example, detected in very small amounts (< 0.2%). Most compounds decreased in concentration from F + 25 until F + 47 except ECG_{term} which was constant around 90 µg/g seed DW and EC_{term} which increased from 0.6 to an average of 6.5 mg/g (data not shown). The seed tannin profile (DW basis) was affected in many ways and PCAs were chosen to highlight differences in overall composition between treatments. Changes were mostly driven by Day T°C, and a good separation of DH and DNH from CB and NH was evident at F + 25 along both 1st and 2nd Principal Components (PC1 and PC2). Treatments heated during the day (DH and DNH) showed an increased %gall and higher concentrations of all subunits, except C_{up} (Fig. 6A). At F + 40, all heating treatments were grouped together, and mDP was increased for DH (6.2), DNH (6.1) and NH (5.7) compared to CB (4.7). Most subunits were also clearly increased in all heated treatments compared to CB, which only had a greater concentration of EC_{term} (Fig. 6B). At F + 47, only a good separation between DNH and CB remained (Fig. 6C). DNH exhibited higher concentrations of all subunits except EC_{term}, as well as increased %gall and mDP compared to CB. A separation between DH/DNH and NH was also observed along PC1 and probably driven by an increase in TT_{seed} and mDP for the treatments under high Day T°C. In summary, the most significantly affected subunit was EC_{term} with an initial increase of 20% under high Day T°C but followed by a decrease of 38% (F + 40) and 22% (F + 47).

3.4.3. Skin polyphenols

Around 30 compounds were detected in the skin at every sampling date as well as some anthocyanins detected at the onset of véraison (F + 47). Of those, 13 metabolites were affected by either Day and/or Night T°C (Fig. 7). Phenylalanine and tyrosine, both amino acid precursors to polyphenols, were increased just after the application of high Day T°C as well as one phenolic acid, fertaric acid. In addition, dihydroquercetin, a dihydroxylated flavonoid, increased throughout berry development under high Day T°C. Flavonols were mostly unaffected by heat except myricetin-3-O-glucuronide and quercetin-3-O-glucuronide which were reduced under high Night T°C. Free flavan-3-ols such as C, EC, EGC and GC were also detected but only trihydroxylated forms were increased under high Day T°C right after treatment application.

4. Discussion

Bunch and berry surface temperatures depend on both background air temperature and energy received through the interception of solar radiation (Cola et al., 2009; Reshef et al., 2018; Smart and Sinclair, 1976; Tarara et al., 2008). The aim of this work was to study the sole effect of temperature on berry physiology and composition, using a chamber-free heating system to avoid changing bunch light exposure. The temperature of air blown onto each bunch related well with the average berry surface temperature as measured by thermal imaging, indicating the capacity of the system to control berry temperature despite variations in bunch light exposure throughout the day. During the day, an average difference of + 8 °C was maintained between heated and control, but the maximum temperature (44.8 °C) reached by berries on day 1 was probably the most critical and resulted in bunch damage, including tissue necrosis. Similar results have been observed in Gamay vines heated at 43 °C with injuries to the pedicel and bunch stem and subsequent berry shrivelling after two days (Matsui et al., 1986).

The level at which grapevines are exposed to high temperature can contribute to variations between results with whole-vine compared to bunch experiments. When using targeted bunch heating, the confounding effects of temperature on leaves, photosynthesis and stomatal

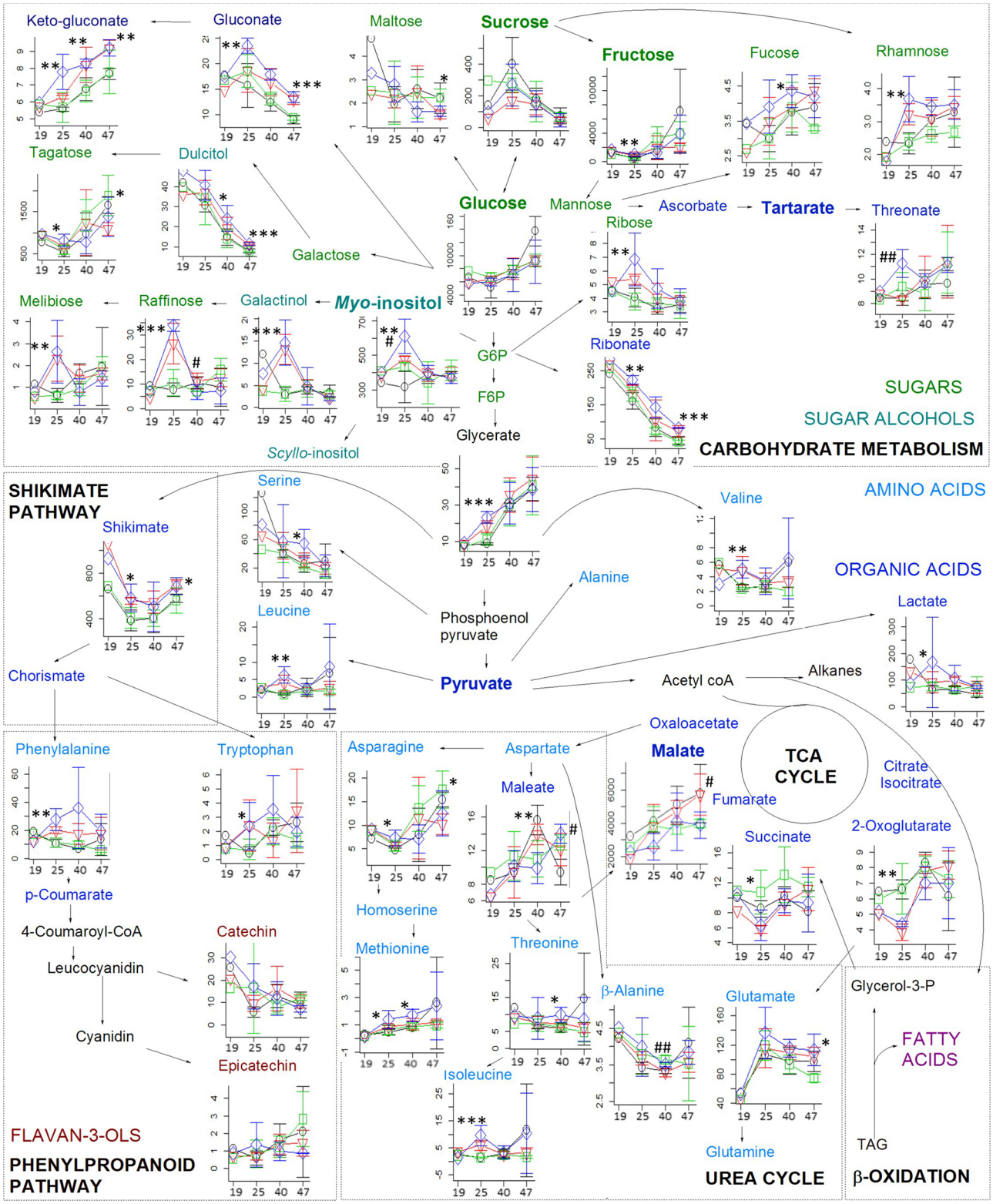

Fig. 3. Schematic representation of the pulp primary (and some secondary) metabolite pathways including tricarboxylic acid cycle (TCA) showing average metabolite abundance (n = 3, mean ± stdev) as affected by heating factors (*; day or #; night temperature or X; interactions) and tested by 2-way ANOVA ($p < 0.05$; 0.01; 0.001) for each treatment (control blower: —o—; day heating: —▽—; night heating: —□—; day and night heating: —◇—) at four sampling dates identified as days after flowering. Symbols (*, #, X) are placed left of F + 25/40 and right of F + 47.

responses are minimised compared to whole-vine experiments where the entire canopy is exposed to heat treatments. Therefore, any effects should be attributable to the immediate bunch microclimate, and not to an interaction with the canopy or loss of leaf function. Berry loss observed in this study would have reduced the number of "sinks" per bunch, and, as often observed with biomass responses of berries following thinning treatments (e.g. Han et al., 2019), high temperature could also have indirectly affected berry growth and metabolism by

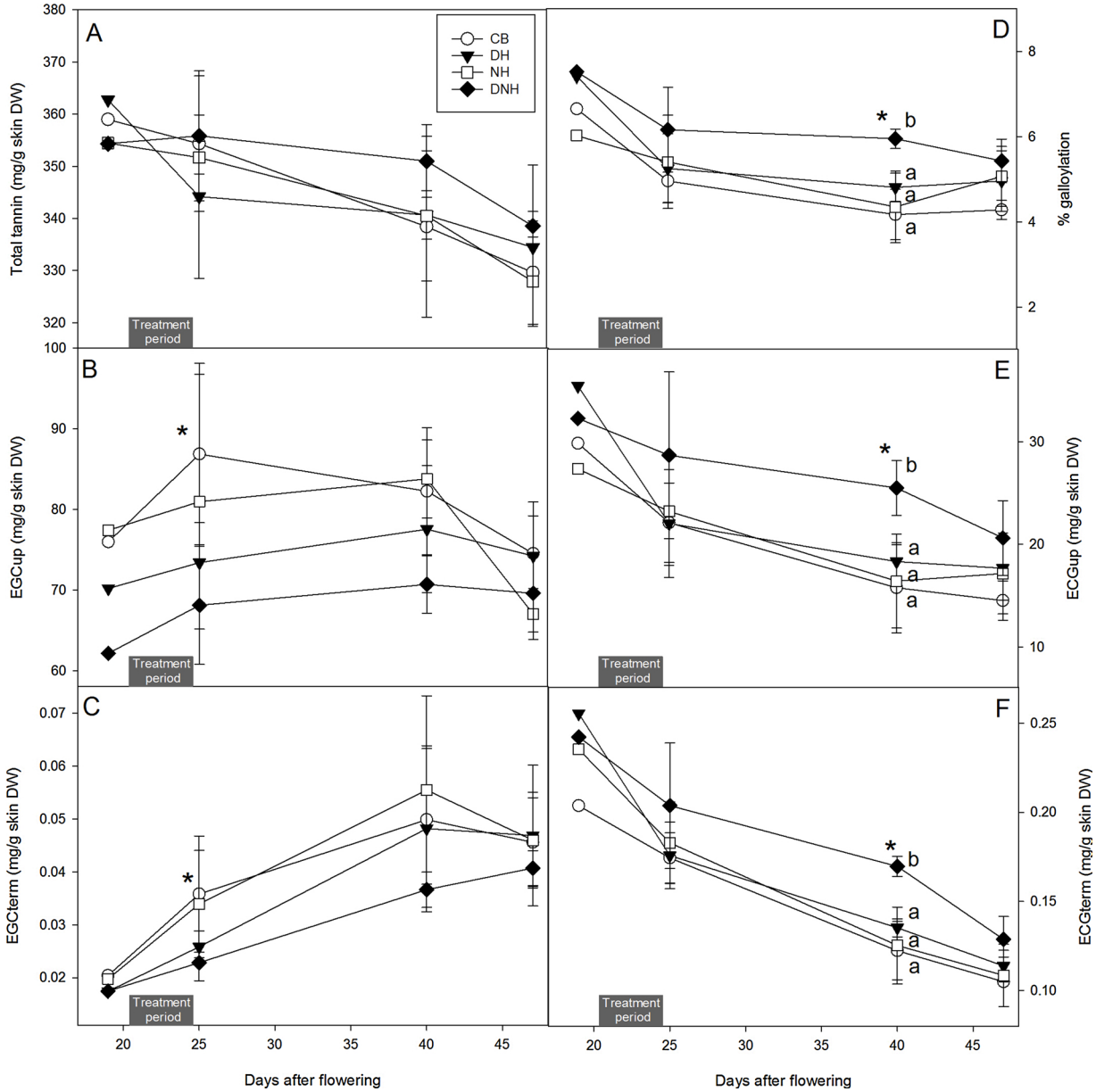

Fig. 4. Effect of treatments (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating) and heat factors (day and night temperature) on skin tannin concentration (A) epigallocatechin upper (B) and terminal (C), epicatechin gallate upper (E) and terminal (F) subunit concentrations and percentage of galloylation (D), (n = 3, mean ± stdev). Significant effects tested by 2-way ANOVA are indicated with * (day), # (night) and X (interactions) for each sampling date ($p < 0.05$, 0.01, 0.001). Significant differences between treatments for each sampling date are indicated with lower case letters ($p < 0.05$).

increasing carbohydrate availability for the remaining fruit. While some interaction between the temperature treatment and carbon supply to the berries can therefore not be ruled out, the dominant effect was from temperature with berries from F + 25 onwards significantly smaller when heated during the day.

In previous experiments, when grapevines were exposed to high temperature during early berry development around setting, growth was differently impacted depending on heat stress intensity. One study conducted on well-irrigated field Shiraz, with whole vines reaching a maximum of 45.7 °C at E-L 31, found berries were significantly smaller (Soar et al., 2009). However, when repeated the second season, maximum temperature only reached 41.5 °C and berry growth was unaffected this time. In another study, no differences were observed for Semillon grapes exposed to 40 °C at berry set for four days (Greer and Weston, 2010). In our study, temperature reached a maximum close to 45 °C and berry growth was immediately disrupted. Collectively, results of the current and earlier studies point to a temperature threshold of 42–45 °C around fruit-set, above which final berry size is irreversibly reduced.

Intra-cluster berry heterogeneity was not considered in this study as sufficient material was needed for chemical analysis. However, berries were carefully sampled so as to be representative of the bunch and most bunches were not yet going through véraison by the end of the experiment. Inter-vine berry heterogeneity at SD0 was particularly high as berries had just set and the flowering date could vary by 2–3 days between vines even though the average per treatment was the same. This source of variation may explain the differences in metabolite concentrations observed at the start of the experiment, and could have also contributed to differences after treatment application. However, upon heating, the magnitude of the differences observed were generally greater and some compounds were immediately significantly increased, with most effects observed just after treatment application. These

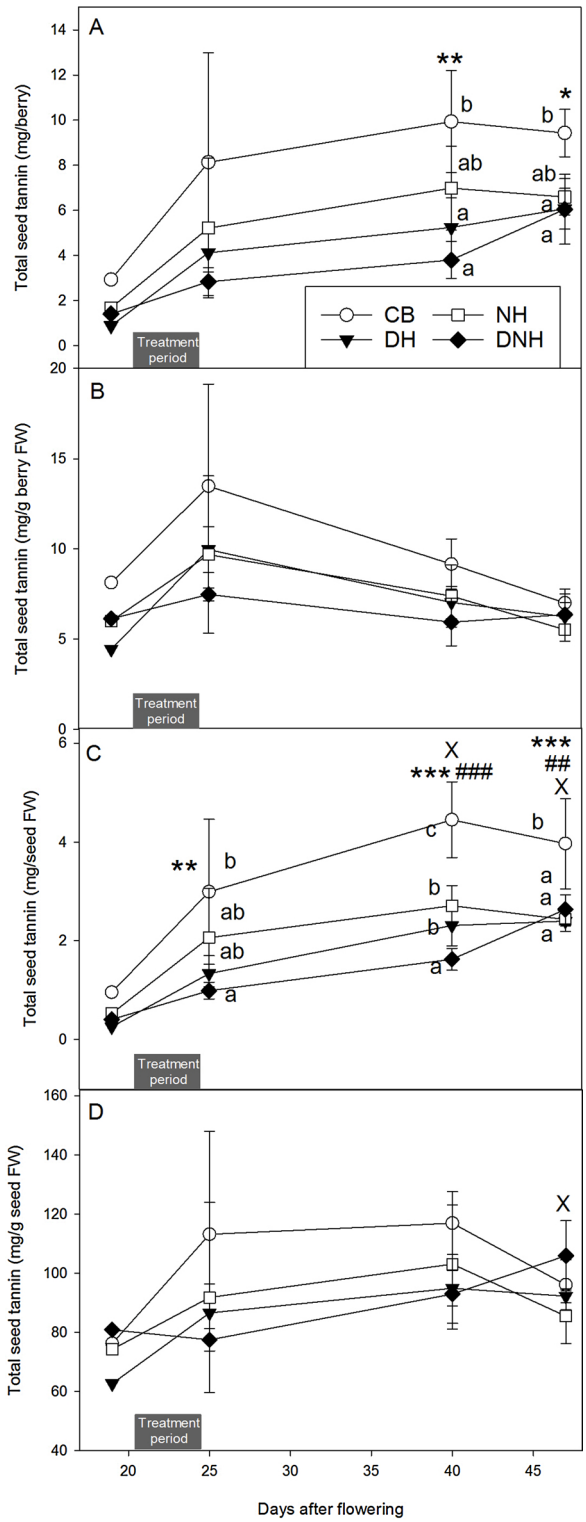

Fig. 5. Effect of treatments (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating) and heat factors (day and night temperature) on seed berry tannin concentration expressed in (A) mg/berry, (B) mg/g berry, (C) mg/g seed and (D) mg/seed (n = 3, mean ± stdev, B,C&D on a fresh weight basis). Significant effects tested by 2-way ANOVA are indicated with * (day), # (night) and X (interactions) for each sampling date ($p < 0.05$, 0.01, 0.001). Significant differences between treatments for each sampling date are indicated with lower case letters ($p < 0.05$).

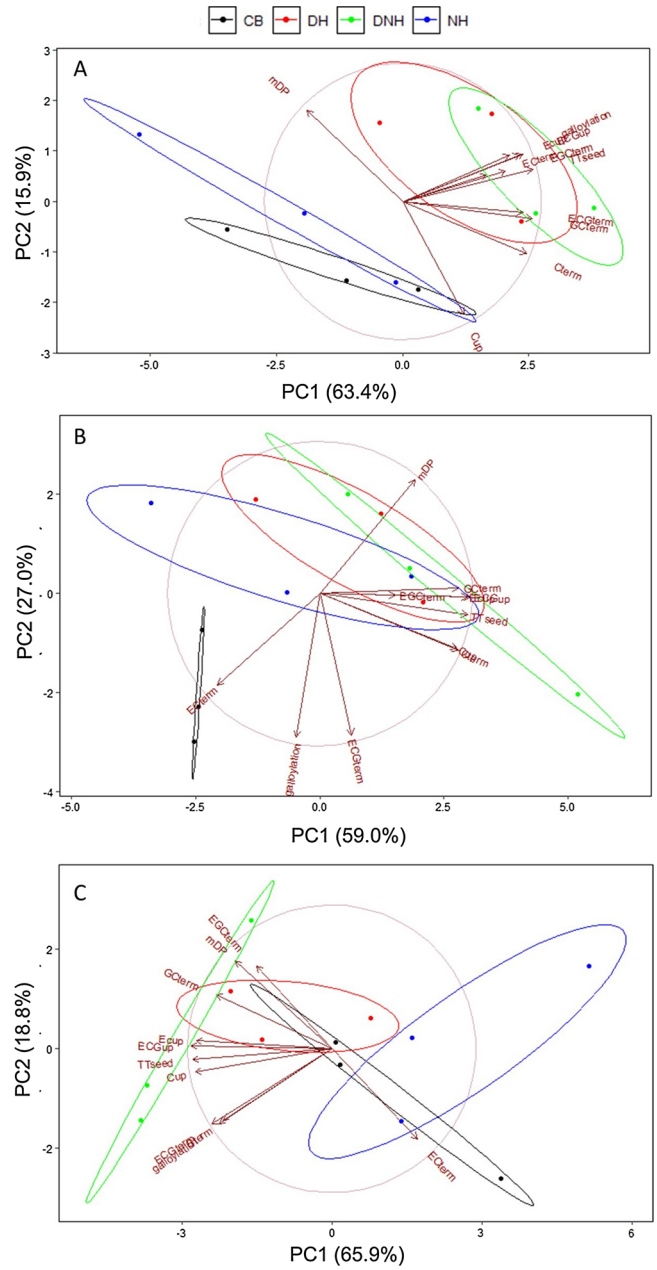

Fig. 6. Principal component (PC) analysis showing the impact of treatments (CB, control blower; DH, day heating; NH, night heating; DNH, day and night heating) on seed berry tannin composition at (A) 25 days after flowering (F + 25), (B) 40 days after flowering (F + 40) and (C) 47 days after flowering (F + 47). Abbreviations used in the legends: C: (+)-catechin; EC: (-)-epicatechin; ECG: (-)-epicatechin gallate; GC: (+)-gallocatechin; EGC: (-)-epigallocatechin; Xterm: X terminal subunit; Xup: X upper subunit; galloylation: percentage of galloylation; mDP: mean degree of polymerisation; TTseed: total seed tannins with all concentrations expressed in mg/g seed DW.

results suggest an increase in biosynthesis by up-regulation of genes coding for the enzyme involved upstream of their synthesis or down-regulation of the genes downstream. Compounds from the *myo*-inositol pathway such as galactinol and raffinose showed a significant increase in abundance just after high Day T°C exposure (F + 25). Previous research investigated the effect of high day temperature (+ 6 or + 9 °C)

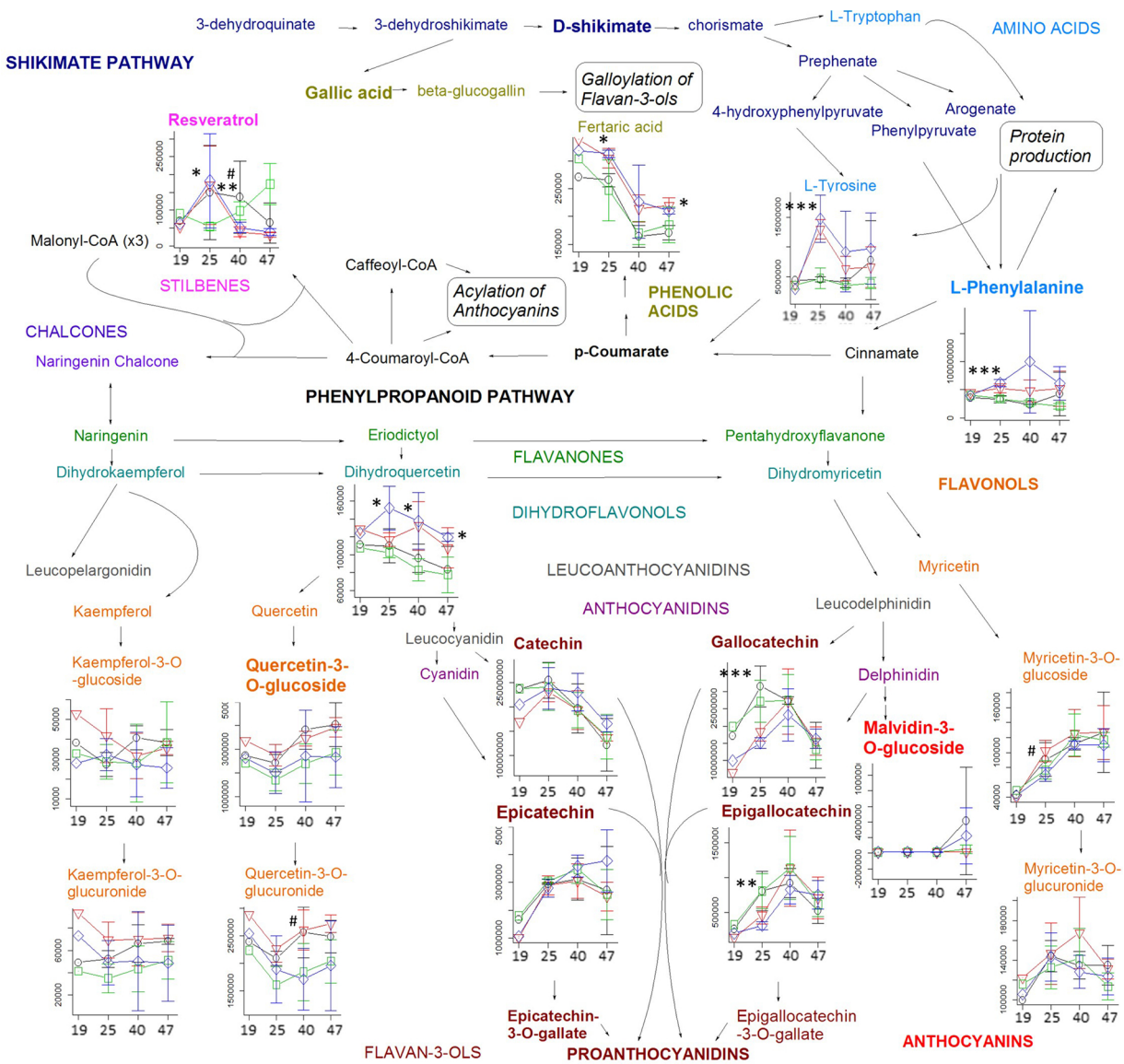

Fig. 7. Schematic representation of the skin shikimate and phenylpropanoid pathways showing average metabolite abundance (n = 3, mean ± stdev) as affected by heating factors (*; day or #; night temperature or X; interactions) and tested by 2-way ANOVA ($p < 0.05$; 0.01; 0.001) for each treatment (control blower: —○—; day heating: —▽—; night heating: —□—; day and night heating: —◇—) at four sampling dates identified as days after flowering. Symbols (*, #, X) are placed left of F + 25/40 and right of F + 47.

for 21 days at two different stages (véraison and mid-maturity) on bunches of fruiting cuttings of Cabernet Sauvignon. The authors found that galactinol concentration was increased after 3 weeks of heat treatment starting at 50% véraison, and the expression of *VvGOLS1*, gene coding for galactinol synthase, was upregulated under heat stress after 1 h onwards (Pillet et al., 2012). In the present study, the whole branch of the *myo*-inositol pathway, including galactinol, was increased under high Day T°C but results could not all be compared to those of Pillet et al. (2012) as raffinose was not detected under their experimental conditions. The metabolic response of the pulp was quite similar to that of another study conducted on Australian Shiraz at E-L 31 using a similar design but for a longer heating period of 11 days (Sweetman et al., 2014), with increases in amino acids, organic acids and *myo*-inositol with high Day T°C. In addition, minor sugars responded to high Day T°C while major sugars were mostly unaffected, in agreement with Sweetman et al. (2014) who found glucose and fructose unchanged.

Despite extensive research on flavonoids and temperature, a limited number of studies have focused on the effect of high temperature on tannin accumulation (Bonada et al., 2015; Cohen et al., 2012a, 2008, 2012b; Mori et al., 2004; Pastore et al., 2017). Among previous work, a number of methods have been employed to measure tannins and other phenolics, with benchtop spectrophotometric methods limiting measurements to total concentrations (Bonada et al., 2015; Mori et al., 2004). Liquid chromatography after polymer cleavage offers more information, but methods using UV detection are limited to the most abundant compounds (Cohen et al., 2012a, 2008, 2012b). With the latest improvements in chromatographic techniques coupled with the use of MS/MS detectors such as a triple quadrupole (Pinasseau et al., 2016), the detection of minor compounds including trihydroxylated flavan-3-ols in seeds or GC_{term} and EGC_{term} in skin has been made possible. Thus, in the present study, flavan-3-ols and tannins were examined in detail to elucidate whether their accumulation was sensitive to extreme high temperature exposure early in berry development. As composition and biosynthesis differ considerably between seeds and skin, tissues were analysed separately (Gouot et al., 2019b; Rousserie et al., 2019).

Surprisingly, in skin, the first barrier protecting against abiotic stresses, tannins were only slightly affected and, apart from a small

change in %gall, ECG and EGC subunits, composition and total skin tannins were mostly unaffected by high day and night temperatures. The effect of temperature on tannins is still not well understood and only one study found that high temperature during berry development increased initial skin tannin accumulation in Merlot bunches, although the effect was no longer evident by véraison (Cohen et al., 2012a). Another study on high temperature during berry ripening on whole vines did not observe any effect on skin tannins by harvest in Sangiovese (Pastore et al., 2017). A lack of knowledge of the exact galloylation process also restricts gene expression studies to known genes involved in the phenylpropanoid pathway (Gouot et al., 2019b). Rienth et al. (2016) suggested that temperature could impact tannin biosynthesis in young green berries, and in particular, galloylation, due to changes in regulation of transcripts coding for UDP glucose-gallic acid-glucosyltransferase, one of the enzymes involved in the addition of gallic acid to flavan-3-ols. Whole microvines were heated with expression was measured in deseeded berry samples and up-regulation associated with cool temperature. Although no detailed tannin analyses were conducted to confirm the impact on concentrations post-treatment, this up-regulation would have probably led to increased tannin galloylation in cooler berries. This contrasts with the findings of the present study, where berry skin galloylation increased when bunches only were exposed to high temperature.

Seeds are expected to be more protected from temperature extremes inside berries than the skin (Saudreau et al., 2007; Wang et al., 2001) and previous work did not observe changes in seed composition with abiotic factors impacting on bunch microclimate (Cohen et al., 2008; Downey et al., 2004; Fujita et al., 2007). However, in the present study, seed tannins were the most affected by high Day T°C with most subunits increased on a DW basis. Overall, seed development was significantly disrupted by high Day T°C with an immediate decrease in seed fresh mass, mirrored by a decrease in berry mass (maintaining the same seed/berry proportion at all SDs). The decrease in seed moisture typically observed during berry development was delayed in heated berries, suggesting that seed growth may have been slowed or interrupted over the treatment period. As a result, seed tannins, which typically decline over berry development, did not decrease as fast in heated berries exhibiting higher concentrations of tannins and most flavan-3-ol subunits on a DW basis. The impact of high Day and/or Night T°C was reflected differently on a FW basis with all treatments heated during the day having a lower tannin berry content as seeds were smaller in those berries. Seeds are important during winemaking as wine mouthfeel can depend on grape seed phenolic maturity as well as extractability and composition of seed tannins (Rousserie et al., 2019). In this study, extrapolation to wine cannot be directly made as the experiment stopped before full maturity but knowing that seeds are affected by high temperature during early development would suggest that wine quality could be affected.

Several studies have suggested that night temperature affects physiology and composition more than day temperature, mostly from work involving whole-vine (Kliewer, 1973; Koshita et al., 2007; Rienth et al., 2014a, Rienth et al., 2014b) but also bunch treatments (Koshita et al., 2007). In the current study however, with heating directly applied on bunches, Night T°C had almost no impact compared to Day T°C, especially on the compounds of interest (pulp flavan-3-ols and skin tannins), except for total tannin content in seeds. The main effect of high Night T°C was a significant decrease in maleic acid, malic acid and β-alanine in the pulp. This finding contrasts with previous studies on Shiraz (Sweetman et al., 2014) and Pinot Meunier microvines (Rienth et al., 2016) which observed an increase in malic acid under high day/night temperature (30/25 °C and 35/20 °C, respectively) before véraison. In our study, potential degradation or reduction of synthesis could be explained by the higher day and especially night maximum temperature regime tested (45/33 °C). While DNH berries exhibited the most differences compared to CB, only few significant interactions between day and night were found, suggesting high Day T°C was the principal factor inducing metabolism changes. Night T°C had almost no effect on its own but could have accentuated the effect of Day T°C and increased bunch damage for DNH vines.

Overall, high temperature only had a small effect on berry tannins with skin tannin only slightly affected and changes in seed tannins most likely an indirect consequence of berry development disruption. The majority of studies would suggest that abiotic factors such as water regimes (Ojeda et al., 2002; Pinasseau et al., 2017) and light (Downey et al., 2006, 2004; Ristic et al., 2007) have more impact on skin tannins than temperature. However, when temperature and water stress have been compared within the same study, temperature was found to have a greater impact on skin tannin than water stress at harvest (Bonada et al., 2015). In the latter, temperature was manipulated for the whole season, while in the present study, the short duration of exposure could explain the lack of long-term response of skin tannins and highlight the ability of Shiraz bunches to restore their metabolism after three days of localised heat stress. However, when berries were exposed to extreme temperatures just above the threshold of survival, necrosis led to complete berry desiccation which would lead to an important loss of yield by harvest. Several mitigation methods are available to minimise the effects of heat events such as irrigation, cover crops, shade cloths or hydrocooling. Extreme heat events represent a greater threat than an increase in average temperature, and it would be thus primordial to reduce bunch temperature during those critical events to prevent irreversible damage.

5. Conclusion

In the present study, extreme high day temperature was found to be critical for berry development. The biochemical response of Shiraz grapevine bunches, locally heated for three days and/or nights after fruit set, was similar to previous experiments conducted on whole-vines or bunches and heated for 11 days. A short heat stress of only three days was sufficient to dramatically reduce berry weight and trigger responses previously reported in grape berries under high temperature. These were likely initiated on day 1 of the heating treatment as bunch temperature rapidly increased and reached values close to 45 °C. Metabolite changes were mostly visible two weeks after treatment application. Skin tannin accumulation during berry development did not seem to be sensitive to extreme high day and/or night temperature with only small effects observed. However, total seed tannins and composition were affected by high Day T°C as seed development was disrupted, leading to smaller berries and seeds with less tannins. Overall it appears that tannins and flavan-3-ols may not have a direct role in berry thermotolerance. Observed changes in tannins were either minor, as opposed to galactinol for example, for which there is stronger evidence in participating in heat stress signalling, or indirect, i.e. disruption of berry development.

Contributions

J.C.G., J.P.S., B.P.H. and C.B. designed the experiment. J.C.G. conducted the experiment, analysed the samples, interpreted the data and wrote the manuscript. C.B. supervised the chemical analyses. All authors reviewed, edited and approved the final version of the manuscript. J.C.G., J.P.S., B.P.H. and C.B. do not have competing interests and take responsibility for the integrity of the work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103866.

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