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Improving industry capacity to manage the yield and wine quality relationship through understanding the influence of vine carbon balance on berry composition



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Abstract

Vine balance is a concept describing the relationship between whole vine carbon uptake and its utilisation, as sugar, for fruit production. It is commonly considered that composition of the berry, and resulting wine, is strongly affected by vine balance. Field manipulations of vine balance were replicated, in three contrasting regions, for three seasons. In addition, the effect of defoliation was simulated, without changing bunch environment, by enclosing whole vines in chambers and supplying them with low CO_2 air to reduce photosynthesis.

Changing vine balance consistently altered the rate of ripening, but did not correlate with treatment effects on fruit composition, where they occurred. Late defoliation extended the maturation period, but *reduced* total anthocyanin content. Crop removal shortened the maturation period, but had little effect on the fruit. Interestingly, early defoliation had no clear effect on vine balance, but resulted in both increased anthocyanin and increased tannin content. The chamber experiment also extended the maturation period, but had no effect on the relationship between sugar and anthocyanins. Overall, there was no conclusive evidence that the changes in vine balance achieved had any significant effect on fruit or wine composition when fruit was harvested at the same sugar ripeness.

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Executive summary

The concept of vine balance is an empirical one and is resistant to a specific definition. However, the importance of balanced vines for quality fruit production is widely acknowledged in the global wine industry and significant time and effort are spent on managing the relationship between crop load and vine vigour. This is often to the detriment of vine yield. Despite this, measurements of vine balance are not often made, and more commonly used metrics, such as the Ravaz index, are retrospective in nature and limited in their applicability across seasons. What the various definitions and metrics have in common, is an acknowledgement that, in essence, vine balance is a source-sink relationship. For the purposes of this project vine balance was defined as the ratio between yield and canopy size.

This multi-disciplinary project presented an opportunity to address the extent to which vine balance directly drives berry composition. By studying the physiology and molecular biology of fruit responses to variations in assimilate supply, whether driven by environmental variability, direct manipulation of crop load or changing carbon dioxide (CO_2) concentrations under controlled conditions, this project aimed to determine the extent to which vine balance directly influences fruit composition.

This project utilised three different approaches to comprehend vine balance: to apply crop and canopy treatments in a consistent manner across trials sites that differed significantly in production aim and climatic conditions, thereby generating fruit from vines that differ in vine balance through multiple causes; to directly manipulate vine carbohydrate supply to the fruit independently of environment; and to not only assess the fruit, and resulting wine, from these experiments, but to utilise molecular biology tools to examine the impact of changes in vine balance on the key genes that regulate the fruit composition. In all cases, physiological maturity of the fruit was defined as a total soluble solids concentration of 24°Brix.

The field component of the project utilised three sites in three states, all with Shiraz vines of a similar age, at Langhorne Creek, Murray Valley and Hilltops. Early defoliation, crop thinning, late defoliation and minimal pruning management strategies were imposed in the same way at each site and reapplied on the same vines in three consecutive seasons shortly before flowering or veraison.

Despite wide differences in vine yield between the sites and the seasons (ranging from 1.1 to 30.1 kg/vine), the impact of the treatments was largely consistent. The early defoliation treatment improved the anthocyanin to sugar ratio and the tannin content of the fruit, but had no effect on vine balance; the crop thinning treatment had no effect on fruit composition, despite drastically reducing yield at all sites; the late defoliation reduced the anthocyanin to sugar ratio, despite elongating the maturation period; and the minimal pruning treatment had no effect on anthocyanin to sugar ratio despite the vines not being fully adjusted to the treatment by the project end.

The direct manipulation of fruit carbohydrate supply was achieved using chambers to reduce CO_2 concentration in the air supplied to the vines by approximately 50%, thereby reducing photosynthesis and carbohydrate availability (simulating a high yield to canopy size vine, without affecting the bunch environment). When applied during the maturation period the

system slowed the rate of sugar accumulation in the fruit by about 50%. However, doing this had no effect on the anthocyanin to sugar ratio of the fruit during maturation or at harvest.

Wine was made in small lots (50 kg ferments) from all the field treatments and using micro-vinification (1 kg ferments) from the chamber treatments. In all cases, the wine composition reflected the anthocyanin to sugar ratio of the fruit used and therefore the effects observed in the fruit were also observed in the wine. In fact, the field effects were often even more exaggerated in the wine. The extraction of anthocyanins from the fruit to the wine generally modified the proportion of different forms of anthocyanins, with treatments T2 (early defoliation) and T4 (late defoliation) impacting on extractability.

The gene expression data supported the results seen in the fruit and wine composition, with no clear effect of vine balance *per se* on the expression of the key regulatory genes for anthocyanin and phenolic compounds. However, in the early defoliation treatment, which did not affect vine balance, but did improve fruit composition, a small increase in *flavonol synthase* (*FLS*) gene expression across sites and seasons was observed in flowers and very young berries. Although this change in gene expression is likely to be a result of increased light exposure of the fruit it could be used to develop a molecular marker for an increase in anthocyanins and tannins at a very early stage of berry development. Interestingly, *UFGT*, which encodes the final step in anthocyanin synthesis, was reduced in the late defoliation treatments over several sampling timepoints, suggesting that the negative impact of this treatment on fruit anthocyanin content was not simply driven by reduced enzyme activity from increased temperature of exposed fruit.

Overall, neither the field management strategies, nor the direct manipulation of fruit carbohydrate supply, supported a significant direct role for vine balance, defined as yield to canopy size ratio, in determining fruit composition at harvest. This was supported by both sensory and chemical analysis of the wines made from that fruit and questions the benefit of using non-selective crop thinning to improve fruit quality.

However, the negative effect of late defoliation and the positive effect of early defoliation do suggest a significant role for bunch environment on fruit, and resulting wine, composition. While this is far from a new concept, the results from this project indicate that focusing vineyard management on bunch environment rather than yield control would be a more successful means to produce fruit, and wine, with a composition typically seen as being of higher quality. Furthermore, the project results suggest that successful implementation of bunch environment management can benefit fruit composition across a wide range of climates and viticultural targets, enhancing the quality of wines produced in any given wine region.

Chapter 1

Project Background

The importance of balanced vines for quality fruit production is widely acknowledged and significant consideration and resources are devoted to managing the relationship between crop load and canopy size. However, commonly used indices, such as harvest yield / pruning weight, are retrospective in nature and often of limited applicability due to inter-seasonal variability. Irrigation and pruning practice remain the most widely used techniques to achieve vine balance in Australian viticulture. Techniques such as early basal leaf removal and, more recently, severe summer pruning have been developed to regulate crop load and control the rate of berry ripening. Mechanical methods provide the option for immediate crop load adjustments and manual bunch thinning can allow precise control over yield. Thus, growers possess a wide range of practical tools for the management of vine balance and fruit quality. However, a clear understanding of the effects of vine balance on fruit composition is still lacking. Furthermore, it is difficult to predict when early canopy or crop load manipulations will benefit fruit quality, or how such methods may be best utilised.

This multi-disciplinary project presented an opportunity to address the extent to which vine balance directly drives berry composition. By studying the physiology of fruit responses to variations in assimilate supply, whether driven by environmental variability or direct manipulation of crop load, the project aimed to inform effective management of the yield and quality relationship within a season, reducing inter-seasonal variability and improving vineyard profitability.

Crop load and vine balance effects on berry composition and wine quality have long been the subject of research interest (e.g. Ravaz 1930, Kliewer and Weaver 1971, Reynolds et al., 1994). Specific ratios for achieving optimal balance have been developed, e.g. 5-10:1 for yield to pruning weight (Bravdo et al., 1985), or 0.3-0.6:1 for kg of pruning weight per metre of canopy (Smart and Robinson 1991). However, these ratios do not directly address berry composition and extrapolations are difficult when environmental and viticultural factors such as water availability, trellis and pruning systems, climate and extent of storage reserves can vary ripening capacity between seasons and vineyards. Sugar accumulation in grapes has been correlated with leaf area and whole vine assimilation (e.g. Intrieri et al., 1997, Kliewer and Dokoozlian 2005), demonstrating the fundamental link between berry ripening and carbohydrate supply. How this influences other measures of fruit quality is more difficult to determine. Clingeleffer et al., (2005) found manipulations aimed at crop control had an effect on maturation, but there was no consistent relationship between yield and wine quality parameters. Dokoozlian et al., (2011) observed that both high and low cropped Cabernet Sauvignon vines had less desirable concentrations of two aroma precursor compounds in berries, compared with those of ‘balanced’ vines. Bunch thinning at véraison significantly improved wine scores and colour in Shiraz and modified the relationship between anthocyanin and sugar accumulation (Smith and Holzapfel, unpublished). The ability to reconcile and explain these differences would be greatly enhanced by a mechanistic understanding of carbon balance effects on berry composition.

Understanding the influence of environment, vineyard management practices and genetic factors on the composition of red wine grapes is critical for the wine industry to match wine styles with market expectations. Manipulation of crop load and vine balance, even in the absence of an exact definition, has been shown by research and experience to influence berry composition, but information on how to consistently achieve desired outcomes for sugar, acids, colour and tannins is lacking.

Project Aims and Performance targets

Utilising multiple field sites, with replicated manipulations of vine balance, and a controlled outdoor experiment to manipulate carbohydrate availability to the berry, the project objectives were to:

- Provide the Australian wine industry with the knowledge and tools to manage the yield, grape composition and wine quality relationship, enabling growers to respond and adapt to conditions within a growing season and changing market segment demands.
- Examine the role of vine balance, defined as the dynamic relationship between vine carbon acquisition and berry carbon demand, in determining berry sugars, acids, colour and tannins and wine quality. Determine whether this role is consistent across three regions representative of large-scale commercial viticulture in Australia.
- Evaluate crop and canopy management options to improve berry colour and tannin development whilst maintaining yield in an Australian viticultural setting and provide an insight into the vineyard management practices required to maximise specific quality attributes.
- Examine the physiological and molecular mechanisms that underlie the impact of vine balance on berry composition and wine quality, by determining canopy size to crop load ratio, estimating internal carbohydrate supply, monitoring berry sugar, acids, colour and tannins and wine quality parameters across three viticultural regions.
- Determine if early season physiological or molecular indicators of crop quality, such as gene activity in the flavonoid pathway, early measures of berry composition or vine carbohydrate reserve status, can be developed as a decision-making tool for crop load and canopy adjustments.
- Determine whether such management options can be utilised to advance the rate of berry colour and flavour development relative to sugar accumulation for better control of harvest date and wine styles.

A 12 month extension to the project was approved in June 2016. This extension added a number of outputs to those planned (see table in Appendix 5.3), but did not affect the project objectives.

Chapter 2

Linking field manipulation of vine balance with fruit composition

2.1 Background

The concept of vine balance is an empirical one and largely defies a clear and specific definition. What the various definitions do have in common though, is an acknowledgement of the role of the canopy in providing carbon for ripening fruit and of the volume of fruit in generating a demand for that carbon. In essence, vine balance is a source-sink relationship.

For a given climate or environment, carbon fixation by the vine depends on photosynthetic capacity of the canopy and light absorption by that canopy. Canopy size is the major determinant for vines that are not subject to significant water or nutrient constraints. Consequently, indices of vine balance typically include canopy size, or a surrogate of it, as well as crop load. The most common example being the Ravaz Index (e.g. Bravdo et al., 1984), which is simply the ratio of yield to pruning weight. As both canopy size and yield (or potential yield) can be measured directly, such a definition of vine balance can be determined objectively. This assists in the reliable and repeatable manipulation of vine balance in the vineyard and an assessment of the effects of this. For the purposes of this study, where leaf area has been calculated from measurements of canopy light interception, ***we have defined vine balance as the ratio between crop load (kg) and canopy area (m^2)***; this also has the advantage of being a fairly widely used viticultural index, if not as common as the Ravaz Index.

Decades of research have demonstrated that the rate of sugar accumulation in the berry and the development of the secondary metabolites, such as anthocyanins, flavonols and condensed tannins (proanthocyanidins) are highly correlated in both field grown vines (e.g. Pirie and Mullins 1977) and artificial environments (e.g. Larronde et al., 1998). Furthermore, Dai et al., (2014) demonstrated that sugar supply to the berry can initiate anthocyanin production, even in the absence of additional hormonal signals. Manipulating vine balance, through canopy or yield adjustments, thereby altering carbohydrate supply to the fruit, clearly has the potential to alter berry composition, but the question is raised as to what extent, if any, the accumulation of sugar and those other metabolites can be separated. If not, then it could be argued that the main effect of altering vine balance is on maturation duration and harvest date; if so, then manipulation of vine balance may allow more targeted efforts to drive fruit composition and different wine styles.

Irrespective of this, the photosynthetic supply of carbon is certainly not the sole driver of berry composition and the potential influence of fruit exposure and canopy microclimate is widely recognised. The concentration and type of phenolic compounds in berry skins, for example, can be influenced by both temperature and light, with temperatures above 35°C being detrimental to anthocyanin production, and light affecting both the type of anthocyanins and accumulation of flavonols (Spayd et al., 2002; Downey et al., 2004). Superimposed on the source-sink and carbohydrate supply component of vine balance is, therefore, an effect of the fruit microclimate, which may vary between vineyards according to specific management practices or differences in climate.

The study described in this chapter utilised replicated field manipulations of vine balance across three different viticultural regions and ran for three seasons. This approach allowed fruit from vines with a range of vine balance values (kg fruit m^{-2} canopy) to be compared with fruit from vines having similar vine balance values, but a different environment, management history or crop/canopy manipulation. We were particularly interested in understanding the mechanisms underlying vine balance effects on fruit composition and, from a practical vineyard perspective, what canopy or crop management techniques can be imposed for reliable and predictable wine quality responses.

2.2 Materials and methods

2.2.1 Field sites

Three trial sites were established in commercial vineyards prior the start of the 2013/14 growing season (Figure 2.1): Cleggetts, Langhorne Creek, SA (35°17'21"S, 139°1'24"E); Deakin Estate, Murray Valley, VIC (34°25'39"S, 142°16'25"E) and Barwang, Hilltops, NSW (35°25'41"S, 148°27'35"E). Each site was part of an existing block, planted with Shiraz vines during the mid-1990s. At the SA site, vines were on their own roots, planted at 2 m spacing, with 3.2 m row spacing, trained to a bilateral cordon and spur-pruned. At the VIC site, vines were on Schwarzman rootstock, planted at 2.44 m spacing, with 3 m row spacing, trained to a double, bilateral cordon and mechanically-hedge pruned with hand clean-up. At the NSW site, vines were on own roots, planted at 2.1 m spacing, with 3.3 m row spacing, trained to a bilateral cordon and spur pruned. All sites were drip irrigated, with irrigation managed across the parent block by the vineyard manager at each site.

Each site utilised a different randomised complete block layout with four replicates, each of which encompassed three rows, providing a 'measurement' row with a buffer row on each side (Figure 2.1). At the Langhorne Creek and Hilltops sites, each replicate included fifteen vines per row, whereas at the Murray Valley site each replicate included twenty vines per row.



Figure 2.1 Aerial images of the three vineyard sites used for the field experiments: Langhorne Creek (left), Murray Valley (centre) and Hilltops (right).

2.2.2 Environmental monitoring

A matched automatic weather station (MEA, Magill, SA, Australia) was installed at each site in open land adjacent to the vineyard block in each case. Each weather station consisted of sensors to monitor global solar radiation, air temperature, relative humidity, wind speed, wind direction and rainfall, recording data at ten minute intervals. In addition, reference evapotranspiration (ET_o) was calculated from the above sensor output with a daily time-step.

Four soil moisture monitoring stations were established within each experimental block under control vines. Each station consisted of sensors at two depths, 0.3 and 0.6 m, recorded at one hour intervals. At Langhorne Creek and Murray Valley the sensors were Delta-T Theta probes (MEA, Magill, SA, Australia), recording volumetric soil water content (VSW), whereas at Hilltops the sensors were gypsum blocks (MEA, Magill, SA, Australia) recording soil tension. The sensors were positioned mid-way between two vines, approximately in the centre of the wetting zone.

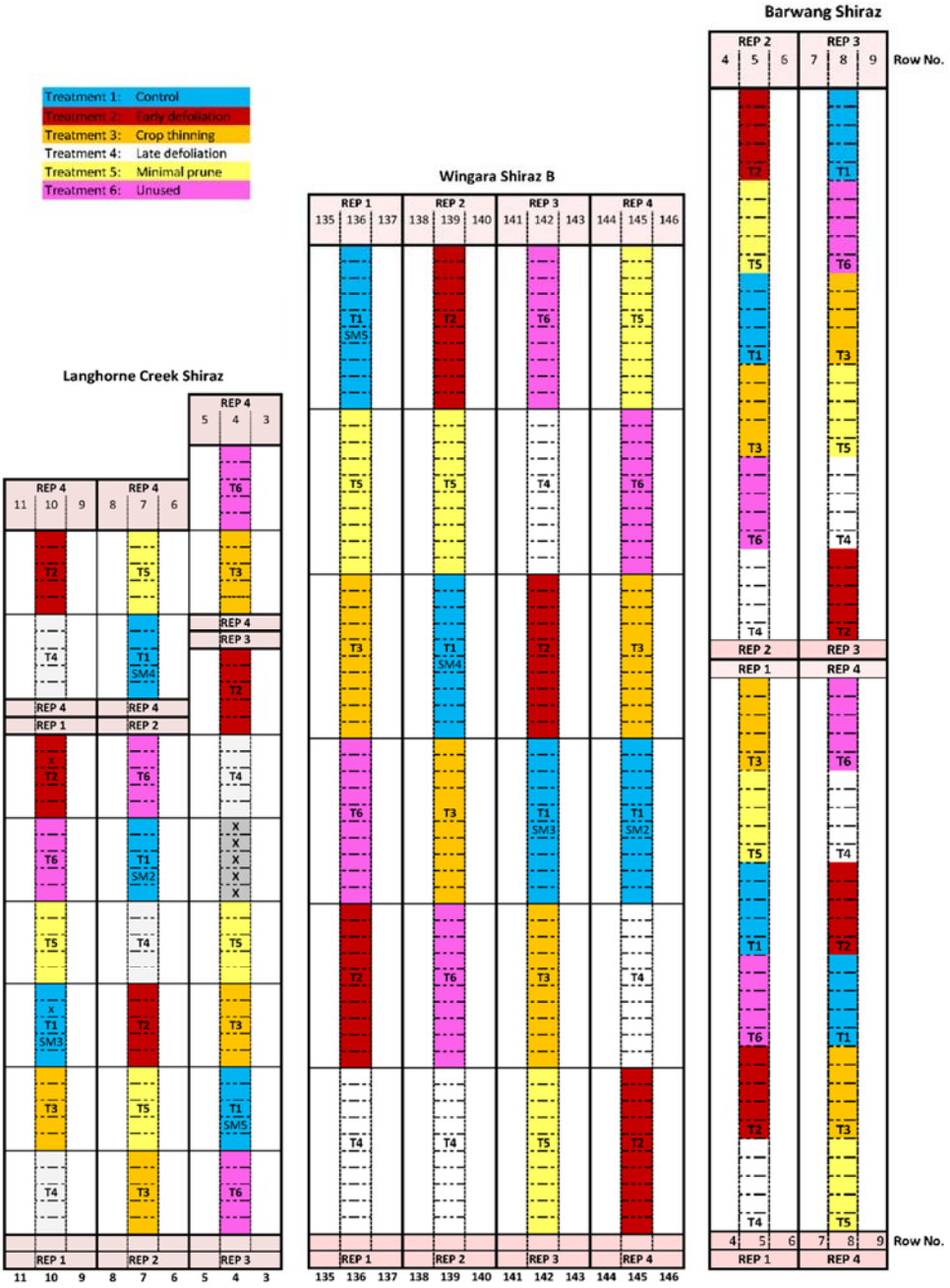


Figure 2.2 Replicated complete block layouts for the three field sites; Langhorne Creek (left), Murray Valley (centre) and Hilltops (right). Measurement panels are coloured blue (control), red (T2: early defoliation), orange (T3: crop thinning); white (T4: late defoliation) and yellow (T5: minimal prune) plots. Unused plots within the designs are marked in pink or grey. The dotted lines within each coloured measurement plot denote a single panel of vines. The letters SM denote a soil moisture measuring station.

2.2.3 Field treatments

Five management regimes were used to manipulate vine balance (ratio of yield to canopy size) and applied as similarly as possible at all three sites.

T1: control – all vines in T1 replicates received the standard management practice for that site.

T2: early defoliation – all fully expanded leaves (approximately the first eight leaves) were removed from each vine in the T2 measurement rows, pre-anthesis, E-L stage 19 (Coombe 1995). The treatment was also applied to all the buffer vines at the Hilltops site and either one or both buffer rows at the Langhorne Creek site, depending on the season. In the 2013/14 season, the T2 treatment was not applied effectively at the Murray Valley site and was only applied to fruiting canes at the Langhorne Creek site. The early defoliation treatment was intended to reduce fruit-set, and thereby crop load, with minimal effect on canopy size.


Langhorne Creek – 2013/14


Murray Valley – 2014/15


Hilltops – 2014/15

T3: crop thinning – all bunches were counted on at least eight vines per site (a minimum of two vines per T3 replicate), averaged and half this number of bunches removed from each vine in the T3 measurement rows pre-véraison, E-L stage 32.


Langhorne Creek – 2013/14


Murray Valley – 2013/14


Hilltops – 2013/14

T4: late defoliation – all vines in the T4 measurement and buffer rows were mechanically hedged pre-véraison, E-L stage 32. The hedging cut the foliage at the edge of the fruiting zone, with approximately half the canopy removed where possible.


Langhorne Creek – 2013/14


Murray Valley – 2013/14


Hilltops – 2014/15

T5: minimal pruning – all vines, measurement and buffer rows, were left un-pruned at the end of the first growing season, with low hanging canes cut about 30-50 cm above the ground prior to budburst.



Langhorne Creek – 2015/16



Murray Valley – 2015/16



Hilltops – 2014/15

The treatments were first applied during the 2013/14 growing season and re-applied to the same vines (Figure 2.2) in the 2014/15 and 2015/16 growing seasons.

2.2.4 Field sampling and fruit composition

Sampling of inflorescences and fruit was undertaken for analysis of gene expression (see Chapter 5) and assessment of fruit composition in each season. Three inflorescences per T1 and T2 replicate were sampled immediately prior to the T2 treatment being applied and four inflorescences per replicate for treatments T1, T2, T3 and T4 were sampled two weeks later (one week alter for Hilltops in 2014/15 and 11 days later for Hilltops in 2015/16). In addition, inflorescences were sampled 24 hrs after the T2 treatment at the Hilltops site in all three seasons. At the other two sites a second set of inflorescence samples, taken 24 hrs after treatment application, only occurred in the 2015/16 season. In all cases, inflorescences from a replicate were combined in a 50 mL plastic centrifuge tube and immediately frozen in liquid nitrogen. The samples were kept in liquid nitrogen until returned to the laboratory and kept at -80°C until use.

During the 2013/14 and 2014/15 growing seasons, developing fruit was sampled from treatments T1-T4 at two weekly intervals, beginning four weeks after the application of the T2 treatments and continuing until harvest. T5 fruit was not sampled during this period. For the 2015/16 growing season fruit was sampled in the same way for the T1 and T2 treatments, but for T3, T4 and T5, sampling began immediately prior to the application of the T3 and T4 treatments. In all cases, four whole bunches were taken from each replicate, placed on ice and all the berries removed from the rachis. The berries were then mixed, split into 40 or 50-berry sub-samples and weighed. Prior to véraison, two 50-berry sub-samples were taken and immediately placed in liquid nitrogen for return to the laboratory, where they were kept at -80°C until use. Post-véraison, four 50-berry sub-samples were taken, one was treated as above, a second was split into skin and seeds, the juice being discarded, before the skin and seeds were separately frozen in liquid nitrogen, and two were kept on ice for return to the laboratory, where they were either processed immediately or frozen and kept at -40°C until processing. Berry fresh weight was calculated for each sub-sample (50-berry weight divided by 50) and defined as the mean of all the sub-samples taken from a given rep on a given date.

One of the two samples returned on ice was squeezed in a zip-lock bag and the resulting juice used to assess total soluble solids (TSS), titratable acidity (TA) and pH. TSS was measured as

°Brix with a digital refractometer, TA and pH were measured using an auto-titrator (841 Titrando, Metrohm, Herisau, Switzerland).

The second of the 50-berry sub-samples returned on ice was used for estimation of berry anthocyanins, phenolics and tannins. The sample was ground over liquid nitrogen using an A11 analytical mill (IKA, Staufen, Germany) in order to keep it frozen. Whole berry total anthocyanin and total phenolic concentrations were determined using the spectrophotometric method of Iland et al., (2000). In brief, a 1 g sub-sample of the frozen powder was weighed and added to 10 mL of 50% acidified ethanol (pH 2), shaken for 1 hr and then centrifuged at 3,500 rpm for ten minutes. A 0.5 mL aliquot of the supernatant was added to 9.5 mL of 1M HCl. After a 3 hr incubation, the absorbance was read at 280, 520 and 700 nm with an Evolution 60S spectrophotometer (ThermoFisher, Waltham, Massachusetts, United States). Total anthocyanin and phenolic concentrations were calculated using the equations given in Iland et al., (2000).

Further aliquots of the acidified ethanol supernatant were used for estimation of total tannin concentration, based on a methyl cellulose precipitation method (Sarneckis et al, 2006). Two 150 µL aliquots were used, one was incubated with 450 µL of 0.04% methyl cellulose solution for three minutes. 300 µL of saturated ammonium sulfate solution was then added to both aliquots, following which they were both made up to 1.5 mL with deionised water. Each sample was mixed and incubated at 30°C for 10 minutes. The samples were then centrifuged and the absorbance read at 280 nm using a spectrophotometer. Tannin concentration was then calculated using the difference between the two samples and comparison with an epi-catechin standard curve.

2.2.5 Vine balance and components

Vine balance was assessed as the ratio between peak canopy size and harvest yield; expressed as kg fruit m^{-2} canopy.

Canopy size was estimated from leaf area index (LAI), row spacing and vine spacing. LAI was measured using a LiCor Li2200 or Li2000 Plant Canopy Analyser (LiCor Nebraska, US) on at least two occasions from late December to early February at each site in each season. Each measurement consisted of two 'above' canopy readings and at least three 'below' canopy readings, taken at three different points within the measurement row, resulting in a single LAI measurement for each replicate. Depending on canopy growth and timing of measurement, either an average of the two measurement dates or data from the date with the highest canopy values, was used. Irrespective of instrument, raw data were adjusted for sensor position and canopy height using the manufacturer supplied FV2200 software.

Fruit maturity for winemaking (see chapter 3) was defined as a juice TSS of 24°Brix. Each treatment was assessed individually during the fortnightly sampling and harvested individually if necessary. Once fruit reached approximately 21°Brix, additional sampling for TSS was undertaken at shorter intervals as appropriate. Typically, two panels were harvested at Langhorne Creek (six vines) and Murray Valley (four vines), whereas all treatment vines were harvested at Hilltops. Additional vines were harvested, depending on season and treatment, to ensure a minimum of 50 kg of fruit was available for winemaking. Harvest weight and bunch number were recorded per panel on a minimum of two panels. Average bunch weight was defined as yield / bunch number.

2.2.6 Photosynthesis

Assimilation under saturating light (A_{sat}) was assessed at each site during each season. In 2013/14 a single measurement session was conducted at each site in the late maturation period. For 2014/15 and 2015/16, two measurements were undertaken, one around véraison and a second during late maturation. In all cases, measurements were conducted between approximately 10 a.m. and 4 p.m. local time on bright, sunny days and made using a 64000 Portable Photosynthesis Analyser (LiCor, Nebraska, USA) at a PPFD of 2,000 µmol m^{-2} s^{-1}, with a reference gas CO_2 concentration of 410 ppm and with a 'block' temperature set to the expected average air temperature during the measurement session, thereby limiting temperature extremes between measurements in the morning and afternoon. A_{sat} was assessed as the mean of approximately nine individual readings taken five seconds apart once the gas exchange readings were stable. In addition to the standard stability definition in the instrument software, no readings were taken unless any changes in stomatal conductance (g_s) were less than 10 µmol m^{-2} s^{-1} min^{-1}. Measurements were made on two sunlit, outer-canopy leaves and two shaded, inner-canopy leaves per replicate on each occasion. A_{sat}, g_s, transpiration (E), inter-cellular CO_2 concentration (C_i), and leaf temperature were all recorded and averaged for the two sunlit or two shaded leaves measured. The A_{sat} value was also used to estimate the maximum rate of carboxylation ($V_{c\ max}$), assuming a respiration in the light value of 10% of A_{sat}.

2.2.7 Trunk non-structural carbohydrates

Prior to budburst in 2014, 2015 and 2016, one vine trunk per replicate was sampled for assessment of non-structural carbohydrate (NSC) concentration. A cordless drill with a 4 mm drill-bit was used at the slowest speed setting to drill to the centre of the trunk in increments to minimise any temperature rise or loss of drill tailings. The tailings were collected, being manually removed from the flutes of the drill bit if required, immediately frozen in liquid nitrogen and then stored at -80°C until analysis. With each sample the drill bit was checked by hand to ensure it was not exceeding approximately 25-30°C.

The trunk wood samples were freeze-dried, ground to a fine powder and analysed enzymatically for glucose, fructose, sucrose and starch content according to Smith and Holzapfel (2009) using commercially available kits.

2.2.8 Statistical analysis

In general, a split-split-plot ANOVA approach was taken with seasons being treated as a within-subjects factor, due to measurements being repeated on the same site/treatment replicates in each season (i.e. repeated measures) and site treated as a within-subjects factor, due to the treatments and measurements being made on the same replicate vines. Treatment was the only variable treated as a between-subjects factor. Wherever possible, the entire dataset (three seasons x three sites x four-five treatments) were analysed together for a given variable. The GLM fitted repeated measures ANOVA function was used in SPSS (IBM, St. Leonards, NSW, Australia) for each analysis. The photosynthesis data included a sun/shade leaf factor, measured on the same vine, thus was treated as an additional within-subjects factor. Any means comparisons made were tested with either the Bonferonni adjustment or using Tukeys HSD test and a probability of 0.05 or less was taken as statistically significant unless otherwise stated.

2.3 Results and discussion

The concept behind the field component of the project was to identify three contrasting vineyards in three different climates, but with the same cultivar (Shiraz) and vines of a similar age and trellis type, then manipulate vine balance in different ways, but in a consistent manner at each site. It was anticipated that this approach would allow any direct effects of vine balance to be separated from potentially confounding site or seasonal effects or interactions. This experimental strategy would provide fruit and associated differences in vine balance, both within and between sites, which could then be examined to determine the extent to which vine balance was directly driving fruit composition.

2.3.1 Climate/Environment

The three sites were chosen based on long-term climate data, but the specific climate conditions during the field seasons were monitored at each site with matched weather stations that were all calibrated prior to use. Differences in climate during the three seasons followed the long-term trends, with mean January temperature (MJT), for example, being highest at the Murray Valley site and lowest at the SA site (Table 2.1). Differences between sites were generally consistent season to season, irrespective of the absolute MJT, but looking at MJT alone disguised site differences in daily (data not shown) and seasonal (Figure 2.3) maxima and minima. For example, Hilltops had the lowest temperatures in spring and autumn, but was hotter than Langhorne Creek during the summer. This resulted in Langhorne Creek and Hilltops having the same whole growing season average daily maximum (approx. 28°C), but a different average daily minimum (approx. 11 and 13°C respectively). Likewise, Murray Valley and Hilltops had the same growing season average daily minimum, but a different average daily maximum (approx. 32°C at the Murray Valley site).

Table 2.1: Mean January temperature (MJT; °C) of the three field sites, Langhorne Creek, Murray Valley and Hilltops, for each of the three field seasons.

Site	2014	2015	2016
Langhorne Creek	21.2	19.5	20.4
Murray Valley	25.5	23.4	24.7
Hilltops	23.5	21.0	22.2

Growing season temperature has long been linked to changes in maturation rate and fruit composition (e.g. Winkler 1974) and direct manipulation of growing temperature within a vineyard has demonstrated the role of growing season temperature in driving key phenological dates (Sommer et al., 2012, Sadras and Moran, 2012). Consequently, the consistent differences in temperature between the three sites would be expected to drive consistent differences in vine phenology and potentially fruit composition.

Other aspects of climate varied between the sites as well, with a small difference in average daily total global solar radiation (GSR; Figure 2.4) and reference evapotranspiration (ET_o: Figure 2.5). The sum of rainfall and applied irrigation varied between sites considerably more than ET_o, with the Murray Valley site applying a much higher rate of irrigation, despite having a slightly lower ET_o than the Hilltops site (Figure 2.5). However, this does not take into account differences in crop evapotranspiration ET_c, largely driven by canopy size (see below).

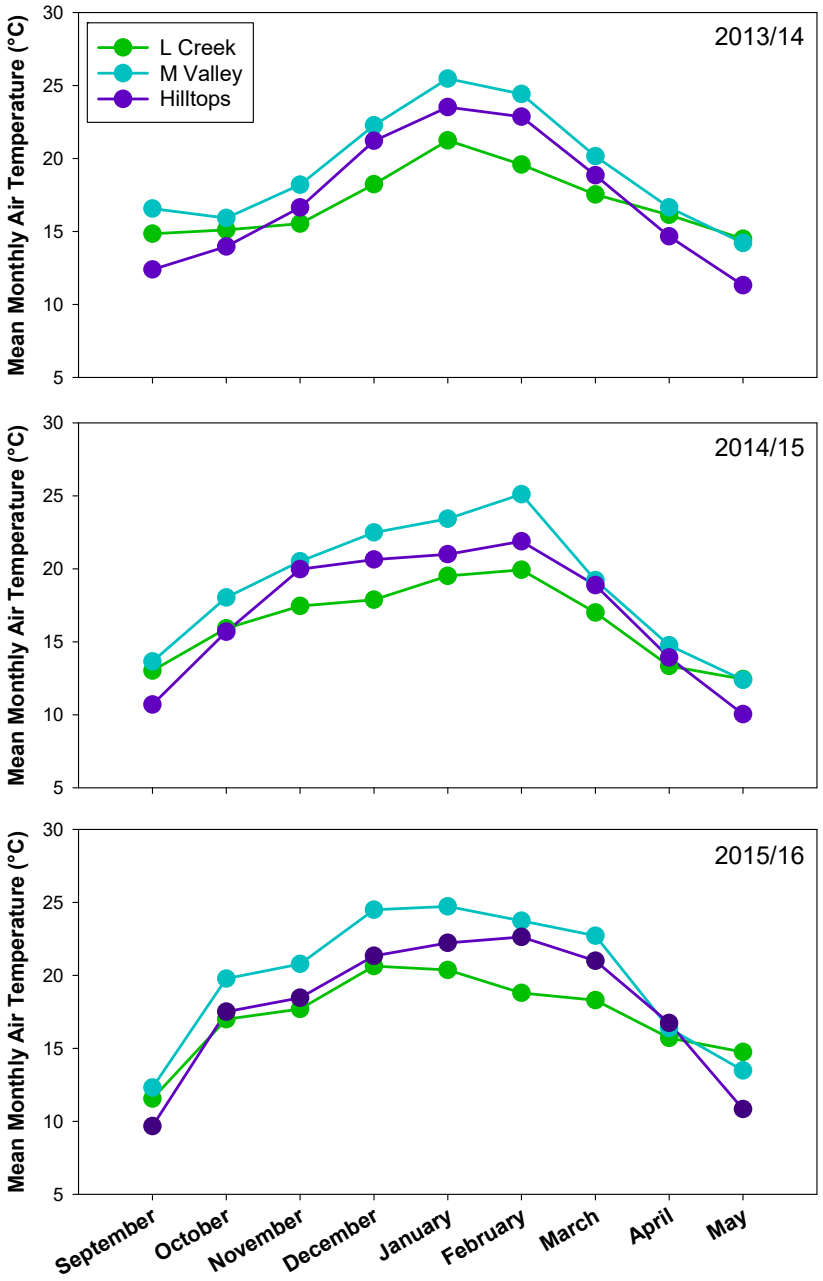


Figure 2.3 Mean monthly air temperatures during the growing season for the three field sites, Langhorne Creek, Murray Valley and Hilltops, for each of the three field seasons 2013/14 (top), 2014/15 (centre) and 2015/16 (bottom).

Volumetric soil water content (VSW) during much of the growing season was similar at the Langhorne Creek and Murray Valley sites (Figure 2.6). Soil tension, rather than soil moisture, was measured at the Hilltops site, but was at a level (<50 kPa) commensurate with the VSW measured at the other sites for most of the season. The maturation period was drier at Langhorne Creek and Hilltops than the rest of the season (and compared to Murray Valley), being driest at Hilltops. Potential treatment effects on soil moisture as a result of differences in canopy size and/or structure were not monitored, but were expected to be small, if present at all.

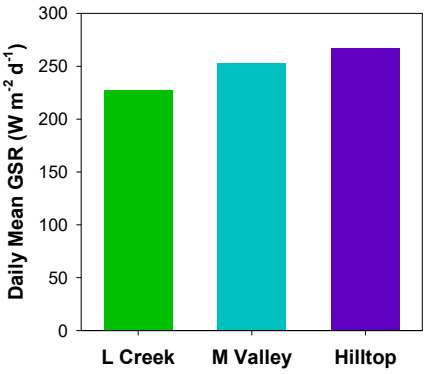


Figure 2.4 Mean daily global solar radiation during the growing season (September-May inclusive) for the three field sites, Langhorne Creek, Murray Valley and Hilltops, averaged across the three field seasons.

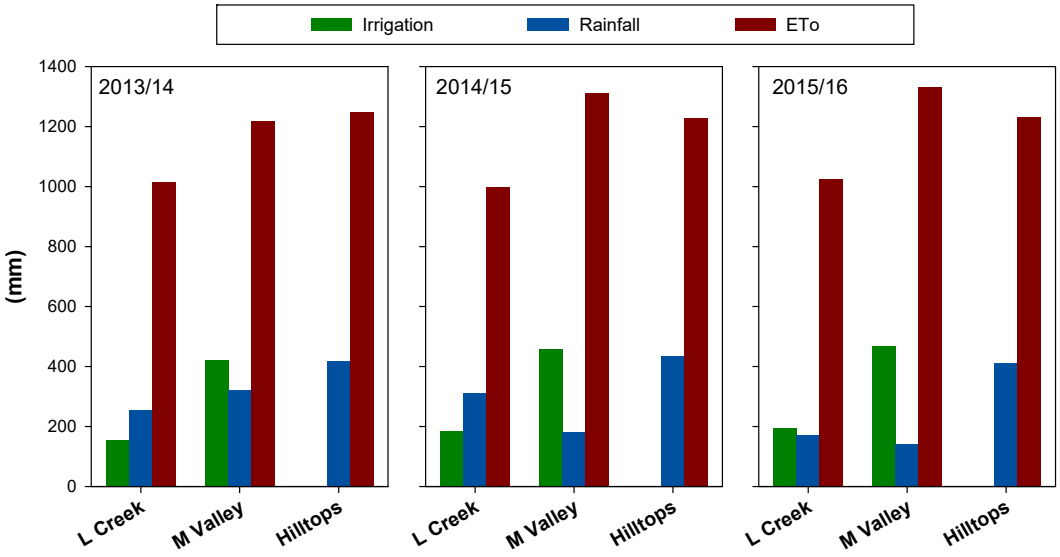


Figure 2.5 Growing season (September to May) irrigation application, rainfall and cumulative ET_o for the three field sites, Langhorne Creek, Murray Valley and Hilltops, for each of the three field seasons 2013/14 (left), 2014/15 (centre) and 2015/16 (right). Irrigation records were not available at Hilltops.

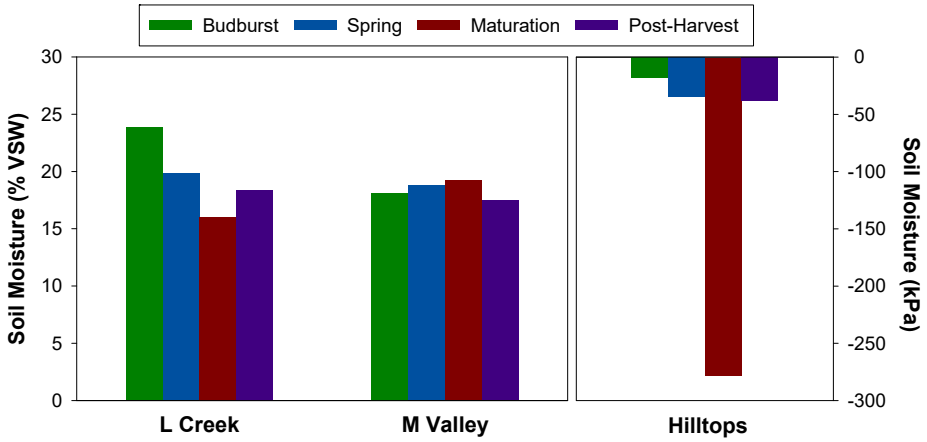


Figure 2.6 Volumetric soil moisture content (Langhorne Creek and Murray Valley) and soil water potential (Hilltops) for key phenological periods; budburst (September), spring (October-November), maturation (December-February) and post-harvest (March-May). Data are means of two depths (30 and 60 cm) at a single position in each of the four control plots at each site, averaged across three seasons.

In summary, as is typical for field experiments, the three seasons of experimental work all experienced differences in climatic conditions. Consequently, any similarities in observed treatment effects, or in relationships between vine balance and fruit composition, between seasons would provide increased confidence in the application of those treatments or the fundamental nature of those relationships. Further, the consistent difference in climate between sites, despite the differences between seasons, would lead to an expectation that climatic effects on fruit composition between sites should be observed in all seasons.

2.3.2 Treatment effects on vine balance and its components

The definition of vine balance used has two components, canopy size and harvest yield. The three sites were chosen to represent, not only a range of climates, but also a range of viticultural management strategies, thus it was anticipated that canopy size, yield and vine balance would vary between sites as well as treatments and may also be affected by season. Indeed, this was the case, with canopy size (expressed as LAI), pruning weight and yield all varying significantly between sites and seasons (Table 2.2). However, to some extent these values co-varied, so vine balance in T1 at Langhorne Creek was not affected by season and only 2014/15 differed from the other seasons at Murray Valley. The three seasons did produce three different T1 vine balance values at Hilltops, but 2014/15 was also the season with the largest variation, compared with the other two.

When the Ravaz index (yield/pruning weight) was calculated for T1 there was a greater impact of season than for vine balance (Table 2.2), indicating a seasonal effect on the relationship between canopy size and pruning weight.

2.3.2.1 T2: early defoliation – The intention of the T2 treatment was to reduce yield with little or no effect on canopy size, thereby decreasing vine balance. The timing and extent of leaf removal was based on the work of Poni et al., (2006) and subsequent studies (e.g. Intrieri et al., 2008 and Tardaguila et al., 2010), who found that early defoliation resulted in a 30-50% decrease in yield with a more limited effect on canopy size, usually reducing the yield:canopy ratio. However, the work in Europe was carried out on vertical shoot positioned vines (VSP), whereas the work described here was carried out on spur pruned vines that were allowed to 'sprawl'. To the best of our knowledge this is the first time early defoliation has been applied to vines managed in this way. The T2 treatment significantly reduced canopy size across all sites and seasons (Table 2.2), with an average reduction of 17% across the whole data set. However, this was matched by the impact on yield, averaging only 16% reduction across the whole data set, resulting in no overall impact on vine balance (Figure 2.7). Interestingly, pruning weight was unaffected, so the Ravaz index was lower under T2 than T1, albeit not in all sites/seasons (Table 2.2).

2.3.2.2 T3: crop thinning – Of the four manipulations employed in this project, crop thinning is the management strategy most commonly employed in the Australian industry, primarily in the cooler climates, but it can also be used in warmer high production regions (Petrie and Clingeleffer, 2006). As would be expected due to the extent of the bunch removal (50%), T3 treatment invariably resulted in reduced yield (Table 2.2, Figure 2.7), although the reduction averaged only 34% across all sites and seasons. The difference was probably in part due to an inability to remove fully half of all bunches in some sites/seasons and in part due to some compensation in berry size (but see below). There was no effect on canopy size and no clear

Table 2.2: Vine balance (yield/canopy size), Ravaz Index (yield/pruning weight) and each of their components for each treatment at each site and each season. Data are means of four field replicates ± standard error. T5 was not implemented in the 2013/14 season, n/c is data not collected, n/a is data unable to be calculated.

Season	Site	Treatment	LAI_{app} (m^2 m^{-2})	Pruning Wt (kg $vine^{-1}$)	Yield (kg $vine^{-1}$)	Ravaz Index (kg kg^{-1})	Vine Balance (kg m^{-2})
2013/14	Langhorne Creek	T1	1.86±0.22	1.59±0.06	5.30±0.86	3.36±0.49	0.45±0.45
		T2	1.49±0.12	1.47±0.08	4.23±0.59	2.88±0.32	0.46±0.46
		T3	1.59±0.13	1.53±0.08	2.30±0.57	1.47±0.24	0.23±0.23
		T4	0.85±0.10	1.40±0.08	5.43±0.69	3.89±0.42	1.01±1.01
		T5	*1.75±0.27*	*n/c*	*n/c*	*n/a*	*n/a*
	Murray Valley	T1	1.73±0.17	1.26±0.07	11.40±1.77	9.19±1.57	0.93±0.93
		T2	1.87±0.09	*1.41±0.09*	*11.63±1.14*	*8.22±0.33*	0.84±0.84
		T3	2.01±0.09	1.36±0.05	9.60±0.84	5.35±1.92	0.68±0.68
		T4	1.31±0.07	1.29±0.05	7.88±1.15	6.27±1.25	0.82±0.82
		T5	*2.16±0.09*	*n/c*	*n/c*	*n/a*	*n/a*
	Hilltops	T1	1.25±0.07	0.71±0.05	1.88±0.15	2.70±0.29	0.22±0.22
		T2	1.02±0.06	0.66±0.05	1.75±0.35	2.71±0.52	0.25±0.25
		T3	1.07±0.07	0.68±0.02	1.17±0.15	1.74±0.26	0.16±0.16
		T4	0.88±0.07	0.43±0.00	2.22±0.20	5.10±0.47	0.36±0.36
		T5	*1.15±0.09*	*n/c*	*n/c*	*n/a*	*n/a*
2014/15	Langhorne Creek	T1	1.47±0.05	1.41±0.07	4.38±0.37	3.16±0.34	0.47±0.47
		T2	1.06±0.11	1.31±0.12	3.18±0.46	2.47±0.36	0.49±0.49
		T3	1.65±0.05	1.76±0.28	2.02±0.44	1.33±0.42	0.19±0.19
		T4	0.99±0.12	0.90±0.09	4.18±0.49	4.69±0.51	0.68±0.68
		T5	1.96±0.36	1.03±0.18	n/c	n/a	n/a
	Murray Valley	T1	2.14±0.16	0.96±0.14	27.78±1.13	30.20±3.33	1.79±1.79
		T2	1.82±0.14	1.22±0.10	23.34±1.81	19.39±1.67	1.78±1.78
		T3	2.48±0.13	0.91±0.03	17.18±1.25	19.12±1.83	0.95±0.95
		T4	1.74±0.18	0.78±0.05	30.78±0.55	40.36±3.19	2.48±2.48
		T5	2.10±0.15	1.35±0.13	n/c	n/a	n/a
	Hilltops	T1	1.37±0.05	0.79±0.04	5.28±0.59	6.77±1.02	0.56±0.56
		T2	1.10±0.12	0.70±0.03	4.95±0.42	7.03±0.34	0.66±0.66
		T3	1.31±0.03	0.83±0.07	3.30±0.15	4.09±0.44	0.37±0.37
		T4	0.95±0.03	0.53±0.01	5.02±0.24	9.44±0.34	0.76±0.76
		T5	n/c	n/c	n/c	n/a	n/a
2015/16	Langhorne Creek	T1	2.25±0.15	1.24±0.08	6.92±1.27	5.65±1.10	0.49±0.49
		T2	1.69±0.19	1.18±0.06	6.90±1.03	6.00±1.12	0.67±0.67
		T3	2.31±0.06	1.21±0.08	5.04±0.52	4.15±0.26	0.34±0.34
		T4	1.37±0.04	0.77±0.04	7.49±0.98	9.68±1.01	0.85±0.85
		T5	3.10±0.49	0.29±0.01	10.48±2.00	35.61±5.36	0.53±0.53
	Murray Valley	T1	2.38±0.18	1.25±0.04	18.12±3.19	14.61±2.69	1.02±1.02
		T2	2.01±0.15	1.29±0.04	13.74±2.17	10.52±1.37	0.92±0.92
		T3	2.79±0.15	1.30±0.04	11.40±0.95	8.74±0.53	0.56±0.56
		T4	1.71±0.09	1.21±0.03	10.16±2.08	8.34±1.57	0.84±0.84
		T5	2.92±0.17	1.17±0.05	13.15±2.67	7.36±1.57	0.61±0.61
	Hilltops	T1	2.39±0.31	1.36±0.18	5.35±0.54	4.07±0.49	0.33±0.33
		T2	1.78±0.31	1.40±0.18	2.93±0.16	2.28±0.48	0.26±0.26
		T3	1.97±0.23	1.32±0.12	3.89±0.25	3.03±0.39	0.29±0.29
		T4	0.92±0.10	1.46±0.21	4.72±0.34	3.44±0.51	0.77±0.77
		T5	2.72±0.27	0.83±0.03	8.00±0.36	9.70±0.45	0.44±0.44

indication of a year-on-year impact on canopy, as might occur over time due to increased reserves for growth following bunch removal (Holzapfel & Smith 2012). As a result, the calculated vine balance was always reduced, as was the Ravaz index (Table 2.2).

2.3.2.3 T4: late defoliation – Summer pruning, equivalent to the T4 treatment used here, has been trialled in Europe as a mechanism to slow maturation (Stoll et al., 2010). Limited trials have previously been attempted in Australia, again on VSP managed vines (Whiting 2012; Savarino et al., 2013). As with the T2 treatment, to the best of our knowledge, the experiments presented here are the first attempt to implement this management strategy on typical Australian 'sprawl' vines, noting that small adjustments to canopy size during the season is common practices in many vineyards, both cool and warm climate. The treatment reduced canopy size during the maturation period by an average of 36% across sites and seasons (Table 2.2, Figure 2.7) and had no effect on harvest yield at the Langhorne Creek and Hilltops sites, with a marginal effect at the Murray Valley site due to accidental removal of some bunches during the treatment implementation. Consequently, calculated vine balance was increased at all sites and in all seasons (Table 2.2), with an average increase of 55%. The same was not true for the Ravaz index, which was not significantly different from T1, as cane material was removed along with the leaves.

2.3.2.4 T5: minimal pruning – The effects of minimal pruning, T5, were only examined in the final season, due to being implemented from the second season onwards. Changing management from spur pruning to minimal pruning has a major effect on growth and the use of reserves in the first season of implementation because of the large change in bud numbers per vine (data not shown) and this will have carry over effects for a number of years. Due to this, measurements were not made in that first season of adjustment (2014/15). Visual observation suggested that, even in 2015/16, the extent of this adjustment process varied between sites. The canopy size data supports this, with the Langhorne Creek and Hilltops sites maintaining a much larger canopy on the T5 vines in 2015/16, but a smaller canopy being present on the T5 vines at the Murray Valley site (Table 2.2), where the pruning system was already semi-minimal and there was a much higher bud number per vine (400-600, data not shown) than at the other sites. Similarly, yield was increased at the former two sites, relative to control, and reduced at Murray Valley. The calculated vine balance, therefore, varied between sites, being close to T1 at Langhorne Creek, reduced at Murray Valley, but increased at Hilltops.

With certain exceptions (T2 at Murray Valley in the 2013/14 season, limited adjustment to T5 at all sites), the treatments were effective in the aim of altering vine balance or its components in a range of ways, with T3 reducing and T4 increasing vine balance. Although T2 did not alter vine balance, it provided a contrast to T1, by having a similar vine balance, but reduced canopy and yield, There was also no indication that repeated application of the treatments on the same vines over three years had a cumulative effect on the vines. Furthermore, the overall treatment effects, averaged across three seasons and proportional to controls, were remarkably similar between the three sites, despite the variation in climate and total yield (Figure 2.7). Consequently, the treatments provided an effective tool to examine the role of vine balance in determining fruit and wine composition.

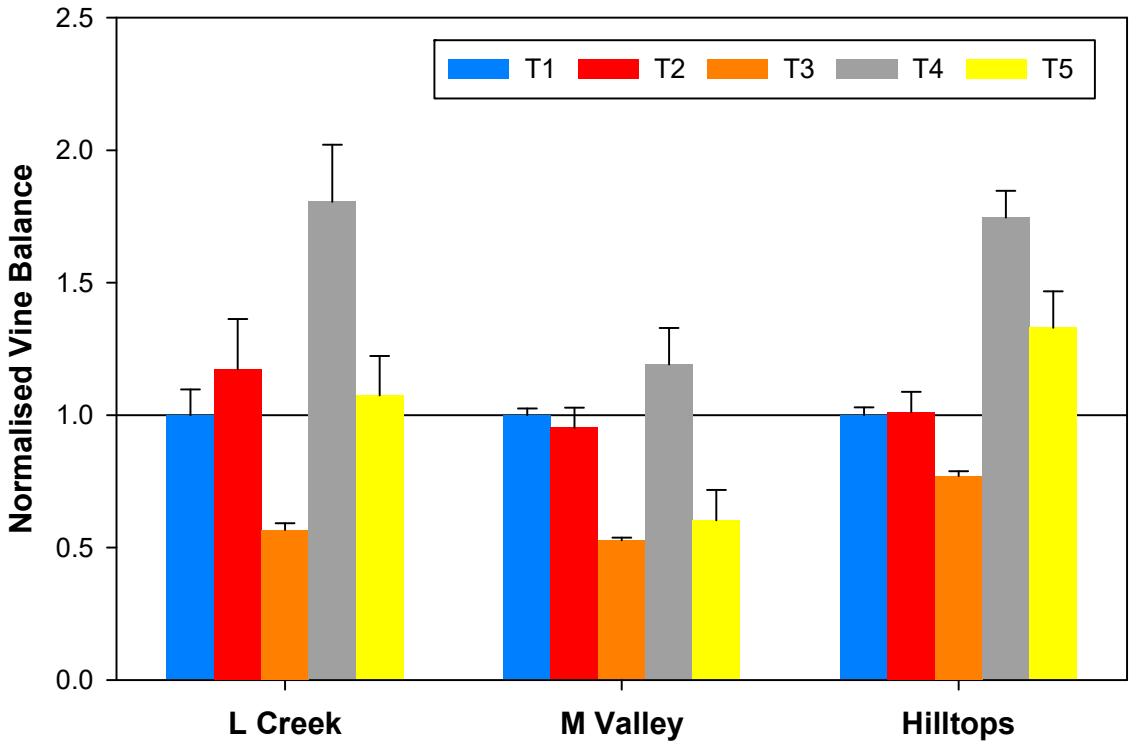


Figure 2.7 Vine balance (yield/canopy size), normalised to the control value at each site, for each treatment and averaged across the three growing seasons, except T5, which was for 2015/16 only. Data are means of four field replicates ± standard error.

2.3.3 The impact on maturation rate of changing vine balance

Vine balance is, by definition, a source-sink relationship, thus altering vine balance through the applied treatments (T3-T5, vs T1 & T2) is likely to affect the carbohydrate supply to the maturing fruit, relative to their requirements. Reducing vine balance (T3) would potentially increase the rate at which carbohydrate could be supplied to the fruit and increasing vine balance (T4) would potentially reduce the rate of carbohydrate supply to the fruit. As all treatments were harvested at the same TSS level (24°Brix), one consequence of this may be a change in the maturation rate and the time at which 24° was achieved (Nuzzo and Matthews 2006).

The precise timing of véraison was not assessed for practical reasons, but the date of harvest (approximate date of achieving 24°Brix), was recorded (Table 2.3). It was apparent that, relative to T1, there were consistent effects, across sites and seasons, of some of the treatments on the timing of harvest. T3 advanced harvest at all sites and T4 delayed harvest at all sites (Figure 2.8), although the effect of T3 at Hilltops was only in one of the three seasons where the more rapid sugar concentration changes in late ripening made it difficult to distinguish from T1 with pre-harvest sampling. There was also an advancement of harvest in the T2 treatment at two of the three sites, despite no effect on vine balance.

Table 2.3: Date of fruit harvest for winemaking, defined as a juice TSS concentration of 24°Brix (13.3°Baumé), for each treatment at each of the three field sites in each of the three growing seasons.

Season	Site	T1 Control	T2 Early defoliation	T3 Crop thinning	T4 Late defoliation	T5 Minimal prune
2013/14	Langhorne Creek	11th March	11th March	11th March	18th March	n/a
	Murray Valley	13th Feb.	13th Feb.	13th Feb.	24th Feb.	n/a
	Hilltops	18th Feb.	18th Feb.	18th Feb.	24th Feb.	n/a
2014/15	Langhorne Creek	2nd March	2nd March	23rd Feb.	2nd March	n/a
	Murray Valley	4th March	27th Feb.	17th Feb.	10th March	n/a
	Hilltops	11th Feb.	11th Feb.	11th Feb.	25th Feb.	n/a
2015/16	Langhorne Creek	1st March	1st March	16th Feb.	8th March	1st March
	Murray Valley	9th Feb.	5th Feb.	5th Feb	10th Feb.	5th Feb.
	Hilltops	15th Feb.	12th Feb.	12th Feb.	18th Feb.	22nd Feb.

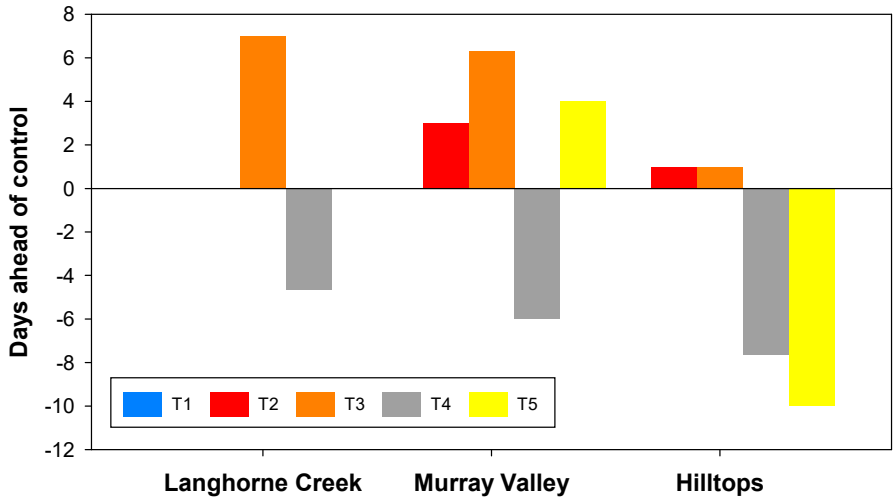


Figure 2.8 Number of days ahead of control (positive) or behind control (negative) of harvest date for each treatment at each site, averaged across the three growing seasons. Control = zero.

Harvest time relative to T1 is only a valid indicator of maturation duration if harvest was at the same sugar level in all treatments as intended. In fact, across sites and seasons, the harvest sugar concentrations were very consistent (Figure 2.9), albeit T4 was typically being harvested at a slightly lower TSS than the other treatments. This was due to the requirement to harvest the fruit prior to the vineyard owners harvesting the rest of blocks used for the experimental work and, at Hilltops, there was additional risk from bird damage if leaving fruit on the vines too long after commercial harvest. However, the implication of this is that the impact of T4 on slowing maturation would be slightly underestimated using harvest date, rather than overestimated.

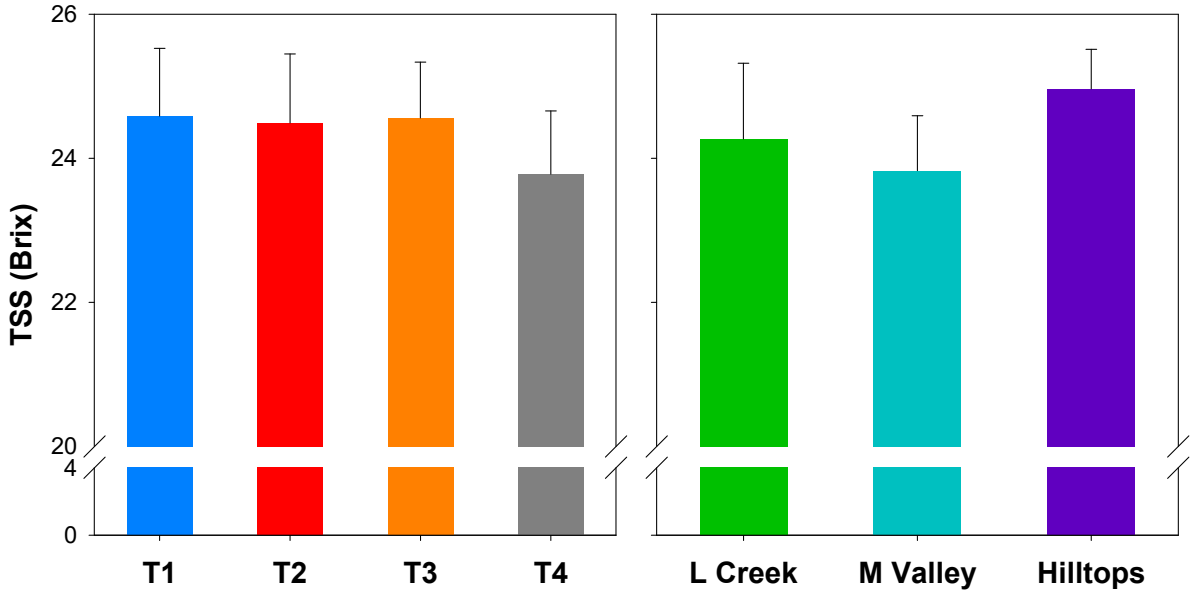


Figure 2.9 Juice TSS concentration at harvest. Marginal means for the four treatments, T1-T4 across all sites/seasons (left) and for the three sites, across all treatments/seasons (right). Data are means ± standard deviation.

2.3.4 Site and treatment impacts on fruit maturation and composition

Berry maturation was tracked from fruit-set to harvest. Figure 2.10 provides berry fresh weight and berry soluble solids for the T1 controls for each site and season. The berry weight data suggests that berry size was greater at Murray Valley and véraison earlier (the inflexion point of the double-sigmoid curve shape) than the other two sites. However, the typical rate of sugar accumulation was highest at Hilltops in all three seasons.

Of the fruit compositional parameters tracked during maturation, the only major impact of the treatments was that of T4 on anthocyanin content (but see comments on TA below). In all cases the T4 treatment delayed and/or reduced anthocyanin accumulation (Figure 2.11, left panels). The effect was typically present from the onset of anthocyanin production, visible at the first measurement time-point. It was possible that this was due to the delayed sugar ripening noted above, however, calculating the anthocyanin:sugar ratio demonstrated that anthocyanin production was reduced in the T4 treated vines, even in comparison with berry sugar accumulation (Figure 2.11, right panels).

Harvesting the treatments at the same sugar content was intended to minimise the impact of the observed changes in maturation rate resulting from the manipulation of vine balance (see above), but also match common commercial viticultural practice, where sugar ripeness is often the primary decision making tool for harvest. In the situation observed in the T4 vines, where anthocyanin content was reduced relative to sugar content, growers would have the option to extend ripening, allowing for a higher sugar and resulting alcohol level, but it was not practical to make additional later harvests within this project.

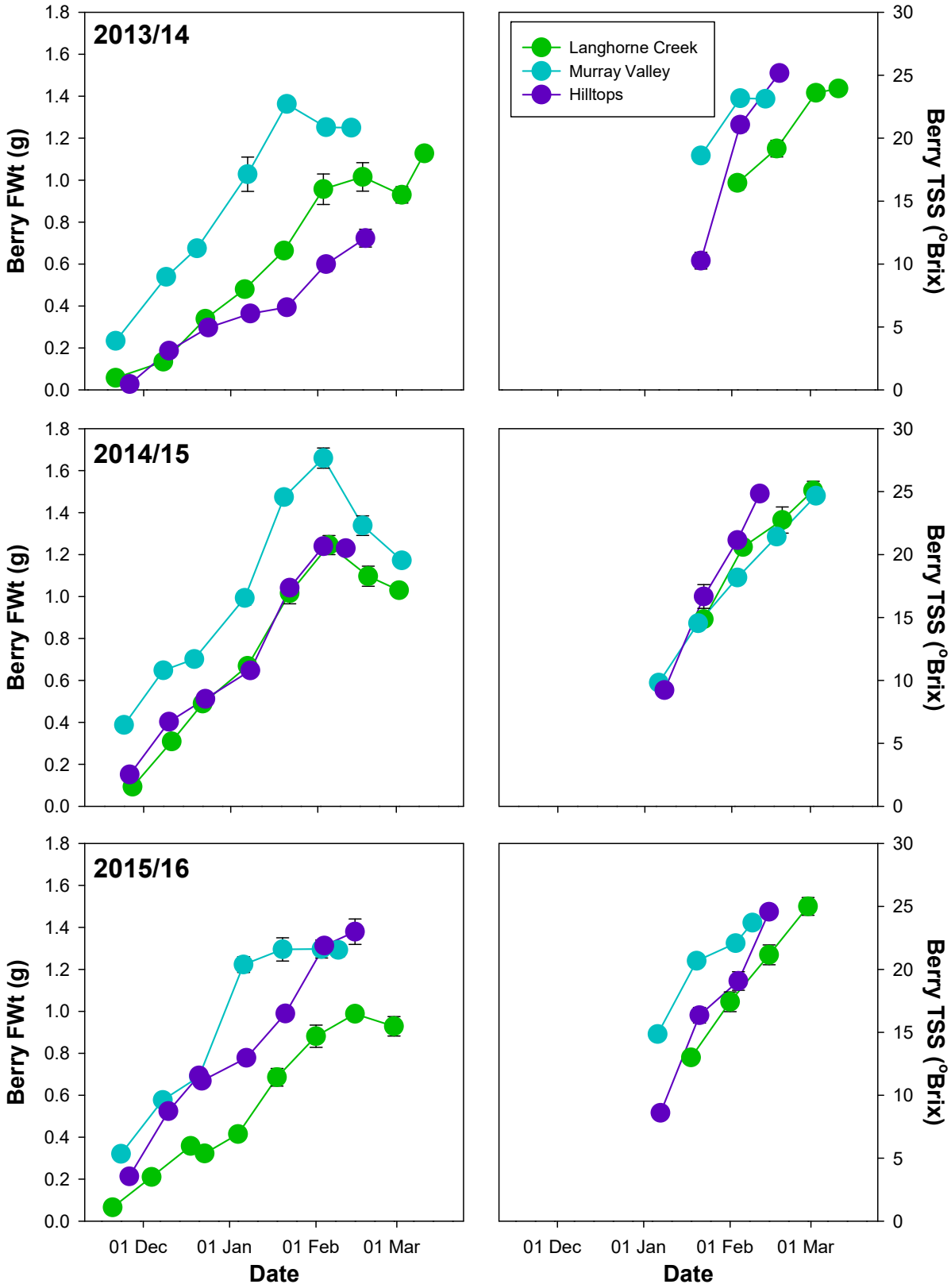


Figure 2.10 Berry fresh weight (FWt, left panels) and total soluble solids (TSS, right panels) for fruit from the control treatment (T1) at each sampling date during berry development (FWt) or maturation period (TSS), for each of the three field sites, during each of the three growing seasons. Data are means ± standard error.

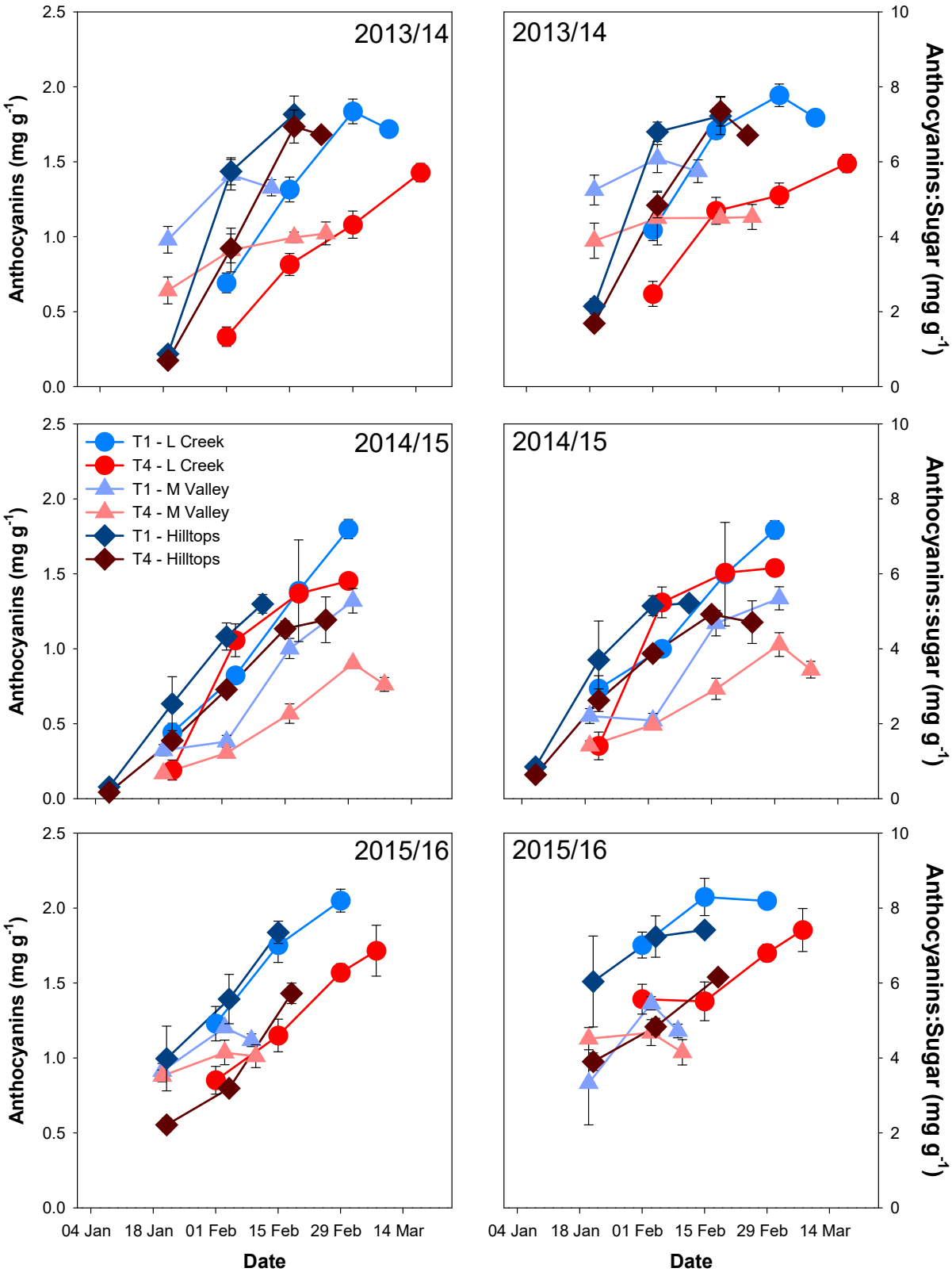


Figure 2.11 Whole berry anthocyanin concentrations (mg per g berry FWt, left panels) and berry anthocyanins:sugar ratio during the maturation period for the control (T1) and late defoliation (T4) treatments at each of the three field sites in each of the three growing seasons. Data are means ± standard error.

Bunch number and weight were estimated from yield, but berry weight was estimated from the samples taken at, or immediately prior to, harvest. As the latter included only four bunches per vine, it was not deemed appropriate to calculate berry number per bunch in the harvest fruit from this data. However, in general, although there were site and seasonal effects on berry weight at harvest, there were no significant effects of T2-T4 (Table 2.4), suggesting that any observed differences in bunch weight were likely to be driven by berry number. Only T5 had an effect on berry weight; reducing berry size at Langhorne Creek and Hilltops. The timing of T3 and T4 application, E-L 32, immediately prior to véraison, meant that cell division within the berry was likely to be complete at the time of application (Harris et al., 1968), so any impact would have been through cell expansion and berry water uptake rather growth *per se*. The effect of T5 on berry weight was likely the result of major changes in vine and bunch architecture (Clingeleffer 2010).

As with berry weight, there were site and seasonal effects on bunch weight and number (Table 2.4), the latter driven by pruning decisions, which affected shoot number (data not shown). Treatment effects on these two parameters were limited though, with a marginally significant reduction in bunch weight in the T2 treatment ($p = 0.051$) and a reduction in bunch number after thinning in T3. Consequently, the observed effect of T2 on vine yield was primarily driven by a reduced bunch weight, rather than a change in bunch number or berry weight. As noted above, this was likely due to reduced berry number per bunch, an effect previously observed with early defoliation treatments (Poni et al., 2006).

Interestingly, across the whole data set, TA at harvest was significantly higher in T3 fruit than the other treatments (Table 2.5). The maturation data (not shown) suggested that this was due to the rate of decrease in TA with maturation time being unaffected by the treatment, thus the shorter maturation period resulted in a higher TA at harvest. In terms of winemaking, this may represent a beneficial attribute of crop removal

The effect of T4 on anthocyanin accumulation during maturation was still visible at harvest, with significantly lower total anthocyanins and anthocyanin:sugar ratio in T4 fruit across the whole data set (Table 2.5). In contrast, there was no effect of T4 on total phenolics or total tannins, whereas both of these measures were higher in the T2 treatment (Table 2.5). There was no significant site by season interaction in these compositional parameters, suggesting that the treatment effects were essentially the same at all three sites, despite the differences in climate and vine yield. The absolute values were all impacted by site, however, and all except total tannin were also affected by season. This suggests that, although there are strong environmental drivers for these compositional parameters, canopy and crop manipulations can be applied irrespective of environment and still have a predictable effect.

The relationship between fruit anthocyanin and sugar concentration is a key quality parameter, particularly as there is an industry perception of reduced maturation duration affecting this relationship. Across the data set as a whole, there was no significant site by treatment or season by treatment interaction that affected anthocyanin:sugar ratio, again suggesting that the canopy and crop load manipulations used had a consistent effect, irrespective of climate.

Table 2.4: Yield and yield components (average bunch FWt, average bunch no., berry FWt) for each treatment at each site and each season. Data are means of four field replicates ± standard error. T5 was not implemented in the 2013/14 season, n/c is data not collected, n/a is data unable to be calculated.

Season	Site	Treatment	Yield (kg/vine)	Bunch Wt (g)	Bunch No.	Berry Wt (g)	Juice TSS (°Brix)
2013/14	Langhorne Creek	T1	5.30±0.76	77.7±5.7	67±6	1.13±0.02	24.0±0.3
		T2	4.23±0.49	66.3±4.7	64±5	1.14±0.02	24.3±0.5
		T3	2.30±0.47	61.3±3.8	37±9	0.93±0.03	24.0±1.2
		T4	5.43±0.59	78.9±5.2	68±3	1.08±0.06	24.0±0.3
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
	Murray Valley	T1	11.40±1.77	82.8±4.1	136±17	1.25±0.02	23.1±0.3
		T2	*11.63±1.14*	*77.5±3.5*	*152±20*	*1.31±0.06*	*23.2±0.5*
		T3	7.63±2.06	99.0±20.4	92±38	1.25±0.03	24.0±0.4
		T4	7.88±1.15	78.2±1.6	101±16	1.21±0.06	22.6±0.1
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
	Hilltops	T1	1.88±0.15	45.8±4.4	41±3	0.72±0.04	25.2±0.1
		T2	1.75±0.35	41.2±6.6	42±2	0.66±0.02	24.7±0.4
		T3	1.17±0.15	43.1±5.8	29±5	0.72±0.02	25.5±0.5
		T4	2.22±0.20	47.8±5.0	47±3	0.76±0.02	25.1±0.2
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
2014/15	Langhorne Creek	T1	4.38±0.37	73.5±3.8	60±5	1.03±0.03	25.1±0.7
		T2	3.18±0.46	53.7±4.8	59±5	1.05±0.01	24.6±0.6
		T3	2.02±0.44	65.3±8.6	32±6	1.11±0.08	24.6±0.5
		T4	4.18±0.49	64.4±8.7	66±3	1.05±0.03	23.6±0.3
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
	Murray Valley	T1	27.78±1.13	64.0±2.0	434±13	1.17±0.02	24.7±0.2
		T2	23.34±1.81	60.1±2.2	392±40	1.32±0.04	24.9±0.5
		T3	17.18±1.25	60.5±1.3	284±20	1.43±0.05	25.8±0.1
		T4	30.78±0.55	65.3±1.3	472±13	1.21±0.08	22.2±0.2
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
	Hilltops	T1	5.28±0.59	100.3±2.6	52±5	1.23±0.01	25.8±0.2
		T2	4.95±0.42	79.6±2.7	62±4	1.35±0.04	24.9±0.7
		T3	3.30±0.15	96.6±6.6	35±3	1.21±0.03	26.5±0.5
		T4	5.02±0.24	88.1±3.3	57±4	1.25±0.06	26.0±0.4
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*
2015/16	Langhorne Creek	T1	6.92±1.27	82.3±1.2	84±15	0.93±0.05	25.0±0.7
		T2	6.90±1.03	56.7±3.7	120±14	0.96±0.03	26.0±0.7
		T3	5.04±0.52	75.4±7.7	69±9	0.93±0.02	24.0±0.7
		T4	7.49±0.98	78.5±5.4	94±7	0.89±0.03	23.0±0.5
		T5	10.48±2.00	28.8±4.0	380±77	0.65±0.05	24.4±0.3
	Murray Valley	T1	18.12±3.19	47.7±1.9	377±63	1.29±0.03	23.7±0.4
		T2	13.74±2.17	45.1±8.5	329±68	1.30±0.06	23.2±0.6
		T3	11.40±0.95	45.5±0.6	251±23	1.36±0.03	23.5±0.4
		T4	10.16±2.08	50.9±2.1	196±34	1.34±0.06	24.4±0.2
		T5	13.15±2.67	58.7±12.6	258±77	1.26±0.04	22.3±0.6
	Hilltops	T1	5.35±0.54	97.4±2.6	55±4	1.38±0.06	24.8±0.7
		T2	2.93±0.16	71.6±2.5	41±1	1.29±0.03	24.7±0.3
		T3	3.89±0.25	102.3±1.8	38±2	1.53±0.01	23.3±0.4
		T4	4.72±0.34	86.4±4.0	55±3	1.21±0.05	23.2±0.6
		T5	8.00±0.36	37.5±0.9	214±13	0.81±0.06	22.3±0.3

Table 2.5: Fruit composition (pH, titratable acidity, total anthocyanins, total phenolics, total tannins and sugar per berry) from fruit collected at harvest for each treatment at each site and each season. Data are means of four field replicates ± standard error. T5 was not implemented in the 2013/14 season, n/c is data not collected, n/a is data unable to be calculated.

Season	Site	Treatment	pH	TA ($g\ L^{-1}$)	Total Anthocyanins ($mg\ g^{-1}$)	Total Phenolics (AU)	Total Tannins ($mg\ g^{-1}$)	Sugar / berry ($g\ g^{-1}$)
2013/14	Langhorne Creek	T1	4.20±0.04	3.50±0.08	1.72±0.04	1.45±0.03	3.39±0.14	0.27±0.01
		T2	4.18±0.03	3.45±0.17	1.93±0.06	1.49±0.03	3.33±0.17	0.28±0.00
		T3	4.30±0.05	3.49±0.12	1.74±0.10	1.47±0.04	3.74±0.25	0.22±0.02
		T4	4.08±0.04	4.07±0.13	1.43±0.06	1.31±0.05	2.94±0.16	0.26±0.01
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
	Murray Valley	T1	4.00±0.08	2.98±0.19	1.33±0.05	1.37±0.08	4.47±0.29	0.29±0.01
		T2	*3.99±0.04*	*2.95±0.17*	*1.27±0.06*	*1.35±0.06*	*4.28±0.43*	*0.30±0.01*
		T3	4.09±0.06	3.09±0.21	1.35±0.03	1.30±0.04	4.52±0.17	0.30±0.00
		T4	4.20±0.04	3.00±0.06	1.02±0.08	1.19±0.08	3.83±0.28	0.27±0.01
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
	Hilltops	T1	3.89±0.05	3.48±0.06	1.82±0.12	1.75±0.17	4.01±0.38	0.18±0.01
		T2	3.76±0.03	3.71±0.11	2.38±0.13	2.22±0.08	5.77±0.48	0.16±0.00
		T3	3.82±0.05	3.90±0.20	2.02±0.07	2.02±0.03	5.06±0.29	0.18±0.01
		T4	3.79±0.02	3.70±0.10	1.68±0.02	2.07±0.03	4.85±0.20	0.19±0.00
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
2014/15	Langhorne Creek	T1	4.14±0.05	4.42±0.19	1.80±0.06	1.64±0.06	4.19±0.30	0.26±0.01
		T2	4.00±0.03	4.60±0.08	1.95±0.08	1.83±0.05	4.87±0.27	0.26±0.00
		T3	3.67±0.03	7.04±0.13	1.79±0.07	1.57±0.07	4.02±0.21	0.27±0.02
		T4	4.02±0.07	4.63±0.19	1.45±0.05	1.48±0.02	3.77±0.45	0.25±0.01
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
	Murray Valley	T1	3.73±0.15	3.29±0.20	1.32±0.08	1.56±0.04	4.98±0.25	0.29±0.00
		T2	3.97±0.13	3.04±0.10	1.36±0.03	1.65±0.02	5.34±0.27	0.33±0.01
		T3	3.80±0.01	3.56±0.05	1.37±0.05	1.65±0.06	4.21±0.13	0.37±0.01
		T4	4.05±0.06	3.22±0.07	0.76±0.05	1.20±0.04	3.86±0.12	0.27±0.02
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
	Hilltops	T1	3.83±0.02	4.35±0.02	1.30±0.06	1.26±0.04	3.39±0.90	0.32±0.00
		T2	3.73±0.03	4.69±0.05	1.32±0.07	1.34±0.06	2.83±0.49	0.34±0.01
		T3	3.91±0.02	4.35±0.09	1.48±0.06	1.42±0.06	2.45±0.28	0.32±0.01
		T4	3.90±0.02	3.73±0.13	1.19±0.15	1.28±0.10	2.36±0.40	0.32±0.01
		T5	*n/c*	*n/c*	*n/c*	*n/c*	*n/c*	*T5*
2015/16	Langhorne Creek	T1	4.03±0.04	4.31±0.16	1.86±0.04	1.58±0.03	3.84±0.16	0.23±0.01
		T2	3.88±0.02	4.36±0.05	2.01±0.06	1.73±0.04	4.23±0.18	0.25±0.01
		T3	3.84±0.03	5.48±0.16	1.88±0.02	1.57±0.04	3.69±0.17	0.22±0.01
		T4	4.06±0.04	3.75±0.15	1.53±0.08	1.49±0.04	3.49±0.17	0.20±0.01
		T5	3.85±0.05	4.12±0.14	1.95±0.15	1.79±0.11	4.15±0.39	0.16±0.01
	Murray Valley	T1	3.81±0.06	3.76±0.15	1.26±0.03	1.41±0.03	4.47±0.18	0.31±0.01
		T2	3.84±0.07	3.50±0.15	1.29±0.03	1.5±0.02	4.86±0.45	0.30±0.01
		T3	3.78±0.06	4.12±0.18	1.29±0.02	1.41±0.02	4.17±0.07	0.32±0.01
		T4	4.19±0.05	3.28±0.17	0.93±0.02	1.24±0.02	3.93±0.16	0.33±0.02
		T5	3.84±0.10	3.58±0.17	1.18±0.04	1.4±0.05	4.05±0.26	0.28±0.02
	Hilltops	T1	3.88±0.04	4.57±0.09	1.65±0.08	1.6±0.08	3.91±0.23	0.34±0.02
		T2	3.83±0.03	4.59±0.06	1.79±0.03	1.71±0.03	4.26±0.07	0.32±0.01
		T3	3.85±0.04	4.63±0.06	1.69±0.06	1.64±0.05	3.69±0.09	0.35±0.01
		T4	3.68±0.03	4.39±0.07	1.44±0.05	1.65±0.02	3.94±0.20	0.28±0.01
		T5	3.72±0.02	4.29±0.04	1.66±0.09	1.8±0.06	4.85±0.10	0.34±0.01

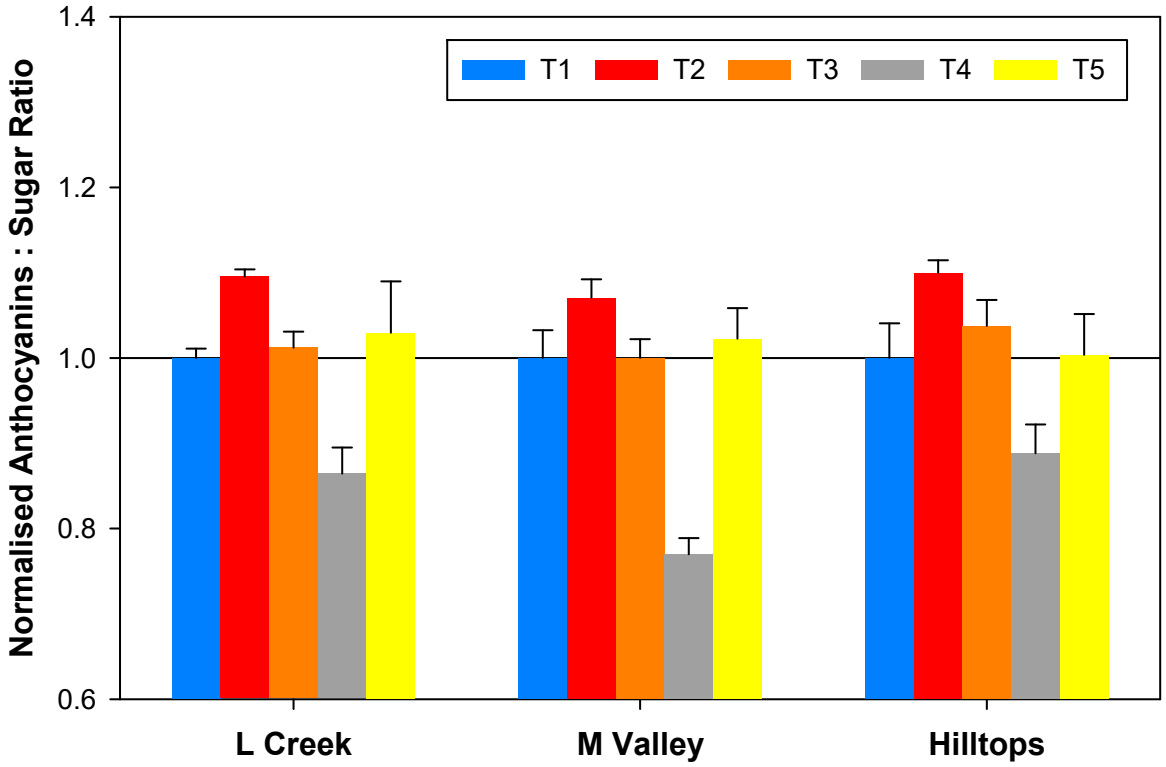


Figure 2.12 Ratio of whole berry anthocyanins to berry sugar content, normalised to the control value at each site, for each treatment and averaged across the three growing seasons, except T5, which was for 2015/16 only. Data are means of four field replicates ± standard error.

Normalising the anthocyanin:sugar ratio to T1 at each site and in each season (Figure 2.12) clearly demonstrates those effects, with T2 consistently improving the ratio by a small amount, T3 having no effect and T4 consistently reducing the ratio. There was no effect of T5, despite the major effects of this treatment on bunch and berry parameters, although T5 was assessed only in a single season.

2.3.5 The role of vine balance in determining treatment effects

A key result of the field work is the contrast between the effect of the T4 late defoliation treatment on berry composition and the lack of any effect of the T3 crop removal treatment (except on juice TA). As both treatments changed vine balance at all three sites, either the T3 treatment did not move vine balance enough to have an effect on fruit composition, or the change in fruit environment as a result of the canopy manipulations was a bigger driver of fruit composition than vine balance.

As anthocyanin content was the berry composition parameter with the most consistent changes as a result of the treatments and fruit is often valued on anthocyanin content, this parameter has been used to test the question as to whether vine balance or some other effect of the treatments was driving the observed changes in fruit composition. Generating a linear regression using the whole anthocyanin data set against calculated vine balance indicates a statistically significant relationship with an adjusted r^2 value of 0.32 (Figure 2.13, Table 2.6). However, it is clear that this relationship was very much driven by the effect of the large crop load at Murray Valley in 2014/15, which accounts for more than half the absolute range in

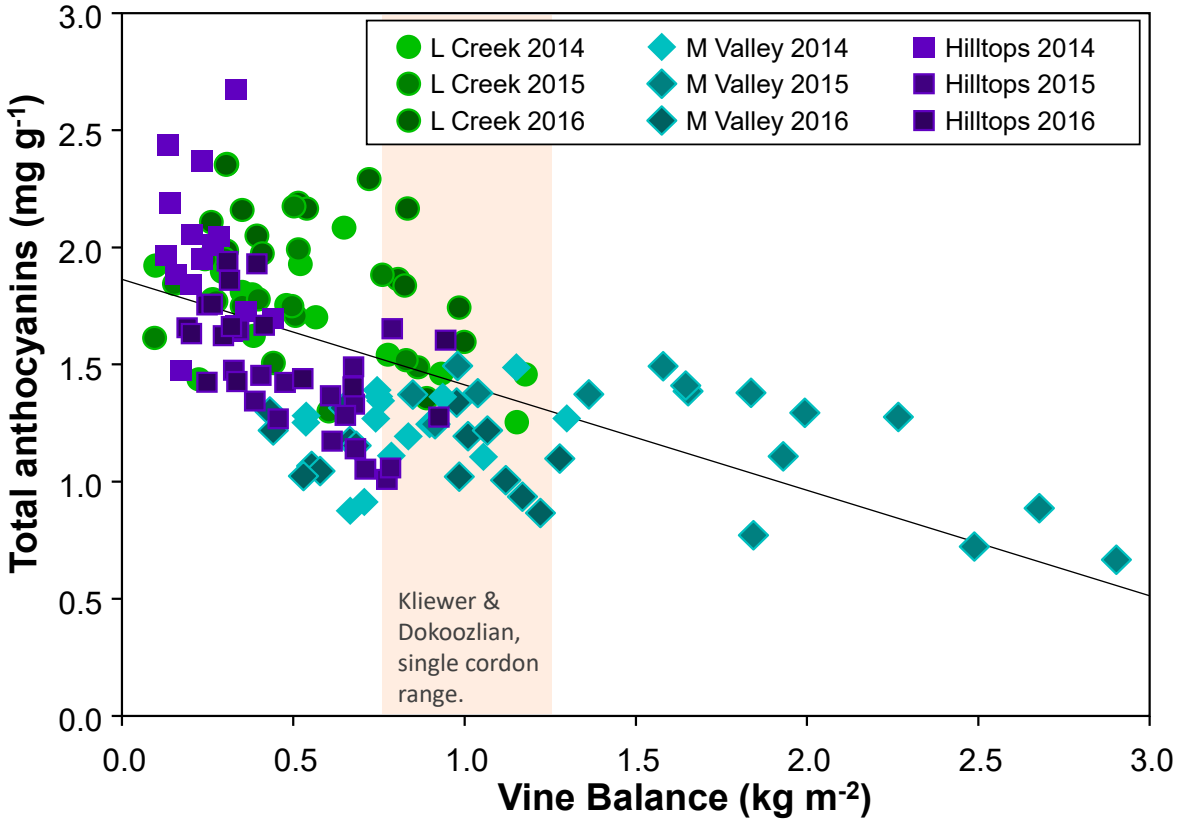


Figure 2.13 The relationship between total anthocyanins of harvested fruit and calculated vine balance across three sites and three seasons. Data are means of four field replicates, shaded area represents the appropriate range suggested by Kliewer and Dokoozlian (2005) for single cordon vines.

Table 2.6: Regression parameters and statistical probability of being different to a slope of zero for various subsets of the anthocyanin and vine balance results.

Data set	adj. R^2	Slope (mg g-1 / kg m-2)	probability	n
All data	0.320	-0.452	***<0.001***	155
All 2014	0.437	-0.803	***<0.001***	47
All 2015	0.418	-0.309	***<0.001***	48
All 2016	0.220	-0.689	***<0.001***	60
All L. Creek	0.115	-0.382	***0.008***	52
All M. Valley	0.100	-0.106	***0.024***	51
All Hilltops	0.405	-1.045	***<0.001***	52
2014: L. Creek	0.326	-0.421	***0.012***	16
2014: M. Valley	0.000	ns	*0.334*	15
2014: Hilltops	-0.018	ns	*0.405*	16
2015: L. Creek	0.037	ns	*0.230*	16
2015: M. Valley	0.498	-0.327	***0.001***	16
2015: Hilltops	0.187	ns	*0.054*	16
2016: L. Creek	0.344	-0.657	***0.004***	20
2016: M. Valley	0.190	-0.222	***0.031***	20
2016: Hilltops	0.163	ns	*0.077*	20

vine balance, and the generally lower anthocyanin content in the Murray Valley fruit. Furthermore, a relationship between vine balance and fruit composition that is only valid across an inter-regional scale is of limited value to a vineyard manager given the task of improving quality in a specific vineyard.

Separating the sites (Figure 2.14), results in a weak relationship between vine balance and anthocyanin content at Langhorne Creek and Murray Valley (adjusted r^2 values both approximately 0.1, Table 2.6), but an improved relationship at Hilltops (adjusted $r^2 = 0.41$). However, even in this case, if the seasons are separated the relationship becomes non-significant for each season (Figure 2.14, Table 2.6).

Consequently, it is difficult to avoid the conclusion that the relationship between yield and canopy size (vine balance) is not a driver of fruit composition at harvest in our data set. It is possible that the choice of sites and the choice of crop manipulation have not covered a large enough range of vine balance to produce an effect on fruit composition, however, the vast majority of the vine balance values recorded were outside of the range suggested by Kliewer & Dokoozlian (2005), including almost all of the values recorded at Hilltops (Figure 2.13). As those authors note, an ideal vine balance will be specific to a region and growing system and the solar radiation received by all three sites is probably higher than many viticultural regions elsewhere in the world. Higher solar radiation is likely to lead to a higher rate of whole vine carbon assimilation and thus the ability to support a larger crop load for a given canopy size.

Although vine balance is a source-sink relationship, it was beyond the scope of the project to make a detailed assessment of vine carbon assimilation and allocation. However, leaf-level photosynthesis was measured during maturation on at least one occasion in each season at each site (Figure 2.15). These measurements demonstrated significant site differences, with Murray Valley having the highest photosynthetic rates, significant seasonal differences and a significant effect of the T4 treatment, increasing A_{sat}. There was also a marginally significant effect of the T2 treatment ($p = 0.060$), also increasing A_{sat}. The seasonal differences in A_{sat} and the effect of T2 were driven by higher stomatal conductance (g_s, data not shown), and at least part of the site differences and effect of T4 were also due to higher g_s. The site and seasonal differences in g_s were probably driven by the differences in soil water availability during the maturation period (Figure 2.6).

As no measurements were made of plant water status, or of treatment effects on soil moisture, it is not possible to definitively ascertain whether the higher g_s in the T4 and/or T2 vines was due to upregulation as a result of the higher relative demand on carbohydrates in those vines or simply because they were using the available soil moisture at a slower rate due to their smaller canopies. However, an estimate of maximum carboxylation rate was made using the A_{sat} data and this indicated a significant effect of the T4 treatment. Thus it appears that the T4 vines maintained a higher photosynthetic capacity during the maturation period than vines in the other treatments, despite the tendency for photosynthesis to decline in grapevine leaves during this period (Kriedemann et al., 1970).

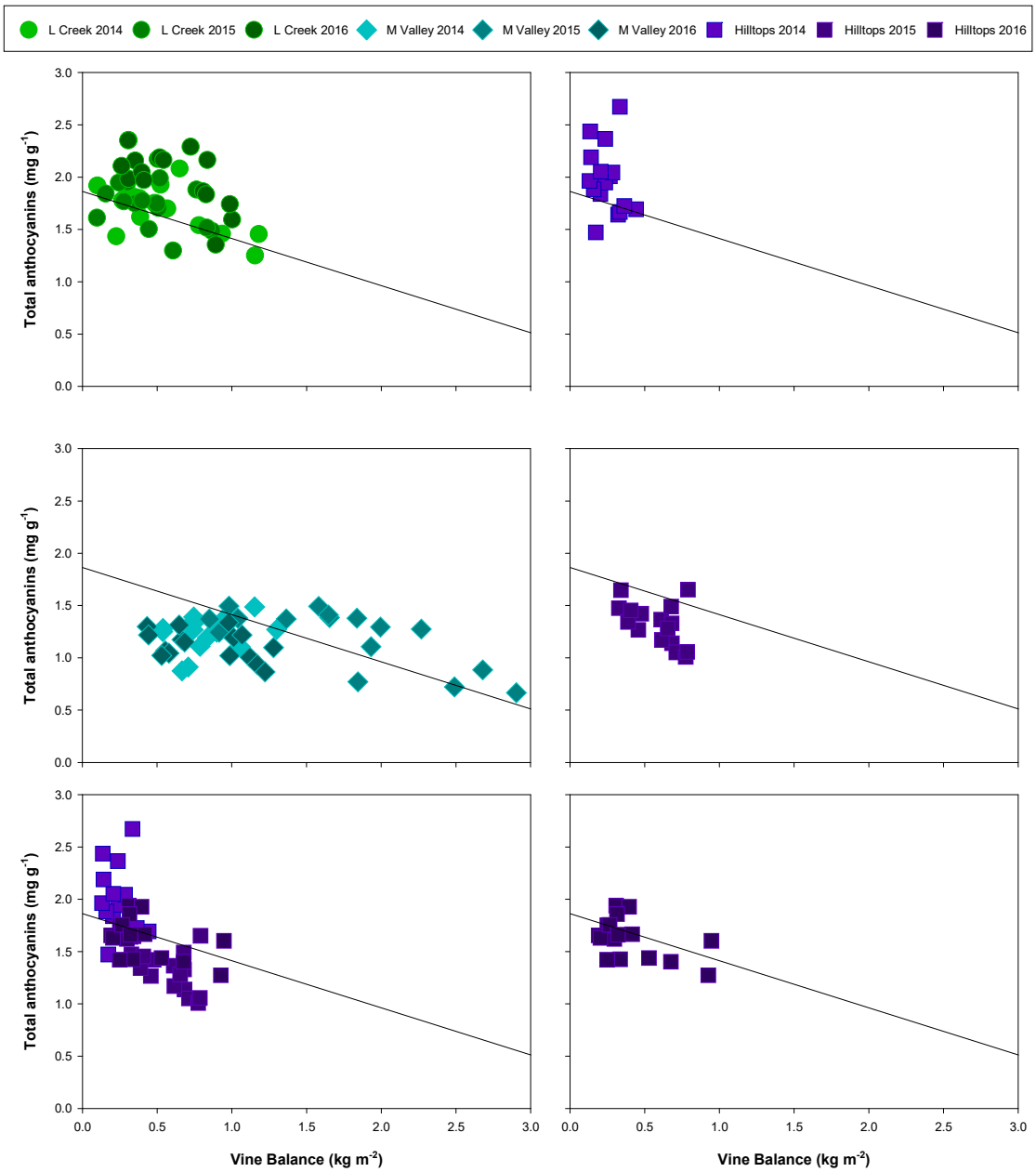


Figure 2.14 Relationship between total anthocyanins of harvested fruit and calculated vine balance for subsets of the total data set: Langhorne Creek (top left), Murray Valley (centre left), Hilltops (bottom left), Hilltops 2013/14 (top right), Hilltops 2014/15 (centre right) and Hilltops 2015/6 (bottom right). Data are means of four field replicates.

Non-structural carbohydrate concentration (NSC) in trunk wood was used to estimate impacts of the treatments, or changes in vine balance, on storage reserves (Figure 2.16). However, no major effects of site, season or treatment on NSC were observed. Earlier work has shown that trunk NSC concentrations were less affected than root NSC if yield level or canopy leaf area is reduced. In addition, the impact on either wood or trunk NSC was generally only observed if severe changes were made to the vines, by either removing all crop mid-ripening or removing all leaves after harvest (Holzapfel et al., 2006). Further, grapevines can refill reserves after harvest in most regions of Australia, indicating the initial treatment effects could have been present at harvest, but not in winter when the NSC were determined in this project.

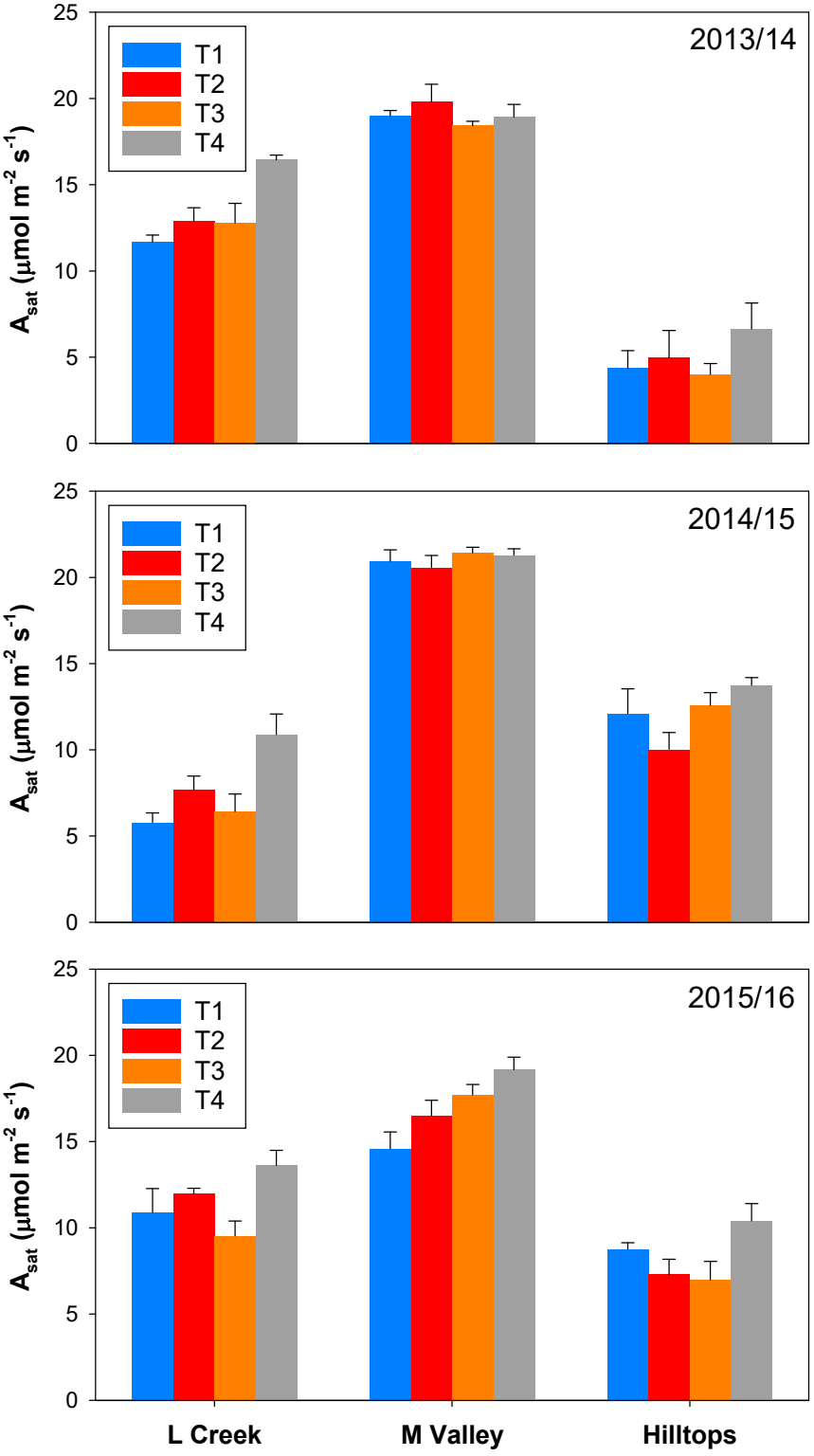


Figure 2.15 Rates of photosynthesis under saturating light (A_{sat}) of single sun exposed leaves, measured in treatments 1-4, pre-harvest at each field site in each growing season. Data are means of four replicates ± standard error.

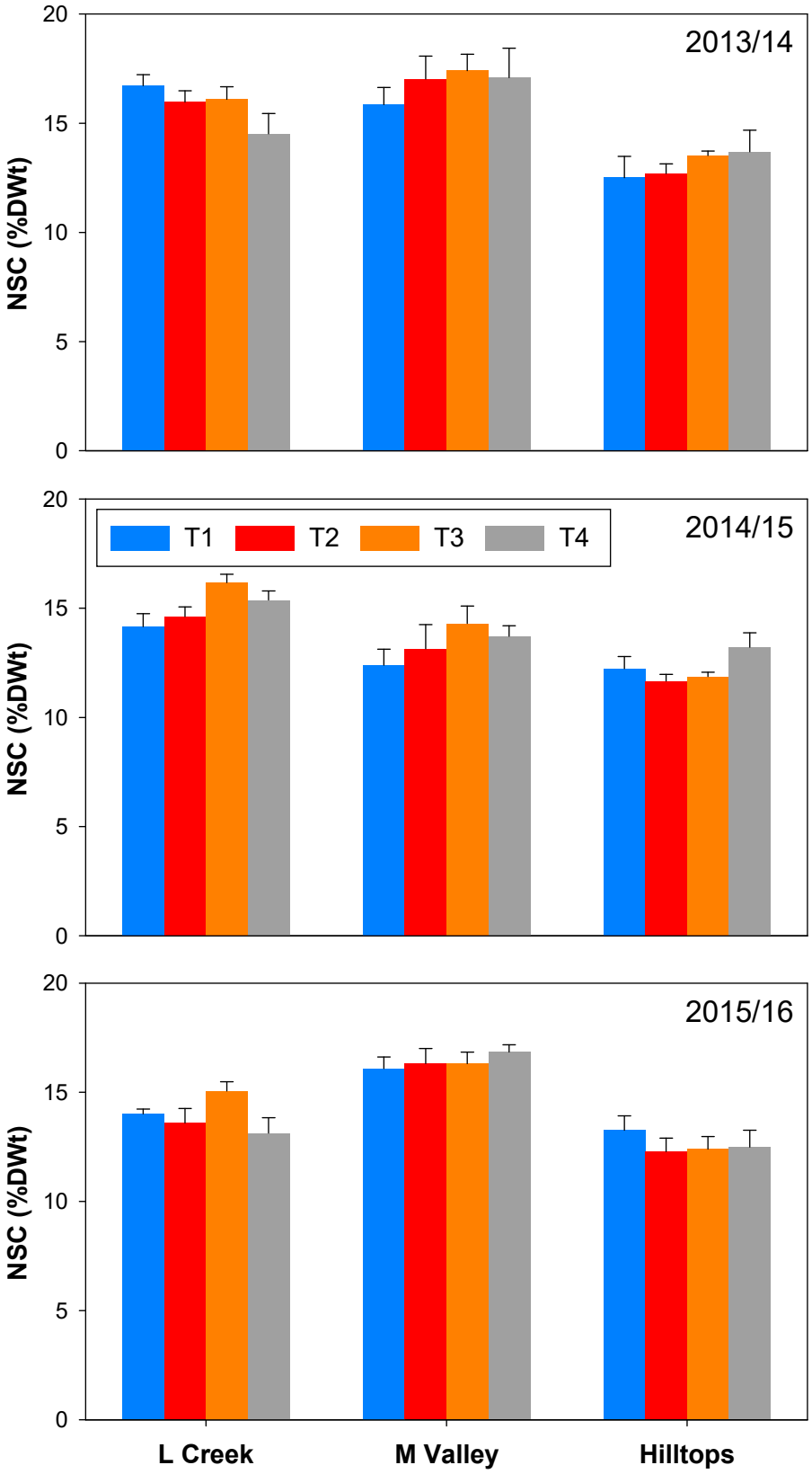


Figure 2.16 Non-structural carbohydrate (NSC) concentration of trunk wood, sampled pre-budburst in each treatment at each field site in each growing season. Data are means of four replicates ± standard error.

2.4 Conclusions

Applying the crop and canopy treatments in a consistent manner across three trials sites that differed significantly in yield and climatic conditions, and doing this over three consecutive seasons, enabled the project to address the question as to whether responses to crop load or canopy manipulations are site or season specific. The value in knowing this is to allow vineyard managers to make decisions based on a degree of certainty as to whether fruit or wine quality responses can be obtained in a predictable and reliable manner. There is a much greater potential scope for widespread adoption than if practices have to be tailored for every specific situation. For T1 to T4, which were all applied over three seasons with as identical methodology as possible, the relative treatment responses have been remarkably similar across the three trial sites. It would be expected that some variation will arise as a result of the differing environments, but the findings suggest that across a common genotype, the physiological responses to changes in fruit exposure and source-sink relations are similar.

Overall, the fruit data do not provide support for the ratio between crop load and canopy size (vine balance) playing a major role in driving fruit composition, a question which is explored further in the subsequent chapters

Chapter 3
Relationship between fruit and wine composition and impact of vine balance

3.1 Background

Fruit quality is a key factor in the production of good wine. However, while vine physiology, berry maturation and composition can be manipulated through canopy management practices, there is less evidence of how this carries through to the resulting wine and/or how this impacts on wine ageing. The aims of this chapter are thus to determine whether differences observed in harvested fruit (Chapter 2) are still apparent in the finished product or are lost through the winemaking process; and to assess whether differences observed at bottling are retained upon ageing or whether the wine evolves differently.

Secondary metabolites such as phenolic compounds are important drivers of berry, and thus wine, quality. These compounds derive from the same precursors: phenylalanine, 3-Malonyl-CoA and 4-Coumaroyl-CoA. In particular, flavonoids are now well known to be important secondary metabolites that vary with biotic and abiotic factors (Castellarin, Bavaresco, Falginella, Gonçalves, & Di Gaspero, 2012; Di Ferdinando, Brunetti, Fini, & Tattini, 2012; Petrussa et al., 2013; Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaujon, 2007). Those compounds are of particular interest in grapes and wine as they are involved in colour, ageing capacity, bitterness and astringency (Adams, 2006; Drappier, Thibon, Rabot, & Geny-Denis, 2017). They have a common C6-C3-C6 backbone and are divided into sub-families depending on their structure and properties: flavonols, anthocyanins, flavan-3-ols and tannins.

In this report, analyses principally focused on anthocyanins, which are synthesised in the skin of the berry (except for Teinturier cultivars where synthesis also occurs in the pulp) and located in the thick-walled hypodermal cells (Adams, 2006). They are end-products of the flavonoid pathway and result from the complexation of anthocyanidins and sugars. While there are only six main anthocyanidins identified in plant organs (mono-hydroxylated: pelargonidin (Pg); di-hydroxylated: cyanidin (C) and peonidin (Pn); tri-hydroxylated: delphinidin (D), petunidin (Pt), malvidin (M)), a multitude of anthocyanins can be generated through glycosylation and subsequent acylation (Terrier, Ollé, Verriès, & Cheynier, 2009). In *Vitis vinifera*, only the di- and tri-hydroxylated anthocyanin families have been detected and 60 to 70% of the total anthocyanins exist in the monoglucosylated form. The remaining 30 to 40% are acylated monoglucosides with non-flavonoid phenolics such as hydroxycinnamates (*p*-coumaric acid, caffeic acid, acetic acid). Pinot Noir only contains monoglucosides while Shiraz contains all forms of anthocyanins. Among the different anthocyanin families, malvidin derivatives constitute the main anthocyanins found in grapes and wine, representing 80% of the total anthocyanins in *V. vinifera* grape cultivars.

Tannins are flavan-3-ol polymers synthetised in the skin and seeds of the berries, and trace amounts can also be detected in the pulp. Differences in size and composition exist between skin and seed tannins. While tannin concentration is higher in seeds (Kennedy, Saucier, & Glories, 2006), tannins found in wine mainly come from the skin.

The proportion of the different families and forms of anthocyanins as well as tannin size and composition are known to mainly depend on the variety and also vary significantly with abiotic factors such as light exposure, water stress, etc. (Downey, Dokoozlian, & Krstic, 2006). Several studies have been recently conducted on the impact of leaf removal, shoot and bunch thinning and variable impacts on flavonoid composition were found. Different results were observed for leaf removal treatments depending on the type (basal vs apical) and time of application (early vs late). Decrease in anthocyanins was sometimes reported (Baiano et al., 2015) but most studies around véraison either observed inconsistent effects across seasons or no effect (Di Profio, Reynolds, & Kasimos, 2011; King, McClellan, & Smart, 2012; Lanari, Lattanzi, Borghesi, Silvestroni, & Palliotti, 2012; Osrečak, Karoglan, & Kozina, 2016; Pastore, Allegro, Valentini, Muzzi, & Filippetti, 2017; Zhang et al., 2017). No effects on grape tannins were found with leaf removal around flowering (before and after) while seasons had a significant effect (Sivilotti et al., 2016). However, early leaf removal led to higher anthocyanin concentrations and had a greater effect than bunch thinning (Bubola, Sivilotti, Janjanin, & Poni, 2017). In this study, bunch thinning, inducing a significant loss of yield, impacted maturation rate but did not impact final grape composition. Effect of canopy management on subsequent wine was sometimes assessed in the studies mentioned above but the inconsistent effects observed on grapes renders interpretation of wine results challenging.

In this project, wine was made from each field replicate on all three sites across three seasons following a standard winemaking protocol. Wine was analysed at bottling by industry standard spectrophotometry methods for colour/phenolics and tannins and by Ultra High Performance Liquid Chromatography-Photo Diode Array (UHPLC-PDA) for anthocyanins; sensory-assessed by winemakers at four months post-bottling; and difference-tested and analysed again by the same Industry standard spectrophotometry and UHPLC-PDA methods at 10 months post-bottling. In this section, results obtained for the wine are compared with those of the harvested fruit in relation to treatments and vine balance.

3.2 Material and Methods

3.2.1 Winemaking

Grapes were picked per treatment, targeting a common sugar level (24°Brix), and spanning a three-week harvesting period between all treatments at the most. Upon arrival at the experimental winery (NWGIC, Wagga Wagga), 50 berries were sampled from each treatment replicate and frozen at -80 °C until further analysis (*see section 3.2.6*). Small scale (50 kg fruit) winemaking was conducted following a standard winemaking protocol (Appendix 1). The protocol did not include malolactic fermentation so as to avoid modifications of fruit sensory characters as well as wine colour and phenolic composition ((Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Izquierdo-Cañas, García-Romero, Mena-Morales, & Gómez-Alonso, 2016) and references therein).

3.2.2 Basic wine analyses

pH and TA were measured by titration with sodium hydroxide (0.1 M, prepared weekly) using a Metrohm Titrando System autotitrator (calibrated daily) run by Tiamo (version 2.3). Measurements were performed on 10 mL juice or wine samples. Prior to analysis, wine samples were degassed with nitrogen to remove dissolved carbon dioxide. The °Baumé was determined with a portable density meter (Anton Paar DMA 35N). Yeast Assimilable Nitrogen

(YAN), Free Amino Nitrogen (FAN), ammonia, acetic acid, glucose and fructose were measured using a Thermo Fisher KonelabTM 20XT Auto analyser and corresponding test kits. The % alcohol was determined using an Alcolyser, DMA 4500 density meter (Near InfraRed) LMWI 40-10 (Anton Paar, Sydney, Australia). Free and total sulfur dioxide were determined as outlined in Barril, Clark, & Scollary (2012), using a FIAStar™ 5000 flow injection wine analyser (FOSS) run by the SoFIA software (version 2.00 Build 13).

3.2.3 Wine analyses by UltraViolet-visible (UV-vis) spectrophotometry

3.2.3.1 'Colour/phenolics' - Wine was adjusted to pH 3.6 and the absorbance was recorded at 520 and 420 nm. From the pH-adjusted wine, three separate solutions were prepared, i.e. with acetaldehyde, with sodium metabisulfite, and with hydrochloric acid, following the method of Iland, Ewart, Sitters, Markides, & Bruer (2000). All measurements were performed on a Shimadzu UV-1700 UV-visible spectrophotometer using 1 mm pathlength quartz cuvettes except for the sample containing hydrochloric acid where 10 mm pathlength UV-transparent semi-micro disposable cuvettes (Brand) were used.

3.2.3.2 Total tannins - Tannins were analysed in duplicates by the Methyl Cellulose Precipitable (MCP) assay adapted from Sarneckis et al., (2006). For each wine, 37.5 µL wine was pipetted into two 1.5 mL Eppendorf tubes (MCP & control) and 450 µL of 0.04% (w/w) aqueous methyl cellulose added to the MCP tube, then left to stand for 2-3 min. Saturated ammonium sulfate (300 µL) and deionised water (600 µL for MCP; 1050 µL for control) were added to each tube, which were then placed in a 30 °C water bath for 10 min. Samples were then centrifuged at 16,000 rpm for 6 min in a microfuge (Microfuge 20, Beckman Coulter) and the absorbance of the supernatant measured at 280 nm in 10 mm pathlength UV-transparent semi-micro disposable cuvettes (Brand) using a Shimadzu UV-1700 UV-visible spectrophotometer. Calibration was conducted using (-)-epicatechin standard solutions (0.01-0.2 mg/L).

3.2.4 Anthocyanin analysis by Ultra High Performance Liquid Chromatography-Photo Diode Array (UHPC-PDA)

Acetaldehyde (20 µL of an aqueous 10% (v/v) solution) was added to 2 mL of wine to release sulfur dioxide-bound anthocyanins. After 45 min, the wine was filtered through a 0.2 µm cellulose acetate filter into UHPLC autosampler vials. The chromatographic analyses were conducted on a Waters ACQUITY Ultra Performance Liquid Chromatograph (UPLC©) connected to a sample manager and a Photo Diode Array (PDA) detector all run by Empower2©. The column was a Waters Acquity BEH C18 (2.1 mm x 50 mm, 1.7 µm); volume of injection was 2 µL, column temperature 40 °C, autosampler 10 °C and flow rate 0.5 mL/min. The elution gradient, adapted from Downey, & Rochfort (2008), consisted of solvent A: 10% (v/v) formic acid in water, and solvent B: 10% (v/v) formic acid in methanol, as follows (expressed in %A): 0 min: 90; 1.5 min: 88; 6 min: 78; 8 min: 75; 9.50 min: 65; 10.5 min: 45; 11.5 min: 0; 14-15.5 min: 90. Chromatograms were recorded over the range 200-500 nm, and anthocyanins were quantified at 500 nm. Calibration was conducted using malvidin-3-O-glucoside standard solutions (0.61-734.67 mg/L).

3.2.5 Wine sensory

3.2.5.1 Winemaker sensory assessment (four months post-bottling) - Sensory assessment of each wine was conducted every year (four months post-bottling) by a panel of winemakers

and researchers at McWilliam's Wines, Griffith, Australia. The tasting was organised in three brackets, one per site, with the 16 wines (20 in season 3) randomised within brackets. The panellists were asked to smell and taste from left to right, and score the following attributes from 0 (absent) to 9 (intense): red fruit, dark fruit and stewed fruit. Mouthfeel and astringency perception were also scored with three attributes: maturity from green (1) to mature (9), texture from low (1) to high (9) and intensity from light (1) to full (9). In addition, preferences were scored from 1 (dislike) to 9 (like).

3.2.5.2 Difference testing (10 months post-bottling) - Differences between the control wine (T1) and each treatment wine (T2-T4 in seasons 1 and 2; T2-T5 in season 3) were assessed by triangle tests, across three days (a site per day) at 10 months post-bottling every year. Several replicates were first combined before pouring in black glasses to avoid colour bias. Panellists (about 25 regular wine drinkers, including professionals) were presented with three (four in season 3) sets of three glasses: within each set, a treatment was compared to the control, and two of the three glasses contained the same wine. Panellists were asked to smell and taste from left to right and identify the wine that was different in each set. Glasses were randomised with regard to set (treatment) and wine (identical wine).

3.2.6 Harvest berry analyses

Frozen harvest berries, sampled at harvest (*see section 3.2.1*) were weighed and then slightly thawed on ice to separate skins from pulp and seeds. Skins were weighed, flash-frozen in liquid nitrogen, and later ground under liquid nitrogen (A11 Basic Analytical Mill, IKA Works). Frozen ground material (100 mg skins ± 5 mg) was extracted using 1 mL of 50% (v/v) aqueous methanol for 20 min on a rotary suspension mixer (Ratek) at 4 °C to minimise oxidation. Samples were then centrifuged for 10 min at 13,000 rpm (Microfuge 20, Beckman Coulter) and the supernatant filtered through a 0.2 µm cellulose acetate filter. The extract (200 µL) was analysed by chromatography as described in *section 3.2.4.*

3.2.7 Statistical analysis

Values are presented as means (average of the four field replicates, unless otherwise stated) ± standard deviation. Significant differences between treatments and the control were determined using R (version 3.2.5) by one-way ANalysis Of VAriance (ANOVA) and pairwise comparison of means by Tukey's test ($p < 0.05$). Significant differences are indicated by different letters or *. Significant differences between bottling and post bottling analyses, indicated by $^{\#}$, were determined using Sigma Plot (version 13.0) and paired t-test (two-tailed). Significance for the difference testing was determined by the Chi^2 test. ANOVA-PCA (Principal Component Analysis) (Bouveresse, Pinto, Schmidtke, Locquet, & Rutledge, 2011; Harrington et al., 2005) and Pearson's pairwise linear correlation coefficients were calculated using Matlab (version R2107b).

3.3 Results and Discussion

3.3.1 Winemaking

Fruit was harvested as close as possible to a target sugar level of 24°Brix for all sites and seasons, and on a 'per-treatment' basis thus resulting in slight variations throughout the span of the project (22-26°Brix/13-15 °Baumé, Table 3.1). Wine was made for each field replicate through a standard protocol at the NWGIC, Wagga Wagga (Appendix 5.1). The resulting wines ranged 13-16% (v/v) alcohol, however, within seasons and sites, only three treatment wines

differed from the control (T3 MV (+0.5) and LC (-1.1) in seasons 1 and 2, respectively, and T5 H (-1.2) in season 3, Table 3.2). Of those wines, only T5 H-S3 was picked at a significantly lower sugar level than that of the control (Table 3.1).

As expected, must pH and TA exhibited an inversely proportional relationship (Figure 3.1), with must pH ranging 3.3-3.8 and TA 3.6-7.4 g/L (T1-T4, Table 3.1), however there was no strong correlation between must °Baumé and pH or TA (Figure 3.1). There was no apparent treatment impact, however, there was a seasonal effect with S3 exhibiting lower pH/higher TA (Figure 3.1). As °Baumé was not particularly correlated with must pH/TA, this was more likely a result of S3 being harvested earlier than S1/2 for most treatments and hence a compressed ripening period resulting in higher acid levels (Table 2.3). In any case, the must was adjusted to about pH 3.6 (final wine pH ranging 3.3-3.7) by tartaric acid additions (final wine TA ranging 5.3-7.3 g/L) as per winemaking protocol. Acetic acid was kept below 0.3 g/L for all wines and there was no difference between any of the treatments and the control (Table 3.2).

Must ammonia and FAN tended to be lower for T2 and T4, with Hilltops exhibiting significant and consistent effects across all three seasons (except for FAN T2-S3, Table 3.1). The resulting YAN was also significantly lower for T2 and T4 at Hilltops in all three seasons (Table 3.1). The timing of defoliation appeared critical as T4 led to a larger decrease in FAN than T2. Interestingly, those effects were only statistically significant at Hilltops where the canopy was relatively small compared to MV, thus for T2, removing eight leaves proportionally reduced leaf area more greatly at Hilltops than at other sites. At LC, where the canopy was larger than Hilltops, but still smaller than MV, the same effect was seen as a trend, but the individual results for T2 were not significantly different from T1. Surprisingly, T3 did not exhibit any impact, although higher nitrogen levels would have been expected due to a reduced sink (i.e., 50% less fruit). There was also no consistent effect on the ratio of ammonia:FAN. In addition, MV exhibited the lowest of FAN and YAN, and seasonal effects were noticeable as correlations for FAN, ammonia and YAN in S3 were different to those observed for S1/2 (Figure 3.2). Overall, the treatment effects on FAN and YAN demonstrated a strong dependence on the canopy for fruit nitrogen content during the maturation period. As the canopy is the primary site for N assimilation, this should not be too surprising, but the greater impact of T4 than T2, suggests that a significant proportion of fruit nitrogen uptake must be occurring post-veraison.

Vine balance did not seem to have any effect on must N parameters in MV, even though this was the site where vine balance was the most changed across all three seasons. However, YAN, and mainly FAN, were affected at Hilltops (Figure 3.2B).

As per the winemaking protocol (Appendix 5.1), YAN was adjusted at the beginning of the winemaking process to minimise differences in fermentation rates and hence ensure similar extraction of grape compounds in the wine. All wines underwent fermentation in about six days on skin, and a further two days after pressing until dryness. Sulfur dioxide adjustments were also conducted throughout winemaking to ensure a free SO_2 of approximately 30 mg/L prior to bottling and minimise variations which may otherwise impact on colour such as through binding of anthocyanins (known as sulfur bleaching).

Table 3.1: Must parameters after crushing. Errors are given as standard deviations (n=4). Significant differences (bolded) are indicated by different letters ($p < 0.05$).

			pH	TA (g/L)	°Baumé	FAN (mg/L)	Ammonia (mg/L)	YAN (mg/L)	Ratio Amm:FAN
Season 1	L Creek	T1	3.73 ± 0.05 a	4.4 ± 0.1 a	13.7 ± 0.2 a	268 ± 38 a	69 ± 10 a	325 ± 45 a	0.26 ± 0.02 a
		T2	3.70 ± 0.07 a	4.4 ± 0.2 a	13.9 ± 0.1 a	215 ± 43 a	57 ± 11 a	262 ± 53 a	0.27 ± 0.01 a
		T3	3.76 ± 0.06 a	4.5 ± 0.2 a	13.8 ± 0.8 a	288 ± 35 a	74 ± 8 a	349 ± 41 a	0.26 ± 0.02 a
		T4	3.66 ± 0.01 a	**5.3 ± 0.1 b**	13.5 ± 0.2 a	237 ± 38 a	64 ± 6 a	290 ± 42 a	0.27 ± 0.02 a
	M Valley	T1	3.63 ± 0.06 a	4.1 ± 0.2 a	13.4 ± 0.2 ab	114 ± 10 ab	67 ± 6 a	169 ± 13 ab	0.58 ± 0.06 a
		T2	*3.61 ± 0.05 a*	*4.1 ± 0.3 a*	*13.3 ± 0.2 a*	*103 ± 11 ab*	*63 ± 6 a*	*155 ± 16 ab*	*0.61 ± 0.02 a*
		T3	3.65 ± 0.04 a	4.1 ± 0.2 a	13.7 ± 0.2 b	123 ± 24 b	65 ± 7 a	177 ± 29 b	0.53 ± 0.05 a
		T4	**3.77 ± 0.05 b**	3.9 ± 0.1 a	13.2 ± 0.1 a	93 ± 5 a	56 ± 2 a	139 ± 7 a	0.60 ± 0.02 a
	Hilltops	T1	3.70 ± 0.06 a	4.7 ± 0.2 a	14.3 ± 0.2 a	228 ± 22 b	66 ± 3 b	283 ± 23 b	0.29 ± 0.03 a
		T2	3.70 ± 0.1 a	4.8 ± 0.3 a	14.6 ± 0.4 ab	**164 ± 26 a**	**50 ± 3 a**	**205 ± 27 a**	0.31 ± 0.05 a
		T3	3.71 ± 0.06 a	4.7 ± 0.2 a	**14.9 ± 0.2 b**	231 ± 27 b	68 ± 2 b	287 ± 28 b	0.29 ± 0.03 a
		T4	3.62 ± 0.02 a	4.5 ± 0.1 a	14.4 ± 0.1 a	**118 ± 7 a**	**51 ± 3 a**	**160 ± 9 a**	**0.43 ± 0.01 b**
Season 2	L Creek	T1	3.73 ± 0.06 b	5.0 ± 0.1 a	14.1 ± 0.5 a	226 ± 43 a	66 ± 8 b	280 ± 50 a	0.29 ± 0.02 a
		T2	**3.58 ± 0.07 a**	5.1 ± 0.1 a	13.5 ± 0.5 a	151 ± 44 a	**42 ± 11 a**	185 ± 53 a	0.28 ± 0.03 a
		T3	**3.60 ± 0.03 a**	**5.9 ± 0.1 b**	13.7 ± 0.4 a	228 ± 36 a	73 ± 9 b	288 ± 37 a	0.32 ± 0.06 a
		T4	**3.60 ± 0.05 a**	5.3 ± 0.3 a	13.4 ± 0.3 a	195 ± 47 a	66 ± 12 b	249 ± 56 a	0.34 ± 0.03 a
	M Valley	T1	3.60 ± 0.1 a	3.7 ± 0.2 a	13.4 ± 0.2 ab	103 ± 33 a	57 ± 8 a	149 ± 40 a	0.58 ± 0.09 a
		T2	3.60 ± 0.1 a	3.6 ± 0.3 a	13.7 ± 0.5 b	87 ± 16 a	50 ± 8 a	128 ± 21 a	0.58 ± 0.09 a
		T3	3.53 ± 0.07 a	**4.4 ± 0.3 b**	13.9 ± 0.5 b	83 ± 7 a	54 ± 8 a	127 ± 12 a	0.65 ± 0.06 a
		T4	3.66 ± 0.05 a	3.9 ± 0.2 ab	12.8 ± 0.2 a	95 ± 6 a	49 ± 5 a	135 ± 7 a	0.51 ± 0.07 a
	Hilltops	T1	3.43 ± 0.08 a	5.5 ± 0.4 b	13.8 ± 0.5 a	128 ± 8 b	56 ± 3 b	174 ± 9 b	0.44 ± 0.04 b
		T2	3.38 ± 0.05 a	5.5 ± 0.2 b	13.7 ± 0.3 a	**103 ± 8 a**	**34 ± 5 a**	**131 ± 9 a**	**0.33 ± 0.05 a**
		T3	3.48 ± 0.05 a	5.2 ± 0.1 b	**14.6 ± 0.3 b**	131 ± 12 b	53 ± 5 b	175 ± 15 b	0.40 ± 0.03 ab
		T4	3.47 ± 0.03 a	**4.3 ± 0.1 a**	14.1 ± 0.3 ab	**94 ± 7 a**	**43 ± 5 a**	**129 ± 10 a**	0.45 ± 0.04 b
Season 3	L Creek	T1	3.43 ± 0.04 b	6.5 ± 0.2 a	14.0 ± 0.3 b	136 ± 27 a	58 ± 12 b	184 ± 18 a	0.5 ± 0.2 ab
		T2	3.38 ± 0.06 ab	**6.6 ± 0.2 b**	14.2 ± 0.4 b	90 ± 32 a	**24 ± 13 a**	110 ± 42 a	0.26 ± 0.04 a
		T3	**3.30 ± 0.03 a**	**7.4 ± 0.1 c**	**12.8 ± 0.2 a**	126 ± 21 a	73 ± 15 b	186 ± 32 a	0.58 ± 0.06 b
		T4	3.44 ± 0.06 b	**5.7 ± 0.3 b**	13.2 ± 0.3 ab	115 ± 28 a	45 ± 14 ab	151 ± 40 a	0.38 ± 0.04 ab
		T5	3.43 ± 0.05 b	**6.6 ± 0.6 b**	13.7 ± 0.9 ab	132 ± 31 a	54 ± 21 ab	176 ± 47 a	0.40 ± 0.08 ab
	M Valley	T1	3.4 ± 0.1 ab	5.5 ± 0.2 a	13.4 ± 0.3 ab	80 ± 30 ab	49 ± 7 ab	121 ± 35 ab	0.7 ± 0.1 a
		T2	3.30 ± 0.05 a	5.6 ± 0.4 a	13.4 ± 0.3 ab	55 ± 10 a	36 ± 14 a	84 ± 21 a	0.6 ± 0.2 a
		T3	3.37 ± 0.06 ab	5.5 ± 0.2 a	13.5 ± 0.3 ab	66 ± 3 ab	53 ± 7 ab	109 ± 9 ab	0.81 ± 0.08 a
		T4	3.45 ± 0.04 b	5.8 ± 0.3 a	14.0 ± 0.4 b	101 ± 13 b	47 ± 2 ab	139 ± 13 b	0.47 ± 0.08 a
		T5	3.36 ± 0.05 ab	6.0 ± 0.2 a	13.1 ± 0.5 a	82 ± 13 ab	63 ± 11 b	134 ± 9 b	0.8 ± 0.3 a
	Hilltops	T1	3.36 ± 0.05 b	6.7 ± 0.2 bc	13.7 ± 0.5 b	174 ± 23 bc	127 ± 12 c	278 ± 33 c	0.74 ± 0.04 c
		T2	**3.54 ± 0.04 c**	5.8 ± 0.1 ab	13.8 ± 0.2 b	160 ± 22 bc	**82 ± 8 ab**	**227 ± 29 b**	**0.51 ± 0.02 a**
		T3	**3.52 ± 0.05 c**	6.3 ± 0.2 ab	13.1 ± 0.3 ab	190 ± 18 c	117 ± 4 c	286 ± 19 c	**0.62 ± 0.05 b**
		T4	**3.25 ± 0.03 a**	7.0 ± 0.2 c	13.2 ± 0.3 ab	**110 ± 11 a**	**75 ± 4 a**	**171 ± 14 a**	0.68 ± 0.05 bc
		T5	**3.22 ± 0.02 a**	**7.8 ± 0.3 d**	**12.6 ± 0.4 a**	137 ± 8 ab	**94 ± 5 b**	214 ± 8 ab	0.69 ± 0.06 bc

Table 3.2: Wine parameters at bottling. Errors are given as standard deviations (n=4). Significant differences (bolded) are indicated by different letters ($p < 0.05$).

			pH	TA (g/L)	Alcohol (% v/v)	Acetic Acid (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Molecular SO_2
Season 1	L Creek	T1	3.71 ± 0.03 a	6.2 ± 0.1 b	14.0 ± 0.3 a	0.25 ± 0.02 a	36 ± 3 a	81 ± 8 a	0.44 ± 0.02 a
		T2	3.69 ± 0.04 a	6.2 ± 0.2 b	14.2 ± 0.4 a	0.22 ± 0.05 a	36 ± 2 a	81 ± 3 a	0.47 ± 0.05 a
		T3	3.70 ± 0.03 a	6.1 ± 0.2 b	13.4 ± 0.8 a	0.22 ± 0.04 a	33 ± 5 a	**97 ± 12 b**	0.41 ± 0.05 a
		T4	3.71 ± 0.05 a	**5.6 ± 0.2 a**	13.3 ± 0.3 a	0.23 ± 0.03 a	36 ± 4 a	95 ± 5 ab	0.43 ± 0.03 a
	M Valley	T1	3.60 ± 0.04 a	5.3 ± 0.3 a	14 ± 0.2 a	0.15 ± 0.01 a	32 ± 4 a	72 ± 3 a	0.51 ± 0.05 a
		T2	*3.60 ± 0.02 a*	*5.4 ± 0.1 ab*	*14.1 ± 0.2 a*	*0.15 ± 0.02 a*	*32 ± 1 a*	*74 ± 3 a*	*0.50 ± 0.02 a*
		T3	3.60 ± 0.03 a	**5.7 ± 0.1 b**	**14.5 ± 0.3 b**	0.14 ± 0.01 a	32 ± 4 a	79 ± 6 a	0.50 ± 0.03 a
		T4	3.61 ± 0.04 a	5.5 ± 0.1 ab	13.8 ± 0.1 a	0.16 ± 0.02 a	31 ± 6 a	75 ± 8 a	0.47 ± 0.07 a
	Hilltops	T1	3.56 ± 0.03 a	5.8 ± 0.2 ab	15.8 ± 0.4 a	0.27 ± 0.05 a	28 ± 5 ab	81 ± 11 ab	0.48 ± 0.07 a
		T2	3.52 ± 0.03 a	6.3 ± 0.2 b	15.4 ± 0.2 a	0.31 ± 0.02 a	22 ± 5 a	64 ± 11 a	0.40 ± 0.09 a
		T3	3.58 ± 0.07 a	5.7 ± 0.4 a	16.1 ± 0.2 a	0.32 ± 0.03 a	27 ± 6 ab	77 ± 14 ab	0.43 ± 0.05 a
		T4	**3.68 ± 0.04 b**	5.4 ± 0.2 a	16.1 ± 0.4 a	0.28 ± 0.07 a	37 ± 3 b	95 ± 5 b	0.48 ± 0.01 a
Season 2	L Creek	T1	3.59 ± 0.01 a	7.3 ± 0.2 b	14.5 ± 0.3 b	0.14 ± 0.04 a	41 ± 2 b	100 ± 4 a	0.65 ± 0.04 b
		T2	3.59 ± 0.01 a	6.9 ± 0.2 ab	13.8 ± 0.1 ab	0.17 ± 0.05 a	40 ± 1 b	93 ± 3 a	0.64 ± 0.02 b
		T3	3.61 ± 0.02 a	7.3 ± 0.2 b	**13.4 ± 0.6 a**	0.09 ± 0.06 a	**35 ± 3 a**	93 ± 5 a	**0.53 ± 0.04 a**
		T4	3.59 ± 0.01 a	**6.9 ± 0.1 a**	14.1 ± 0.4 ab	0.11 ± 0.03 a	40 ± 1 b	96 ± 4 a	0.64 ± 0.01 b
	M Valley	T1	3.3 ± 0.1 a	6.2 ± 0.4 bc	14.2 ± 0.5 ab	0.09 ± 0.08 ab	23 ± 5 a	69 ± 15 a	0.69 ± 0.08 a
		T2	**3.5 ± 0.1 b**	5.9 ± 0.1 ab	14.7 ± 0.6 b	0.04 ± 0.04 a	**37 ± 3 b**	**89 ± 7 b**	0.8 ± 0.1 a
		T3	3.44 ± 0.07 ab	6.4 ± 0.1 c	15.1 ± 0.4 b	0.18 ± 0.03 b	30 ± 4 ab	80 ± 7 ab	0.67 ± 0.02 a
		T4	**3.52 ± 0.03 b**	**5.6 ± 0.2 a**	13.4 ± 0.3 a	0.12 ± 0.09 ab	**32 ± 2 b**	**88 ± 3 b**	0.6 ± 0.04 a
	Hilltops	T1	3.63 ± 0.02 b	6.8 ± 0.3 b	14.8 ± 0.6 ab	0.14 ± 0.03 ab	40 ± 1 b	98 ± 2 a	0.59 ± 0.02 ab
		T2	3.62 ± 0.02 ab	6.9 ± 0.2 b	14.6 ± 0.5 a	0.19 ± 0.03 ab	42 ± 1 b	96 ± 2 a	0.63 ± 0.02 b
		T3	3.62 ± 0.03 b	6.9 ± 0.1 b	15.6 ± 0.3 b	0.19 ± 0.03 b	40 ± 1 b	103 ± 5 a	0.59 ± 0.03 ab
		T4	**3.54 ± 0.06 a**	**6.3 ± 0.2 a**	14.9 ± 0.2 ab	0.10 ± 0.06 a	**32 ± 6 a**	92 ± 16 a	0.57 ± 0.03 a
Season 3	L Creek	T1	3.63 ± 0.03 a	6.9 ± 0.2 ab	13.9 ± 0.3 a	0.15 ± 0.02 a	40 ± 3 a	94 ± 6 ab	0.58 ± 0.05 a
		T2	3.63 ± 0.05 a	6.9 ± 0.2 ab	13.9 ± 0.4 a	0.12 ± 0.01 a	40 ± 2 a	81 ± 5 ab	0.58 ± 0.03 a
		T3	3.61 ± 0.01 a	7.1 ± 0.2 b	13 ± 0.8 a	0.16 ± 0.03 a	40 ± 2 a	96 ± 4 b	0.61 ± 0.02 a
		T4	3.62 ± 0.01 a	6.5 ± 0.2 ab	13.3 ± 0.3 a	0.14 ± 0.01 a	37 ± 2 a	91 ± 5 ab	0.55 ± 0.03 a
		T5	3.60 ± 0.03 a	6.8 ± 0.3 ab	13.5 ± 1.1 a	0.19 ± 0.06 a	37 ± 3 a	89 ± 9 ab	0.58 ± 0.04 a
	M Valley	T1	3.5 ± 0.1 a	6.3 ± 0.2 a	14.2 ± 0.4 ab	0.14 ± 0.02 ab	28 ± 7 a	70 ± 17 ab	0.59 ± 0.06 a
		T2	3.47 ± 0.09 a	6.33 ± 0.05 a	13.8 ± 0.4 ab	0.12 ± 0.01 a	29 ± 5 a	63 ± 9 a	0.60 ± 0.04 a
		T3	3.54 ± 0.09 a	6.1 ± 0.2 a	14 ± 0.4 ab	0.14 ± 0.01 ab	32 ± 7 a	71 ± 12 ab	0.56 ± 0.04 a
		T4	3.61 ± 0.01 a	6.5 ± 0.2 a	14.6 ± 0.3 b	0.13 ± 0.01 ab	35 ± 1 a	91 ± 4 b	0.54 ± 0.01 a
		T5	3.52 ± 0.05 a	6.2 ± 0.5 a	13.2 ± 0.8 a	0.16 ± 0.01 b	31 ± 3 a	70 ± 5 ab	0.58 ± 0.07 a
	Hilltops	T1	3.63 ± 0.02 a	6.6 ± 0.3 a	13.9 ± 0.6 b	0.14 ± 0.02 ab	38 ± 2 a	99 ± 6 a	0.55 ± 0.04 a
		T2	3.63 ± 0.01 a	6.4 ± 0.1 a	14.1 ± 0.3 b	0.12 ± 0.01 a	39 ± 2 a	94 ± 2 a	0.57 ± 0.04 a
		T3	3.63 ± 0.02 a	6.3 ± 0.2 a	13.3 ± 0.4 ab	0.13 ± 0.01 ab	38 ± 3 a	94 ± 4 a	0.56 ± 0.02 a
		T4	3.56 ± 0.03 a	6.28 ± 0.05 a	13.4 ± 0.4 ab	0.16 ± 0.02 ab	35 ± 3 a	87 ± 6 a	0.59 ± 0.02 a
		T5	3.53 ± 0.03 a	6.1 ± 0.1 a	**12.7 ± 0.7 a**	0.15 ± 0.03 b	31 ± 1 a	98 ± 4 a	0.56 ± 0.03 a

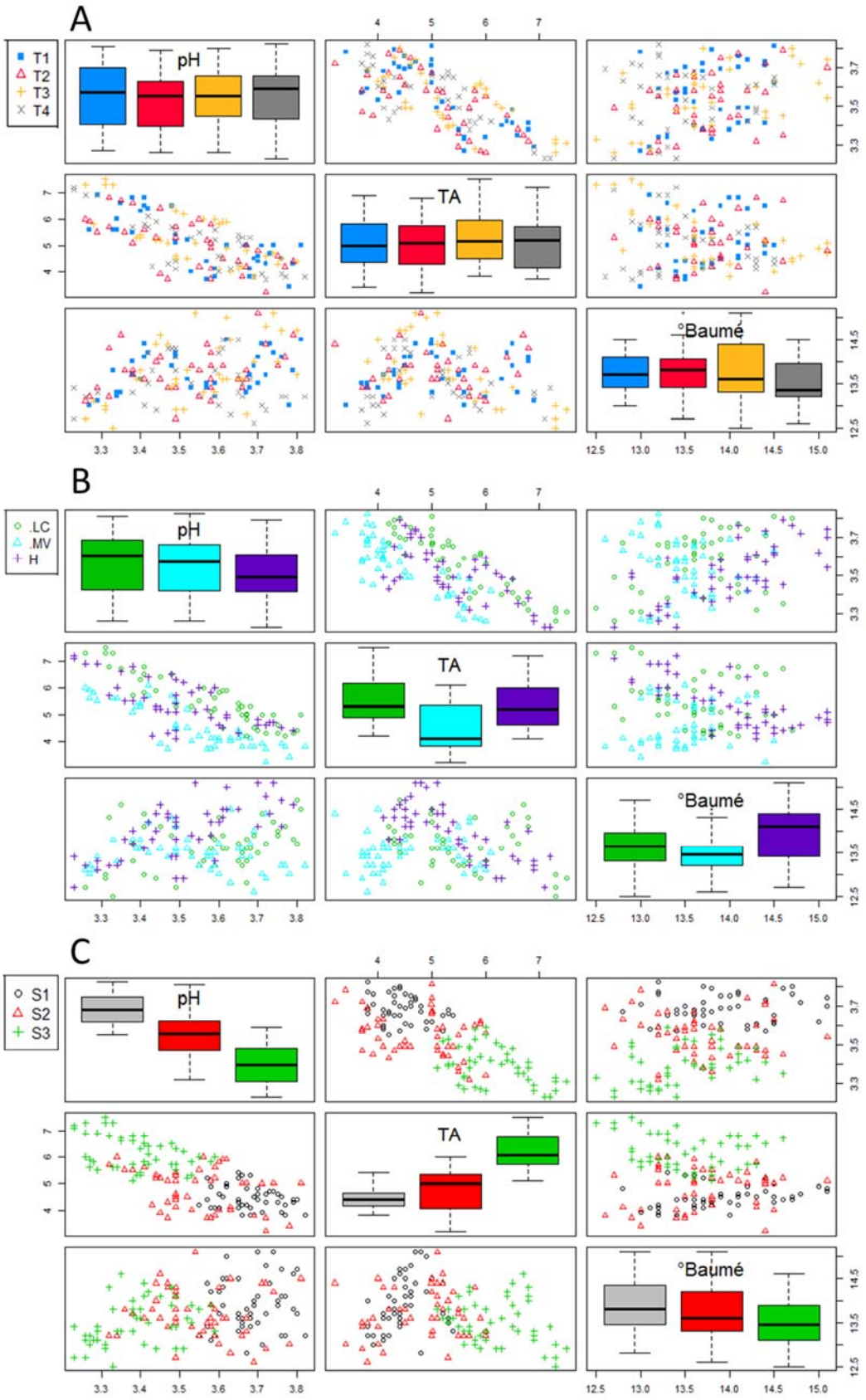


Figure 3.1 Scatterplot matrices with boxplots (diagonal) for must pH, TA and °Baumé per treatments (A), sites (B) and seasons (C). T5 was not included as data were only collected for one season.

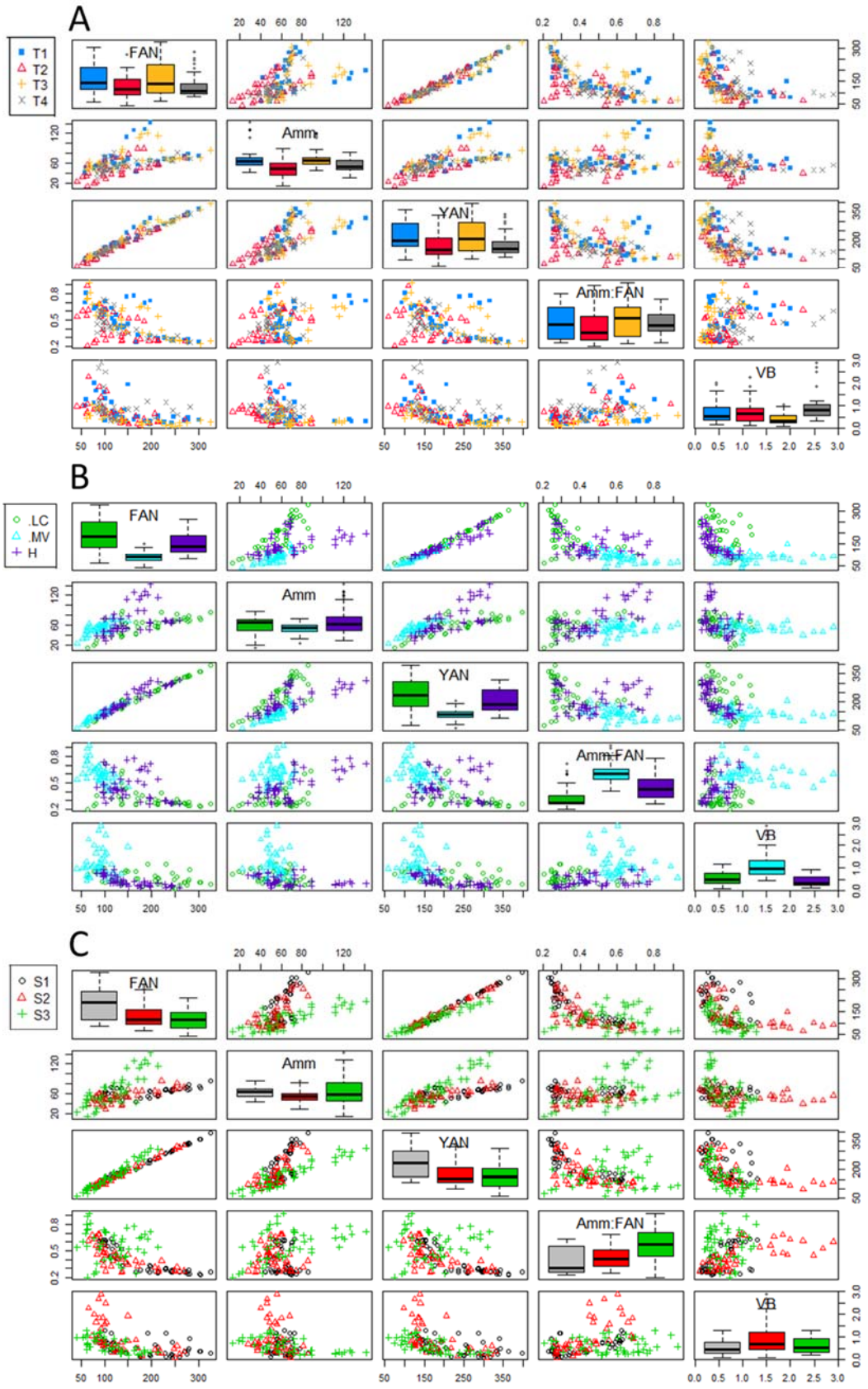


Figure 3.2 Scatterplot matrices with boxplots (diagonal) for must FAN, ammonia, YAN, ammonia:FAN ratio and VB per treatments (A), sites (B) and seasons (C). T5 was not included as data were only collected for one season.

3.3.2 Wine phenolic composition

Wines were analysed at bottling for colour/phenolics parameters and tannins using industry standard methods (spectrophotometry), as well as anthocyanins by UPLC-PDA to gain further details on some of the key compounds comprising this family of flavonoids. Analyses were repeated 10 months post-bottling, a common timeframe before commercial release, to assess the effect of short-term bottle ageing. In addition to tannins, parameters most commonly used in the industry to describe red wine colour and phenolics include wine colour density, a measure of the colour intensity of red and yellow/brown pigments; colour hue, describing the tint or shade of the colour, and often used to assess colour change during ageing; total red pigments, including free anthocyanins and anthocyanin adducts with small tannins and fermentation product; and total phenolics, encompassing flavonoids and non-flavonoids. Together with UPLC-PDA anthocyanins, those industry parameters were not only compared between bottling and 10 month post-bottling, but were also compared to analyses performed on harvest fruit using industry standard methods (total anthocyanins, total phenolics and tannins, Chapter 2), as well as further analyses of anthocyanins on harvest berry skins by UPLC-PDA presented in this Chapter.

When comparing fruit and wine composition, it must be noted that while compounds located in the skin are readily extracted during the winemaking process, compounds located in seeds are much less extracted. Hence, as industry standard analyses of harvest fruit were conducted on whole berries (Chapter 2), anthocyanins, located in skins, can be readily compared, whether whole berry to skins or whole berry/skins to wine parameters, but fruit and wine comparison of total phenolics and tannins, both originating from skin and seeds, must be treated carefully. In fact, berry total phenolics and tannins were not very well correlated with those parameters measured in wine, and were thus excluded from the scatterplots presented in Appendix 5.2. Anthocyanins measured by the spectrophotometry industry standard method on whole berries (B antho S in Appendix 5.2) and by UPLC-PDA on skins (B antho LC in Appendix 5.2) were, as expected, reasonably well correlated together, and with wine colour density, total red pigments and UPLC-PDA anthocyanins at bottling (WB CD, WB TRP and WB antho LC in Appendix 5.2). The strongest correlation between berry and wine colour parameters was obtained for UPLC-PDA skin anthocyanins (B antho LC) and wine colour density (WB CD) (adjusted $r^2 = 0.800$, $p < 0.001$), which was maintained 10 months post-bottling (adjusted $r^2 = 0.746$, $p < 0.001$ with WPB CD, Appendix 5.2).

As observed in harvest berries, treatment effects in wine were most apparent for T2 (shifted to higher values) and T4 (shifted to lower values), which seemed more noticeable in the wine than in the berries (Appendix 5.2A). More detailed discussion of treatment effect with regard to the control is presented for individual parameters below. Site variations and seasonal effects were also observed (Appendix 5.2B-C). Similar to MV fruit exhibiting lower anthocyanin concentrations at harvest, MV wines were generally lower in colour density, total red pigments, UPLC anthocyanins as well as total phenolics both at bottling and upon ageing (Appendix 5.2B). Season 1 was higher in wine total red pigments (anthocyanins to a lesser extent), tannins and especially total phenolics, which seemed to have impacted the relationship with colour density (Appendix 5.2C). Interestingly, anthocyanins were not particularly higher in season 1 fruit. Seasonal effects were less apparent upon ageing. Colour hue appeared to be affected by both sites and seasons (Appendix 5.2B-C), and did not correlate with any other parameters.

3.3.2.1 Wine colour and phenolics - Wine colour density (A_{420} + $A_{520,}$ Figure 3.3) was higher for T2 wines at LC/MV in both seasons 2 and 3, and T3 wines at MV/H in season 2. On the other hand, wine colour density was lower for T4 at LC/MV in season 1 and H in season 3. T5 wines also exhibited less colour density at H in season 3. All treatment replicates but three (T2 H-S1, T4 LC-S1, and T3 MV-S2) decreased in wine colour density over 10 months. Loss in colour density during bottle ageing was the least prominent for T4 wines, however as those wines exhibited lower colour density at bottling, their decrease was relatively the same as that of other treatments (Appendix 5.2). Differences observed at bottling for T2 and T4 were still apparent 10 months later, in addition to T2 H-S1 (higher), and T4 H-S1 and LC-S2 (lower), both treatments affecting five out of nine wines each by then.

Wine colour hue ($A_{420}/A_{520,}$ Figure 3.4) was much less impacted by treatments than colour density, and only T2 (lower) in LC/MV-S2, and T4-MV (higher) and T5-H (higher) in season 3 were significantly different to the control. All treatment replicates considerably increased in colour hue over 10 months. As wine ages, its colour becomes less attributable to free anthocyanins and red-coloured oligomers (measured by A_{520}), and more to large yellow/brown polymeric pigments (measured by A_{420}), thus noticeably affecting colour hue (Somers & Evans, 1977). However bottling and post-bottling values did not correlate very strongly (Appendix 5.2), indicating that respective compositional changes occurred in an unrelated manner. Of the four wines that were different at bottling, only two retained their difference (T2 MV-S2, lower; T5 H-S3, higher) 10 months later. By post-bottling, T3 in MV/H-S2 were also found different, although MV was lower and H was higher.

Total red pigments ($A_{HCl/520}$, Figure 3.5) were significantly lower for six out of the nine T4 wines across sites and seasons (all sites-S1, LC-S2, and LC/H-S3). T3 exhibited lower total red pigments at LC but higher at MV in season 2. T2 was higher at MV in season 2 and LC in season 3. T5 was lower at Hilltops in season 3. All treatment replicates, but two (T2 and T5 H-S3), decreased in total red pigments. This is due to the concentration in free anthocyanins, main contributor to total red pigments, halving during the first year of ageing as they undergo various reactions (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Mercurio, Dambergs, Herderich, & Smith, 2007), as observed in the concentrations measured by UPLC-PDA in this project (*see section 3.3.2.3*). The decrease in red pigments contributed to the increase in colour hue mentioned above. Of the six T4 wines that were different at bottling, only four remained lower than the control, with H-S1 and LC-S3 not significantly different after 10 months. Differences observed at bottling for T2 and T3 were retained. T5 H-S3 also remained lower than the control, but in addition to LC-S3 which exhibited higher red total pigments.

Total phenolics ($A_{HCl/280}$ - 4, Figure 3.6) were less impacted by treatments than total red pigments. T2 wines displayed higher total phenolics in H-S1, LC/MV-S2 and LC-S3, while T3 wines were only higher in H-S1, and MV-S2. T4 wines exhibited lower total phenolics in LC/MV-S1. T5 wines were lower in LC/H-S3. Total phenolics were noticeably higher in season 1 (Appendix 2C and Figure 3.6), but decreased in all treatment replicates upon ageing (Figure 3.6). Total phenolics also decreased for nine out of the 16 wines in season 3, with T1 and T2 at all sites (Figure 3.6). Differences observed at botting for both T2 and T3 wines in H-S1 and MV-S2, as well as T2 LC-S3, were retained 10 months later; however, T2 wines in LC-S2 were

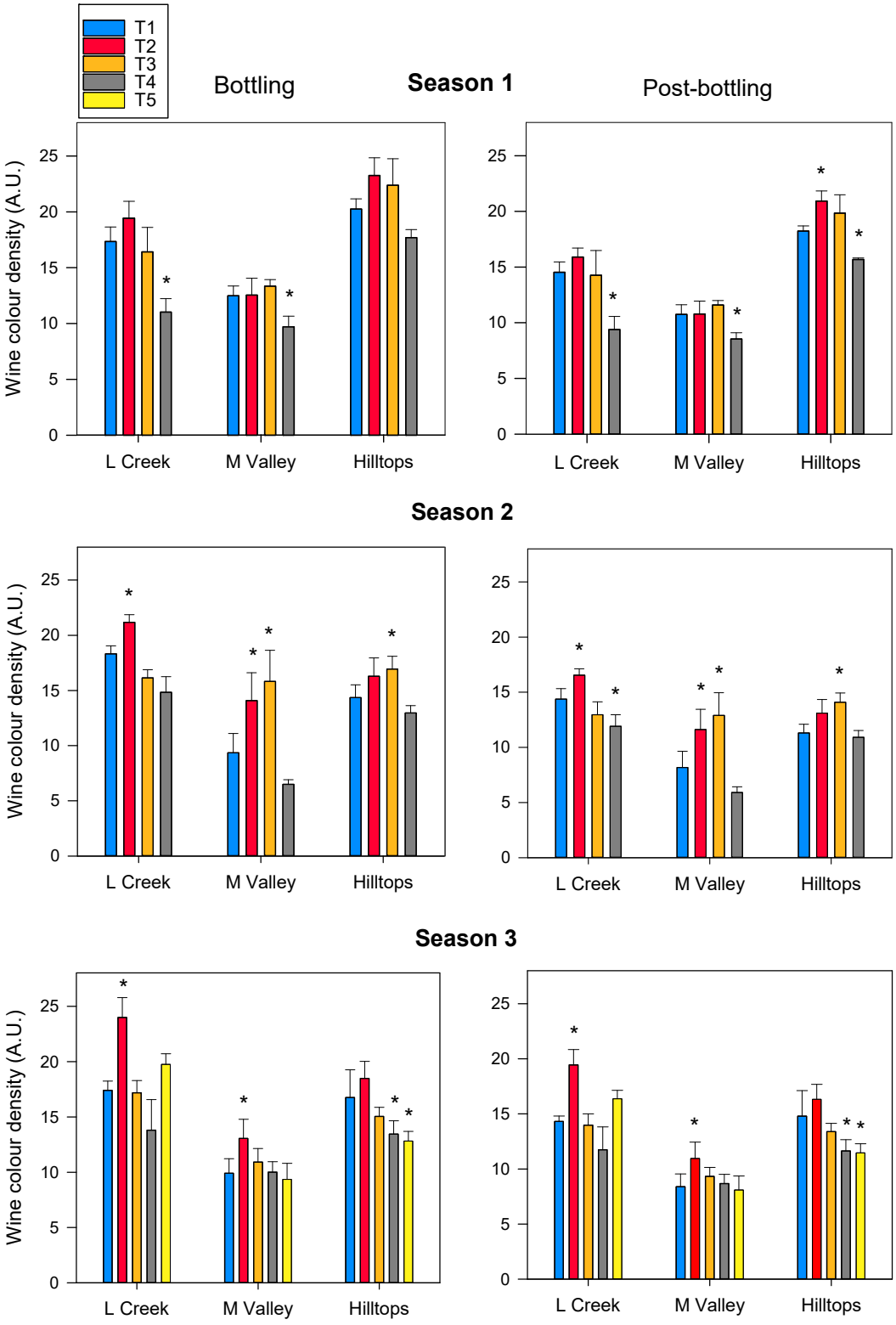


Figure 3.3 Wine colour density at bottling and 10 months post-bottling. Significant differences between treatments and control are indicated by * ($p < 0.05$). All treatment replicates significantly decreased between bottling and 10 months post-bottling ($p < 0.05$), except T2 H-S1, T4 LC-S1 and T3 MV-S2. Error bars represent the standard deviation (n=4).

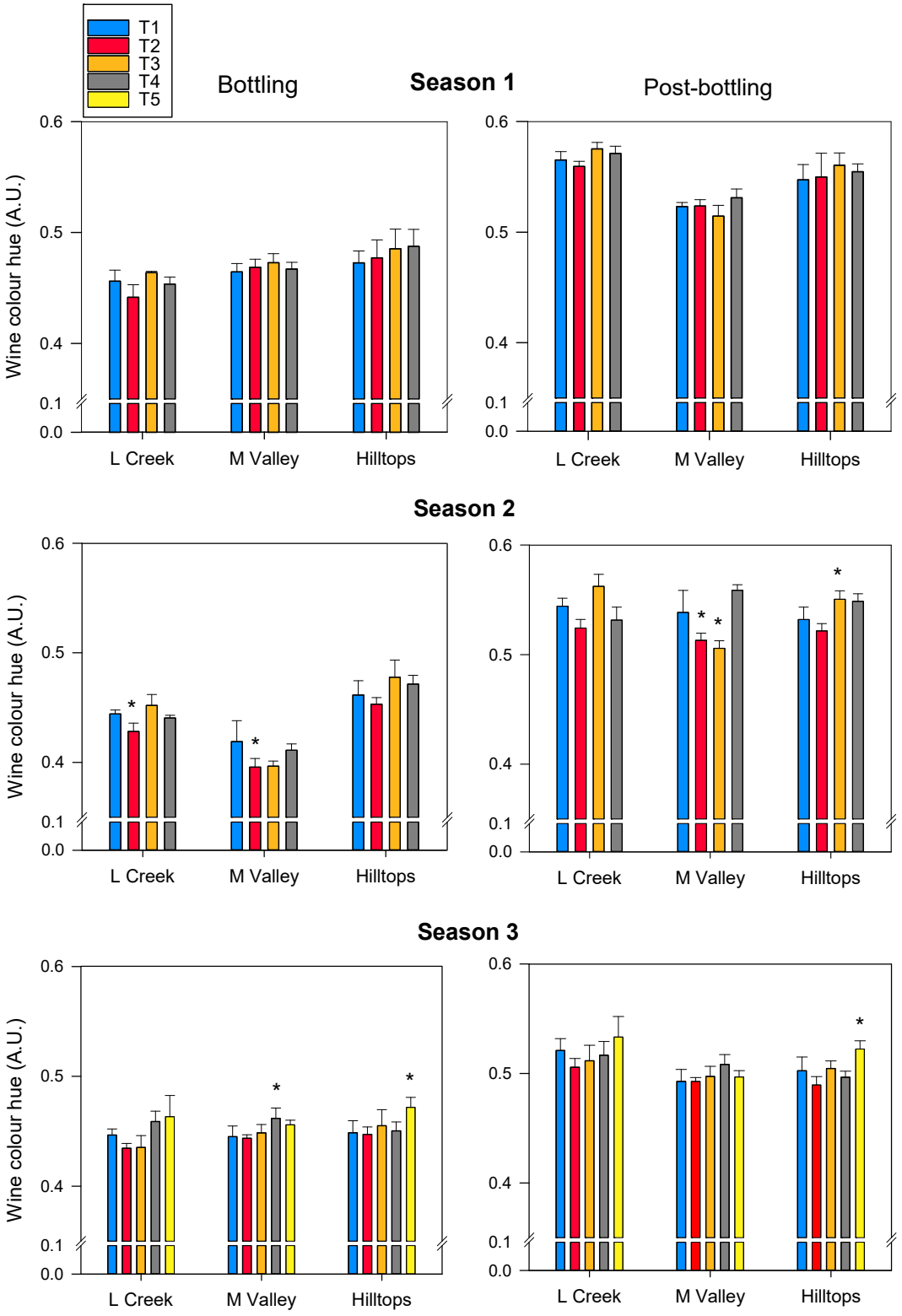


Figure 3.4 Wine colour hue at bottling and 10 months post-bottling. Significant differences between treatments and control are indicated by * ($p < 0.05$). All treatment replicates significantly increased between bottling and 10 months post-bottling ($p < 0.05$). Error bars represent the standard deviation (n=4).

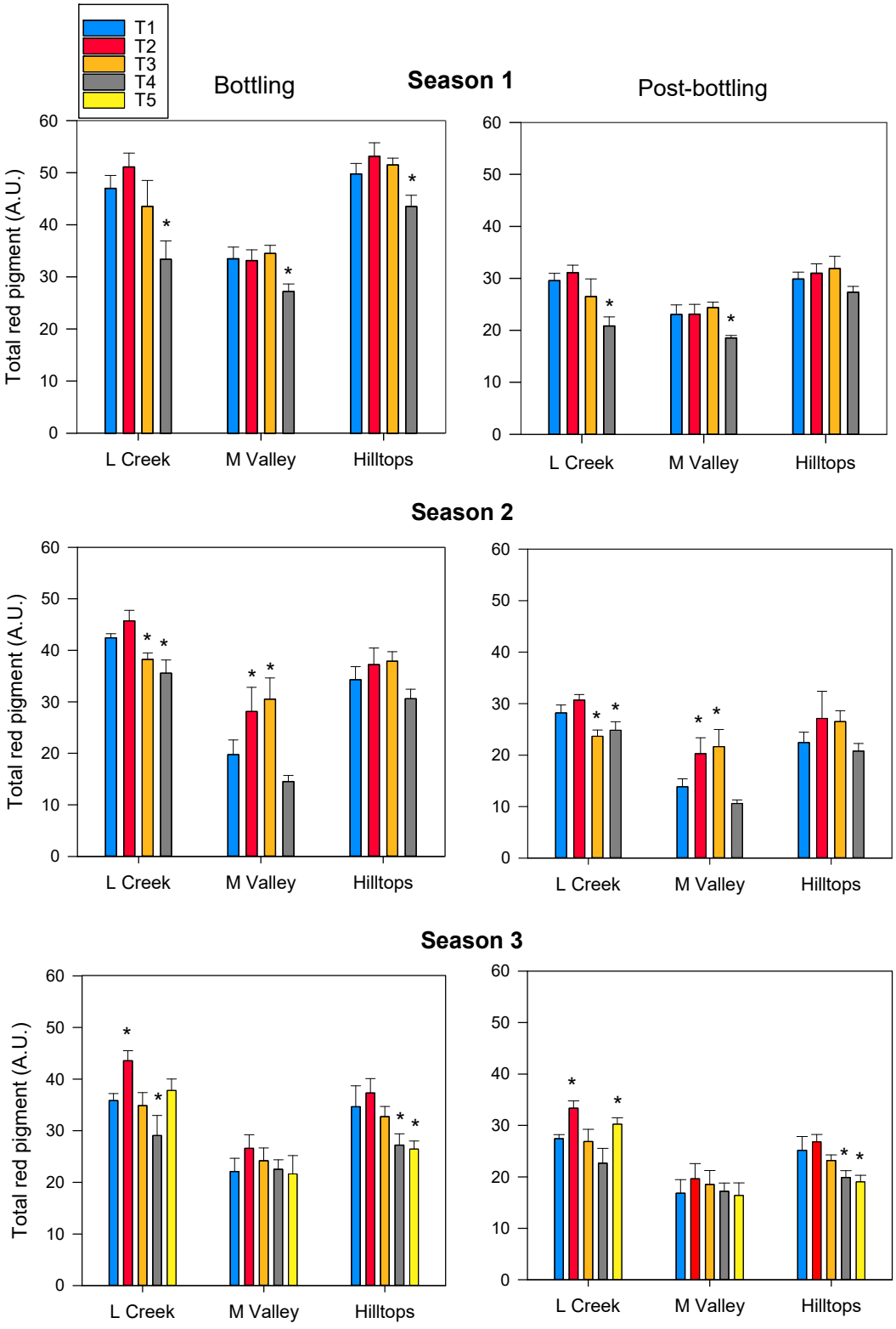


Figure 3.5 Total red pigments at bottling and 10 months post-bottling. Significant differences between treatments and control are indicated by * ($p < 0.05$). All treatment replicates significantly decreased between bottling and 10 months post-bottling ($p < 0.05$), except T2 and T5 H-S3. Error bars represent the standard deviation (n=4).

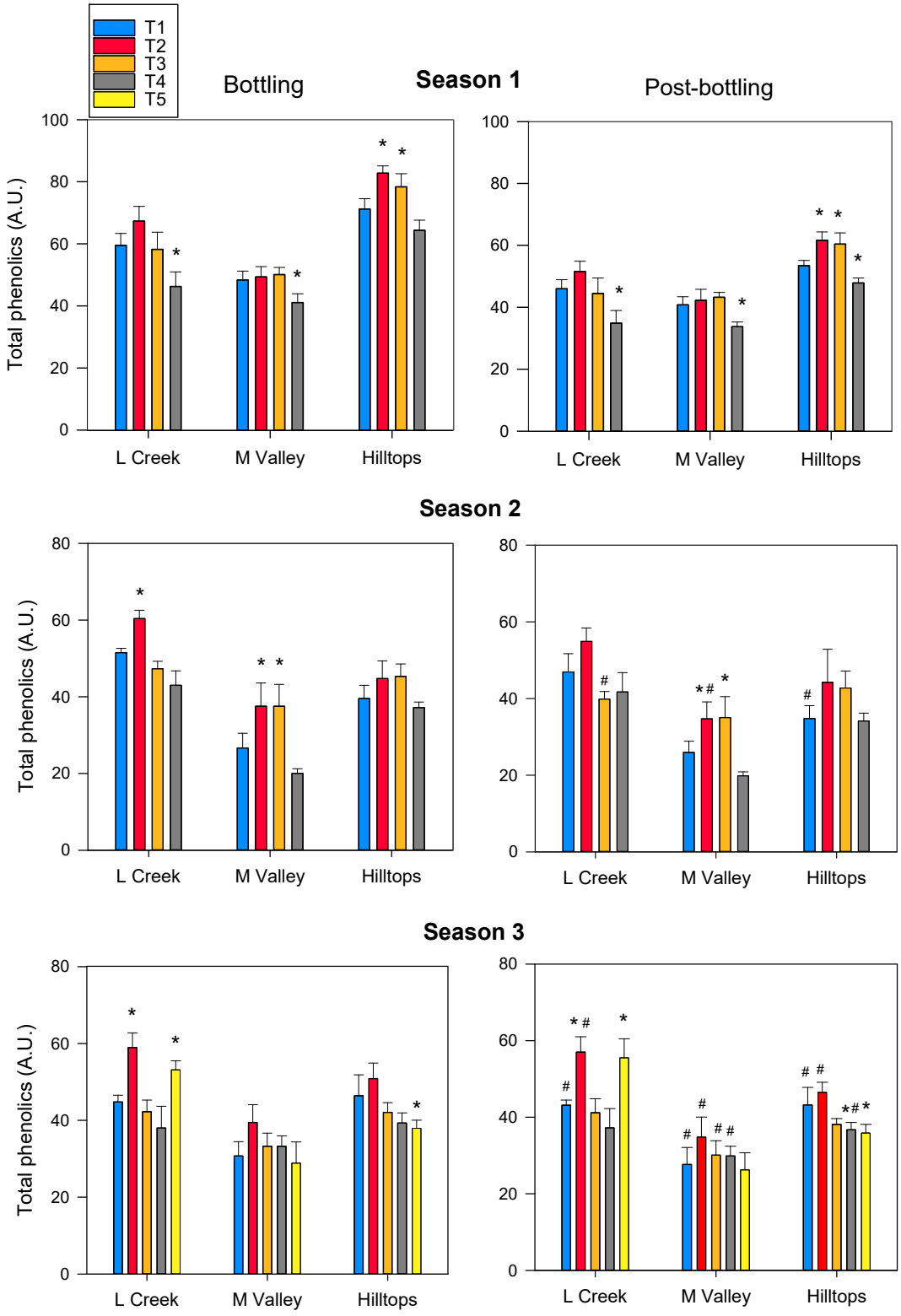


Figure 3.6 Total phenolics at bottling and 10 months post-bottling. Significant differences between treatments and control are indicated by * ($p < 0.05$). Differences in treatment replicates between bottling and 10 months post-bottling were observed for all wines in S1 and as indicated by # in S2 and S3 ($p < 0.05$). Error bars represent the standard deviation (n=4).

no longer different to the control. Lower total phenolics in T4 wines were conserved after 10 months in bottle, with H-S1/3 also both exhibiting lower phenolics upon ageing.

Among the four parameters described above, the main treatment impact was observed for T4 with lower total red pigments at bottling; the treatment effect however diminished upon ageing. Wine colour density was also most impacted by T4 (lower), and T2 (higher), especially after 10 months in bottle. The most notable treatment correlation between parameters was observed for T4, with five out of the six wines displaying lower total red pigments at bottling exhibiting lower wine colour density 10 months post-bottling. Total phenolics and wine colour hue were largely unaffected by treatments.

3.3.2.2 Wine tannins - Tannin are known to vary with seasons and regions (Smith, Mercurio, Dambergs, Francis, & Herderich, 2008) and this was observed in this project. Concentrations varied between seasons, with season 1 exhibiting the highest concentrations, and season 2 the lowest (Appendix 5.2C and Figure 3.7). Variations were also observed between sites, although not consistently across seasons. The most significant treatment impact at bottling was that of T2 exhibiting tannin concentrations higher than the control in six out of nine wines across sites and seasons (Figure 3.7). Other treatments did not display significant effects at bottling, with only T3 and T5 wines lower in tannin concentrations at Hilltops in season 3 (Figure 3.7).

Tannin concentrations remained well correlated upon ageing (Appendix 5.2) and generally did not change, except as discussed below (Figure 3.7). Higher tannin concentrations observed for T2 at bottling were still apparent 10 months later, with LC/MV-S3 treatment replicates exhibiting increased tannins, and with LC-S2 and H-S3 also significantly higher than the control 10 months later; H-S2 was however no longer different to the control, although each replicate increased in tannins (Figure 3.7). The lower tannin concentration for T5 at Hilltops in season 3 was retained, while T3 at Hilltops in season 3 was no longer different to the control (Figure 3.7). Although T3 and T4 in MV-S3 were not significantly different to the control, each treatment replicate increased in tannins over 10 months (Figure 3.7).

It must be noted that there is debate as to whether the MCP assay measures all sizes of tannins, with dimers and trimers being questioned. In addition, the MCP assay precipitates pigmented polymers, i.e., anthocyanin-tannin adducts (Sarneckis et al., 2006). Thus as wine ages, it contains longer tannins and more pigmented polymers, which would be measured by the MCP, and would account for the increase in tannins between bottling and 10 months post-bottling analyses.

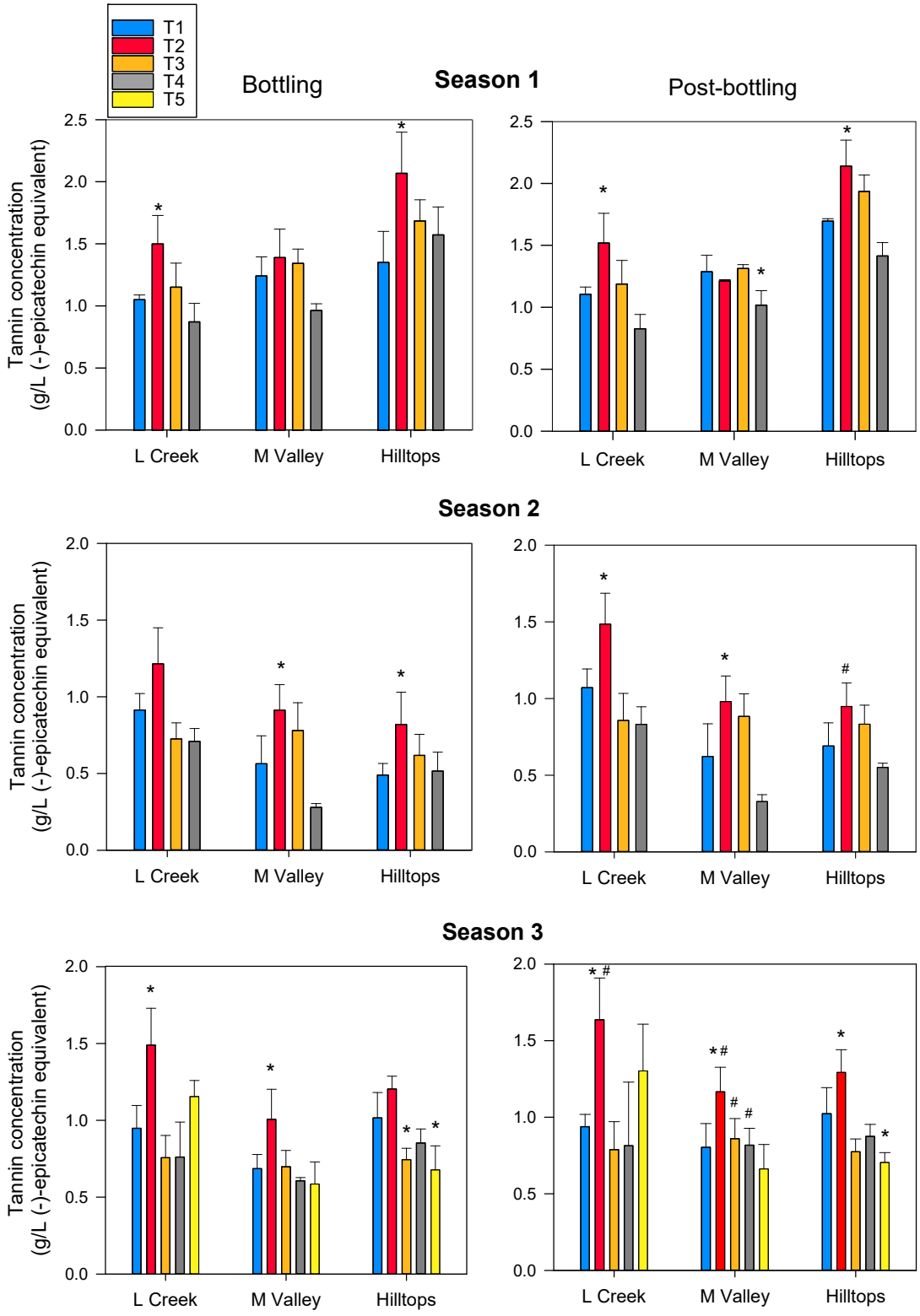


Figure 3.7 Wine tannin concentration at bottling and 10 months post-bottling. Significant differences between treatments and control are indicated by * ($p < 0.05$). Significant differences in treatment replicates between bottling and 10 months post-bottling are indicated by # ($p < 0.05$). Error bars represent the standard deviation (n=4).

3.3.2.3 Wine anthocyanins by UPLC-PDA - Total berry and wine anthocyanins were relatively well correlated at bottling and post bottling (Figure 3.8). While total berry anthocyanins and % monoglucosides exhibited a positive relationship, % malvidins were somewhat negatively correlated with total anthocyanins concentrations, and even more so with % monoglucosides (Figure 3.8). Those relationships tended to disappear in the wine and upon ageing. The amount of malvidins was proportional between berries and wines, although the range was compressed and slightly shifted from 30-75% in the fruit to 55-85% in the wine, with a slight decrease upon ageing for all replicates. Similar results were observed for the proportion of monoglucosides, from 30-75% in the fruit to 50-75% in the wines, with slightly more variations upon ageing (Figure 3.8 and Table 3.3). Those results thus highlight that the anthocyanin profile changed from the fruit to the wine but remained relatively similar upon ageing.

As mentioned above (*see section 3.3.2*), total anthocyanins were largely impacted by site, with MV exhibiting lower concentrations in berries and wine (Table 3.3). Interestingly, the proportions of monoglucosides and malvidins were also impacted by site, with MV exhibiting the lowest proportion of monoglucosides and the highest proportion of malvidins. Seasons did not seem to impact on those parameters.

Total anthocyanins and proportions were also affected by treatments to a small extent. In berries, T4 had consistently lower total anthocyanins but this was only significant in four cases out nine. In general, T2, T3 and T5 did not exhibit significant or consistent differences (data not shown). Differences in proportion of monoglucosides and malvidins were inconsistent across seasons and sites. In wines at bottling (Table 3.3), T2 generally exhibited increased anthocyanin concentrations although there was only one significant difference in berries (H-S1), while T4 was lower, consistent with berry results in most cases. T5 wines also tended to be lower while no differences were observed in berries. The total anthocyanin content of T3 wines varied depending on the season or site. Treatments did not seem to consistently impact the proportion of monoglucosides and malvidins. Total anthocyanin concentrations halved over 10 months in bottle and differences observed at bottling were still apparent upon ageing (Table 3.3).

Differences between fruit and wine observed for T2 and T5 could be explained by variations in extraction due to berry skin thickness, cell death, remodelling of cell wall and/or location of anthocyanins at the cellular level, as has been reported for tannins (Cadot, Chevalier, & Barbeau, 2011). However, no measurements were conducted on berry physiology to prove those hypotheses in this project. In addition, T2 and T4 were found to impact on the extractability of total anthocyanins, monoglucosides and malvidins (T2 only) compared to T1.

Differences between bottling and post bottling could be explained by different reactivities of anthocyanin forms and/or families as they form adducts with fermentation by-products and other phenolic compounds, including tannins, upon ageing (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Reactions of anthocyanins are supported by the observed decrease in free anthocyanins measured in the wine. In addition, the production of new pigments such as pyranoanthocyanins, visitins and co-pigments with tannins may contribute to colour stabilisation and 'weakening' of treatment differences observed 10 months after bottling.

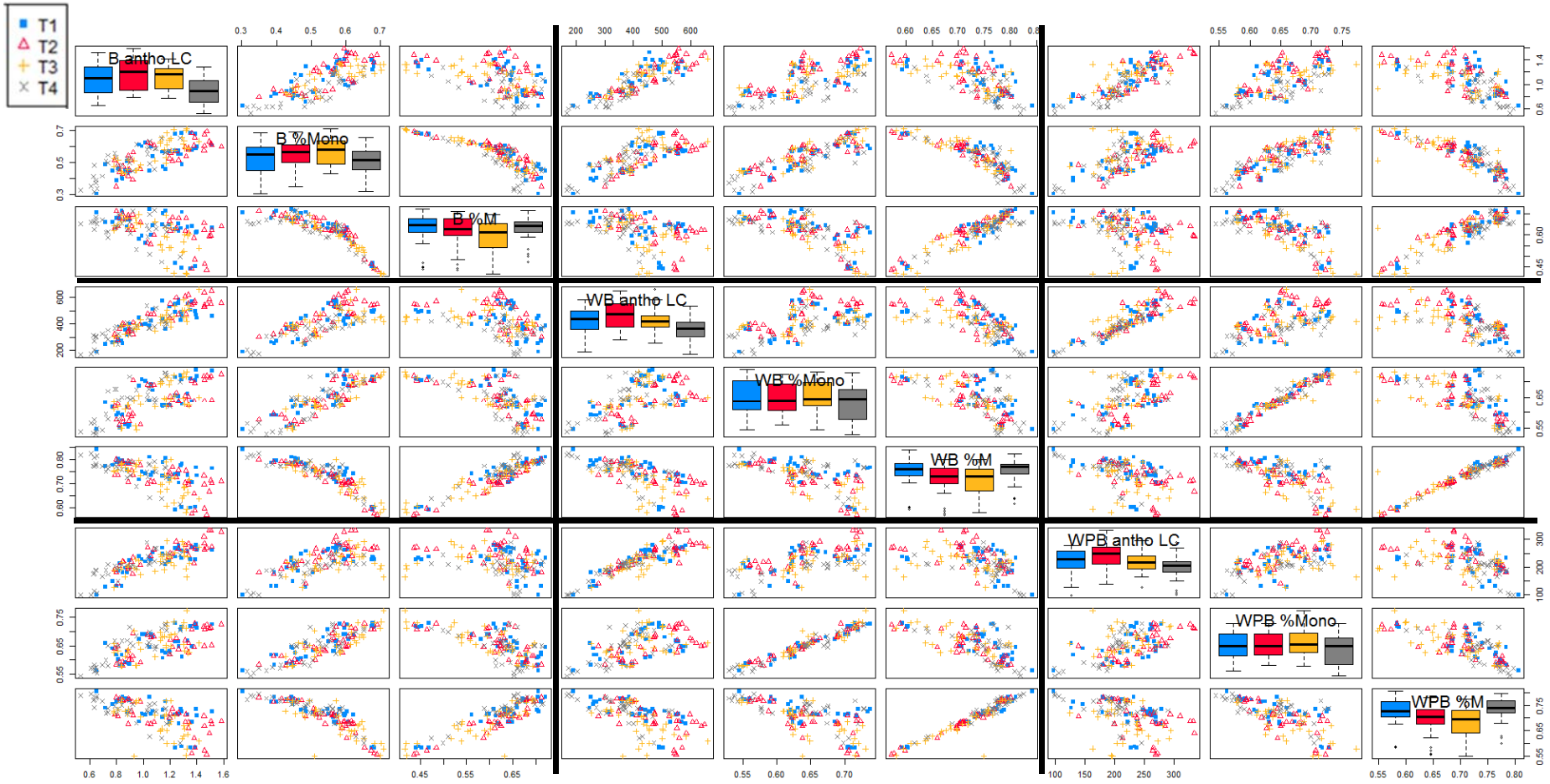


Figure 3.8 Scatterplot matrices with boxplots (diagonal) for berry (B) and wine at bottling (WB) and 10 months post-bottling (WPB) for total anthocyanins (antho LC), % monoglucosides (% Mono) and % malvidins (% M) measured by UPLC-PDA per treatments. T5 was not in included as wine data were only collected for one season.

Table 3.3: Wine anthocyanins measured by UPLC-PDA. Errors are given as standard deviations (n=4). Significant differences (in bold) are indicated by different letters ($p < 0.05$).

			Bottling			Post bottling		
			Total antho (mg/L)	**% Monoglucosides**	**% Malvidins**	**Total antho (mg/L)**	**% Monoglucosides**	**% Malvidins**
Season 1	**L Creek**	T1	542 ± 21 bc	66.0 ± 0.9 b	74.0 ± 0.5 a	261 ± 7 b	66.9 ± 0.8 a	71.7 ± 0.7 a
		T2	594 ± 29 c	65.5 ± 0.5 ab	72 ± 2 a	277 ± 19 b	66.9 ± 0.7 a	70 ± 2 a
		T3	486 ± 47 b	**64 ± 2 a**	72.8 ± 0.8 a	**221 ± 26 a**	66 ± 3 a	70 ± 2 a
		T4	**401 ± 35 a**	64.9 ± 0.6 ab	74 ± 1 a	**215 ± 16 a**	67 ± 1 a	73 ± 2 a
	M Valley	T1	373 ± 19 b	55.6 ± 0.4 bc	78.3 ± 0.2 a	208 ± 13 b	58.6 ± 0.6 bc	77.3 ± 0.3 a
		T2	381 ± 23 b	57 ± 1 c	78.2 ± 0.3 a	209 ± 9 b	59.5 ± 0.4 c	77.1 ± 0.5 a
		T3	381 ± 15 b	54.9 ± 0.5 b	77.8 ± 0.5 a	208 ± 18 b	58.0 ± 0.1 ab	76.6 ± 0.6 a
		T4	**311 ± 9 a**	**54 ± 1 a**	77.5 ± 0.4 a	**179 ± 11 a**	**57.3 ± 0.8 a**	77.5 ± 0.8 a
	Hilltops	T1	563 ± 24 a	63 ± 1 a	72 ± 1 bc	260 ± 26 a	64 ± 1 a	69 ± 1 bc
		T2	599 ± 50 a	63 ± 1 a	69 ± 2 ab	253 ± 32 a	64 ± 1 a	66 ± 2 ab
		T3	572 ± 68 a	64 ± 1 a	**66 ± 2 a**	252 ± 16 a	64 ± 2 a	**63 ± 2 a**
		T4	509 ± 30 a	63 ± 1 a	74.6 ± 0.7 c	268 ± 17 a	64 ± 1 a	72 ± 1 c
Season 2	**L Creek**	T1	494 ± 7 b	70.9 ± 0.5 ab	60.0 ± 0.5 a	232 ± 5 b	72.0 ± 0.6 ab	58.4 ± 0.2 a
		T2	**551 ± 13 c**	71 ± 1 ab	58 ± 1 a	**268 ± 4 c**	72 ± 1 ab	57 ± 1 a
		T3	**437 ± 17 a**	72 ± 1 b	60 ± 2 a	**194 ± 22 a**	74 ± 3 b	58 ± 2 a
		T4	**426 ± 36 a**	69 ± 1 a	**65 ± 3 b**	217 ± 20 ab	70 ± 1 a	**63 ± 3 b**
	M Valley	T1	244 ± 37 a	58 ± 2 ab	80 ± 2 b	135 ± 34 a	60 ± 3 ab	78 ± 2 b
		T2	**340 ± 39 b**	58.6 ± 0.9 b	79.3 ± 0.6 b	**192 ± 20 b**	60 ± 2 ab	77.2 ± 0.8 b
		T3	**377 ± 47 b**	**62 ± 1 c**	**75 ± 1 a**	**183 ± 18 b**	65 ± 4 b	**67 ± 8 a**
		T4	182 ± 16 a	55 ± 1 a	81.7 ± 0.7 b	105 ± 7 a	56 ± 1 a	79 ± 1 b
	Hilltops	T1	416 ± 24 ab	69.6 ± 0.9 b	74 ± 1 b	228 ± 8 b	68.6 ± 0.9 b	71 ± 2 bc
		T2	459 ± 32 b	69.3 ± 0.8 b	71 ± 2 ab	**252 ± 18 c**	68.6 ± 0.7 b	69 ± 2 ab
		T3	440 ± 20 b	69.7 ± 0.6 b	71 ± 1 ab	240 ± 4 bc	68.0 ± 0.7 b	**67 ± 2 a**
		T4	367 ± 12 a	**67.4 ± 0.3 a**	**77.6 ± 0.3 c**	**205 ± 7 a**	**66 ± 1 a**	74 ± 1 c
Season 3	**L Creek**	T1	449 ± 17 ab	73 ± 1 ab	76 ± 2 b	266 ± 9 ab	72 ± 1 ab	73 ± 1 b
		T2	**555 ± 25 c**	71.7 ± 0.5 a	**71 ± 3 a**	**326 ± 11 c**	71.7 ± 0.7 ab	68 ± 3 ab
		T3	466 ± 45 b	71 ± 1 a	**67 ± 2 a**	272 ± 34 b	72 ± 1 a	**65 ± 2 a**
		T4	376 ± 43 a	72.3 ± 0.6 a	76 ± 3 b	218 ± 23 a	72 ± 1 ab	73 ± 4 b
		T5	454 ± 38 ab	75 ± 1 b	72 ± 1 ab	246 ± 29 ab	74 ± 1 b	67.7 ± 0.2 ab
	M Valley	T1	277 ± 31 a	62 ± 1 b	78 ± 2 b	160 ± 28 a	63 ± 2 b	75 ± 2 b
		T2	324 ± 34 a	61 ± 2 b	**73 ± 2 a**	187 ± 34 a	63 ± 2 b	**71 ± 2 a**
		T3	296 ± 29 a	62 ± 1 b	75.5 ± 0.2 ab	178 ± 36 a	64 ± 1 b	72.8 ± 0.7 ab
		T4	276 ± 26 a	**58.1 ± 0.8 a**	76.3 ± 0.9 ab	172 ± 17 a	**58.9 ± 0.8 a**	73 ± 1 ab
		T5	260 ± 28 a	61 ± 1 b	77 ± 2 b	151 ± 17 a	63 ± 2 b	74 ± 2 ab
	Hilltops	T1	412 ± 47 bc	62.6 ± 0.8 a	80 ± 1 b	237 ± 21 bc	62 ± 2 a	75 ± 3 a
		T2	453 ± 25 c	63.4 ± 0.8 a	**75.7 ± 0.9 a**	253 ± 13 c	63 ± 1 ab	72 ± 1 a
		T3	380 ± 25 ab	64 ± 1 a	79 ± 2 b	218 ± 25 bc	64 ± 2 ab	75 ± 3 ab
		T4	356 ± 24 ab	64.3 ± 0.3 a	79 ± 0.7 b	210 ± 11 ab	64.9 ± 0.2 ab	75.8 ± 0.6 ab
		T5	**315 ± 20 a**	64.3 ± 0.1 a	**83.7 ± 0.7 c**	**174 ± 11 a**	**65.3 ± 0.7 b**	**80 ± 1 b**

3.3.3 Wine sensory assessment

3.3.3.1 Sensory evaluation - Wines were presented each year four months post-bottling to winemakers at McWilliam's Wines, Griffith, to allow regular discussion with Industry partners and conduct sensory assessment. While the sensory assessment panel was too small (3-5) to provide strong statistical results, panellists were experienced tasters, and it highlighted that preference was most correlated with tannin texture, itself strongly correlated with tannin maturity and intensity, as well as dark fruit. Red fruit however was negatively correlated with dark fruit and stewed fruit (data not shown).

Overall, T2 wines were the most different to the control, exhibiting more dark fruit characters and increased tannin texture, intensity and maturity, and were generally preferred (Figure 3.9). T3 wines were similar to T1, except in Murray Valley, season 2, where it exhibited the same characters as T2 wines and was preferred (Figure 3.9). T4 wines scored similarly or lower than the control (LC/MV-S1/S2, Figure 3.9), showing less developed characters (less stewed fruit and more red fruit). T5 did not exhibit any consistent impact on season 3 wines.

There was no consistent correlation between differences in sensory attributes and must (Table 3.1) or wine parameters (Table 3.2), highlighting that sensory differences were due to treatments and not slight variations in fruit ripeness or winemaking. On the other hand, the increased tannin texture, intensity and maturity observed for T2 wines (Figure 3.9) was in accordance with higher tannin concentrations measured in those wines (Figure 3.7).

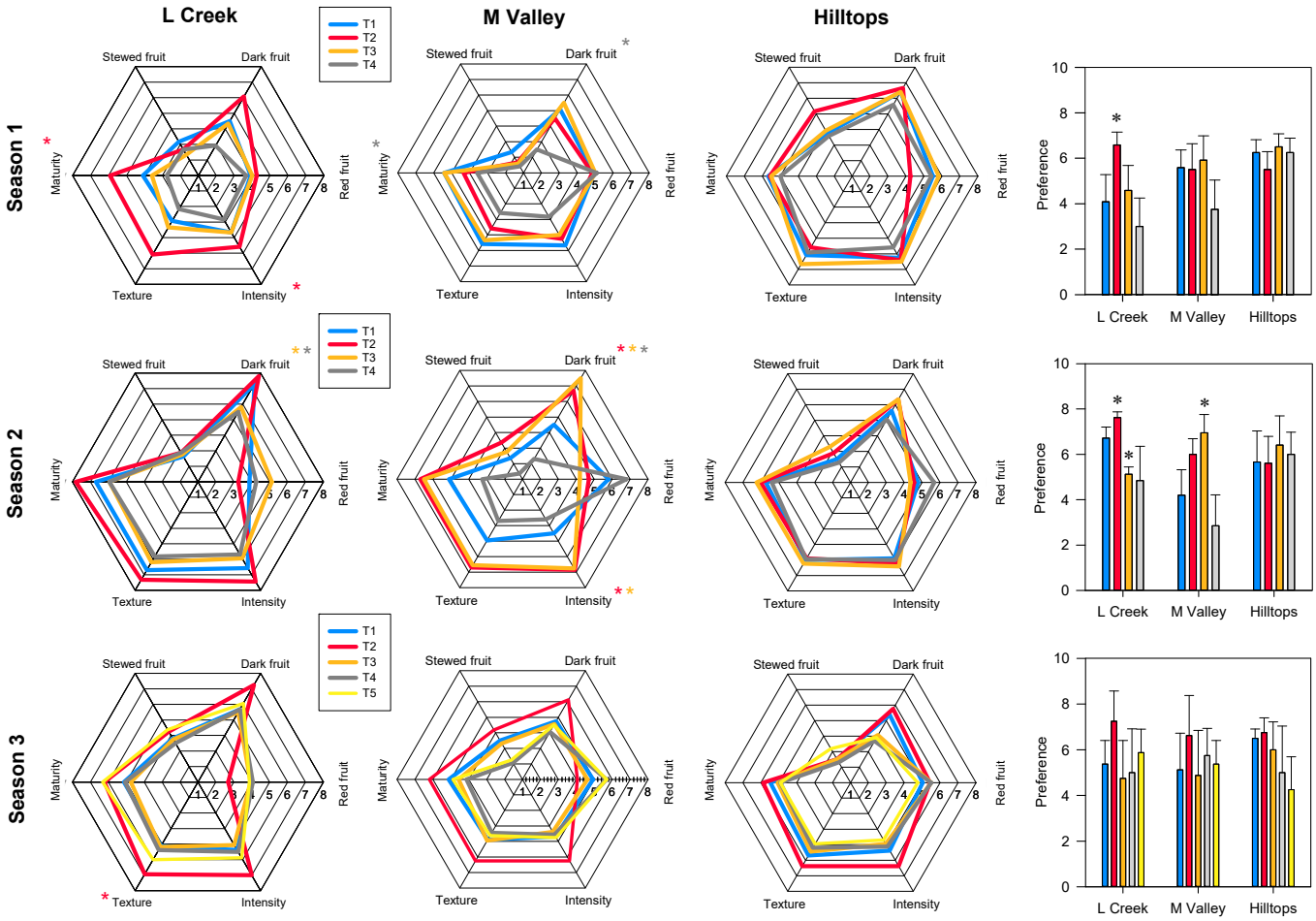


Figure 3.9 Wine sensory assessment plots and preferences. Significant differences between treatments and control are indicated by * ($p < 0.05$). Error bars represent the standard deviation (n=4).

3.3.3.2 Difference testing - Difference testing was conducted on the wines 10 months post-bottling together with Industry standard ('colour/phenolics' and tannins) and UPLC-PDA (anthocyanins) analyses (see section 3.3.2).

Wines that were found different were generally wines that exhibited increased dark fruit and tannins at the four months post-bottling sensory assessment (T2: LC-S2, LC/MV-S3, T3: MV-S2, Table 3.4 and Figure 3.9). Differences in Hilltops wines were for different treatments each year and did not correlate with any particular attribute from the four-month post-bottling sensory assessment. In line with the four months post-bottling sensory assessment, T5 was found different at all sites, although no correlations with particular attributes were observed (Figure 3.9). Panellists anecdotally reported T5 as greener/herbaceous. It must be noted that whether a wine was found different or not was most often determined by one or two panellists, except for T2 LC-S3 and T5 H-S3, both significantly different to the control at *p<0.01*, and T2 MV-S1, T3 H-S1 and MV-S3, and T4 across all sites in season 3 and Murray Valley in season 1, all clearly similar to the control (Table 3.4).

Table 3.4: Triangle tests post-bottling. Significant differences are indicated by * ($p < 0.5$), ** ($p < 0.1$) and *** ($p < 0.01$); NS: not significant.

Treatment Comparison		L Creek		M Valley		Hilltops	
Season 1	T2 vs T1	12/26	NS	4/26	NS	14/26	S*
	T3 vs T1	11/26	NS	12/26	NS	4/26	NS
	T4 vs T1	13/26	NS	7/26	NS	12/26	NS
Season 2	T2 vs T1	12/23	S*	12/24	NS	11/24	NS
	T3 vs T1	10/23	NS	14/24	S*	11/24	NS
	T4 vs T1	11/23	NS	10/24	NS	13/24	S*
Season 3	T2 vs T1	19/27	S***	14/27	S*	11/25	NS
	T3 vs T1	13/27	NS	7/27	NS	13/25	S*
	T4 vs T1	7/27	NS	9/27	NS	9/25	NS
	T5 vs T1	15/27	S*	14/27	S*	18/25	S***

3.3.4 Overall wine differentiation and correlations with vine balance

An ANOVA-PCA (Principal Component Analysis) was run on the wine phenolic parameters presented above. ANOVA-PCA combines the use of matrix decomposition by ANOVA and by PCA and allows to overcome individual's variability that is larger than that of treatments and which would otherwise masks treatment effects in a regular PCA (Bouveresse et al., 2011; Harrington et al., 2005). As highlighted above and in Chapter 2, site and/or season variability was evident in all measured parameters and a PCA run on the data resulted in site and season separation only. However, an ANOVA-PCA (Figure 3.10) highlighted treatment separation.

To perform the analysis, four factors were chosen: season, site, treatment and replicate, the latter also selected as a factor as the block layouts were the same every year and some variation between samples could be due to the location in the vineyard. The data was also centred-reduced prior to analysis. As expected, biplots of individuals for the factors 'site' and 'season' showed a clear vintage and terroir effect (data not shown). On the plot of scores and biplot of individuals per treatments (Figure 3.10A and C), T2 and T4 were clearly distinct from the control (T1) in opposite directions along PC1 when plotting the ellipse (95% confidence).

Separation was driven by a general increase in all phenolic parameters and a decrease in colour hue for T2 and vice-versa for T4. T3 was close to T1 and the treatment with the most spread data points, reinforcing the previous observations that T3 yielded inconsistent results. PC2 was mainly driven by colour hue and also total tannins but did not separate the treatments (Figure 3.10B-C). T5 was not included in the ANOVA-PCA as the statistical analysis requires a balanced design and hence data for all seasons, however wine was only made in season 3 for T5.

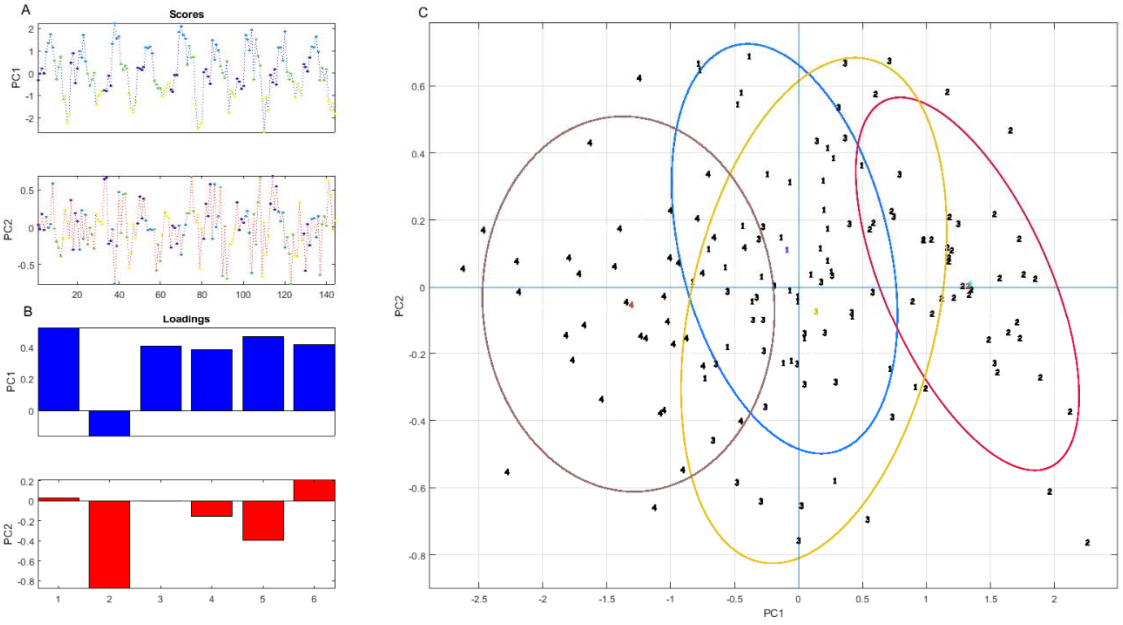


Figure 3.10 ANOVA-PCA applied to wine phenolic parameters at bottling and results showing scores on PC1 & PC2 (A), loadings (1: colour density, 2: colour hue, 3: total red pigments, 4: total phenolics, 5: total tannins, 6: antho LC) on PC1 & PC2 (B), and biplot of individuals for the factor "treatment" (C).

The ANOVA-PCA was thus successfully used to remove the effect of site and season and extracted a consistent effect of both leaf removal treatments at each site and in each season on wine phenolic composition at bottling. The same analysis was performed 10 months post bottling (data not shown), however treatment differences were reduced upon ageing.

Aside from treatment effects, correlations between vine balance (VB) and each parameter mentioned above (must, berry and wine composition, sensory) were tested using pairwise linear correlation coefficients. The calculated Pearson's coefficients (r values) between each pair of parameters are represented in the matrix heatmap (Figure 3.11), where negative correlations are blue and positive correlations are red. Weak correlations were observed between sensory parameters and vine balance as well as pH must, berry tannins (B tannin), and wine colour hue post bottling (WPB CH). The highest positive correlations for vine balance were with wine % malvidins at bottling (WB %M, r = 0.4659) and post-bottling (WPB %M, r = 0.4662), while wine total red pigments post-bottling was the most negatively correlated (WPB TRP, r = -0.6858) with vine balance (Figure 3.11). Thus, this parameter was chosen to illustrate the possible relationship between vine balance and wine colour parameters in general, as they were all correlated together, except colour hue (*see section 3.3.2*).

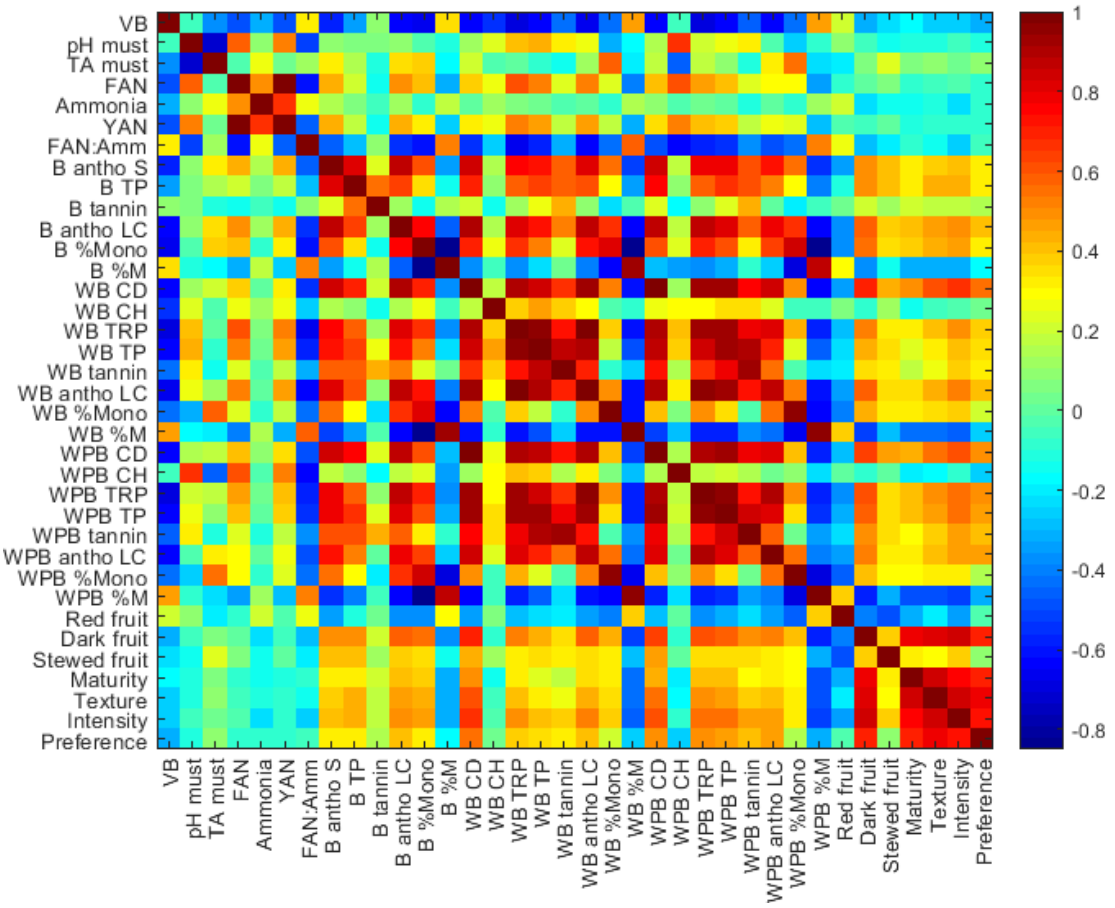


Figure 3.11 Correlation matrix heatmap of Pearson's r values obtained by pairwise comparison of all must, berry and wine parameters presented in this Chapter.

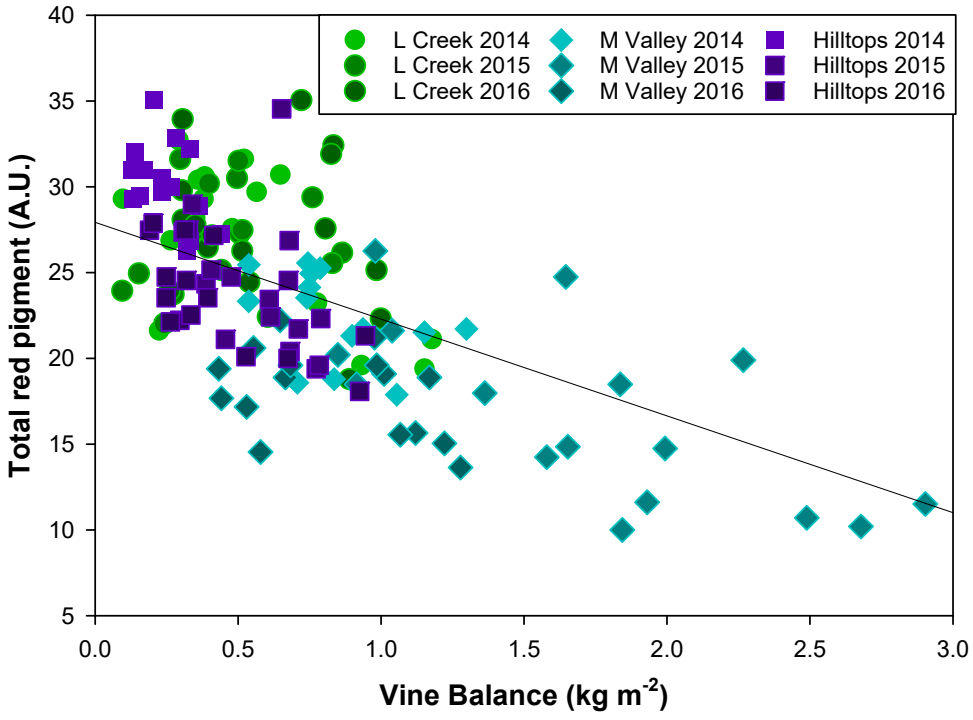


Figure 3.12 Relationship between wine total red pigments 10 months post-bottling and calculated vine balance.

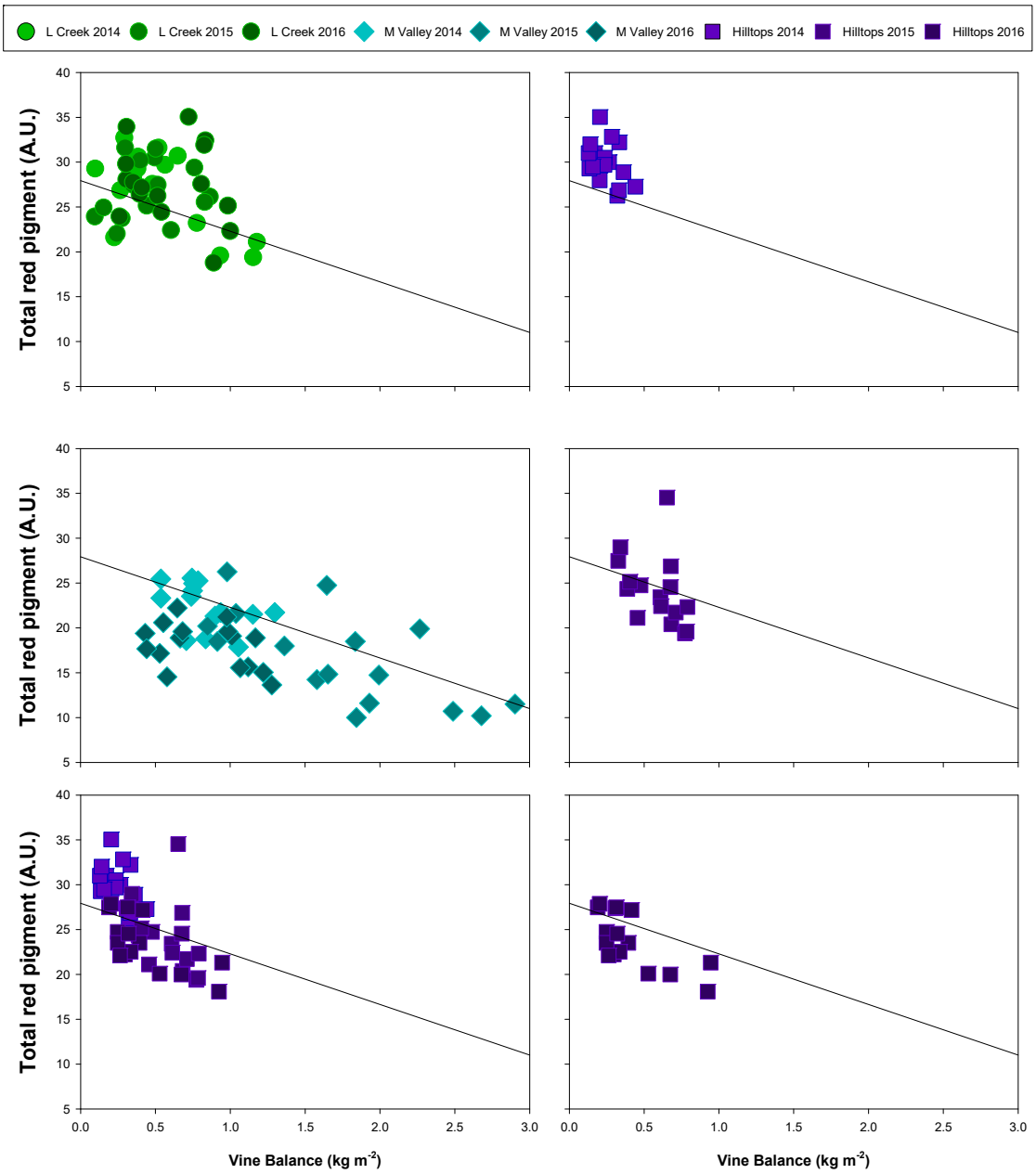


Figure 3.13 Relationship between wine total red pigments 10 months post-bottling and calculated vine balance for subsets of the total data set: Langhorne Creek (top left), Murray Valley (centre left), Hilltops (bottom left), Hilltops 2013/14 (top right), Hilltops 2014/15 (centre right) and Hilltops 2015/6 (bottom right). Data are means of four wine replicates.

The correlation between wine total red pigments post-bottling and vine balance (Figure 3.12) was stronger than that observed for total anthocyanins in the fruit with an adjusted r^2 value of 0.4734. However, as observed for the fruit, the relationship was largely driven by sites (MV) and seasons (Figure 3.13).

3.4 Conclusions

As observed in the fruit, treatments impacted on the wine, especially T2 (early defoliation), with increased colour/anthocyanins and tannins, and T4 (late defoliation), with decreased colour/anthocyanins. T3 (crop removal) mostly had no effect or inconsistently, if any. T5 (minimal pruning) was also inconsistent, most likely as the treatment was only applied for two

seasons and such a drastic canopy management practice can require several seasons until vines re-establish a stable production pattern. Interestingly, differences observed for treatments seemed to be more apparent in the wine than in the fruit, with the extraction of anthocyanins from the fruit to the wine impacting on the proportions of monoglucosides and malvidins (also extracted differently for T2 and T4). While some colour/phenolic parameters were more impacted at bottling and others 10 months post-bottling, the highest correlation with vine balance was obtained for total red pigments 10 months post-bottling. However, when looking at the effect of vine balance, any relationship with the wine parameters was, similar to the fruit, mainly driven by site and season. Regardless, a mechanistic understanding of the treatment impacts was furthered in Chapter 4, where carbohydrate supply was manipulated without affecting canopy to separate carbohydrate supply/vine demand from bunch microclimate.

Chapter 4

The effect of targeted carbohydrate supply manipulation on berry composition and relationship between key metabolites.

4.1 Background

When direct adjustments to crop load are made with field grown grapevines, one of the main underlying effects is on the internal relationship between photosynthetic carbohydrate supply and the carbohydrate requirement for ripening fruit. If the amount of fruit on the vine is reduced during the berry development period, then the relative availability of carbohydrates will be higher for the remaining fruit. This will typically have the effect of increasing the rate of berry sugar accumulation, and for varieties known for over cropping, or in unusually high yielding seasons, crop thinning may be used to ensure adequate ripening or improve quality parameters (Petrie and Clingeleffer 2006). Conversely, if sufficient leaf area is removed to reduce canopy light interception, the supply of carbohydrates is reduced relative to the fruit demand and potentially slows the rate of berry sugar accumulation. This approach offers a possible method for countering climate change induced increases in ripening rate and berry sugar content (e.g. Stoll et al., 2013).

In addition to possible effects on the rate of berry sugar accumulation, crop load can also influence other berry compositional parameters. There is a general perception that low yield is required for high quality wine, and that fruit and wine quality from more heavily cropped vines will be inferior. While there are examples from the scientific literature that either support or contradict this view (Matthews 2015), a link between the rate of berry sugar accumulation and the production of secondary berry metabolites would be expected. However, a question that remains unclear is the extent to which carbohydrate supply can uncouple, or modify the relative accumulation of various primary and secondary metabolites. Flavonoids, with anthocyanins as an important example for red grape composition, are derived from a metabolic pathway that utilises an estimated 20% of a plant's assimilated carbon (Haslam, 1993). Anthocyanin production may therefore be directly linked to the supply of carbohydrates to the ripening fruit, and thus vary proportionally. Alternatively, gene regulation may respond to limited carbohydrate or precursor supply such that the important primary metabolites take priority over the metabolically expensive secondary metabolites.

A number of aspects of berry secondary metabolism are also known to be influenced by light and temperature, so any adjustment in crop load that changes bunch exposure may also modify berry composition. Some examples include anthocyanin profile responses to light and temperature (Spayd et al., 2002) and quercetin responses to light in both red and white grape varieties (Downey et al., 2004, Friedel et al., 2016). For more direct adjustments of vine balance within a season, removing leaves, shoot trimming or shoot thinning may alter both fruit exposure and carbohydrate balance. For longer term adjustments of vine balance that will span many seasons, such as pruning practice, nutrition or irrigation, both parameters may also be varied, but it becomes even more difficult to attribute resulting fruit composition responses. Although this is the production system grape growers and winemakers have to work with, understanding how carbohydrate supply modifies fruit composition, and making

a distinction from exposure effects, would provide a more informed basis for more targeted management practices.

If carbohydrate supply does not change the relationship between accumulation of sugars and secondary metabolites of interest, then the duration of the ripening period rather than yield *per se* becomes the most important consideration. Likewise, if fruit exposure is the main influence on composition, there may be opportunities to increase yield without detriment to quality, providing light conditions within the canopy remain favourable. Conversely, if carbohydrate supply does modify the relationship between berry sugar accumulation and secondary metabolites, then managing the yield or internal carbohydrate supply and demand balance becomes a more critical factor for regulating fruit composition. However, from a research perspective, a difficulty in addressing these questions is that effects of carbohydrate supply and fruit exposure are not easy to fully separate in the field. Where the timing of berry development events is also important, such as tannin gene expression after flowering and anthocyanins at véraison, it is also difficult to specifically manipulate carbohydrate balance within the vine for short periods.

To investigate the specific influence of carbohydrate supply on berry sugar accumulation, and examine subsequent effects on other grape and wine quality parameters, a more controlled experimental system was therefore devised to manipulate whole vine carbohydrate relations by reducing the supply of CO_2 using an enclosed chamber system. These chambers were designed to fit large pot-grown grapevines that were planted several years prior to the start of the project. A series of experiments was then undertaken over a period of three season to target different stages of berry development. The first objective was to reduce whole vine photosynthesis, and reduce the rate of berry sugar accumulation without changing fruit exposure. The effects on sugar transporters (Chapter 5) and other primary metabolites were also examined. The second objective was then to determine how anthocyanins and tannins, as a key secondary metabolites in red grapes, responded to changes in berry sugar accumulation. This included the profile of anthocyanins, the analysis of expression of genes involved in anthocyanin synthesis, and the final influence on wine quality following small scale fermentations.

4.2 Materials and Methods

4.2.1 Whole vine controlled CO_2 chamber system

4.2.1.1 Experimental approach - Carbohydrate production in grapevines is determined by the photosynthetic capacity of individual leaves, and the total amount of light intercepted by the canopy. The photosynthetic rate (A) is determined by the internal leaf CO_2 concentration (intercellular CO_2 concentration, or C_i), which in turn depends on external or ambient CO_2 concentration (C_a) and the stomata regulated control of gas exchange between the leaf and atmosphere. The response of A to C_i follows a well characterised relationship, where A will initially increase at a near linear rate with CO_2 concentration, and then slow as the limiting factor transitions from the carboxylation rate of RuBisCO to electron transport and the rate of regeneration of ribulose-1,5-bisphosphate. With an infrared gas analyser that controls CO_2 supply to a leaf under saturating light conditions, the photosynthetic response to CO_2 supply across a range of concentrations can be measured and plotted as an A:Ci curve (Figure 4.1).

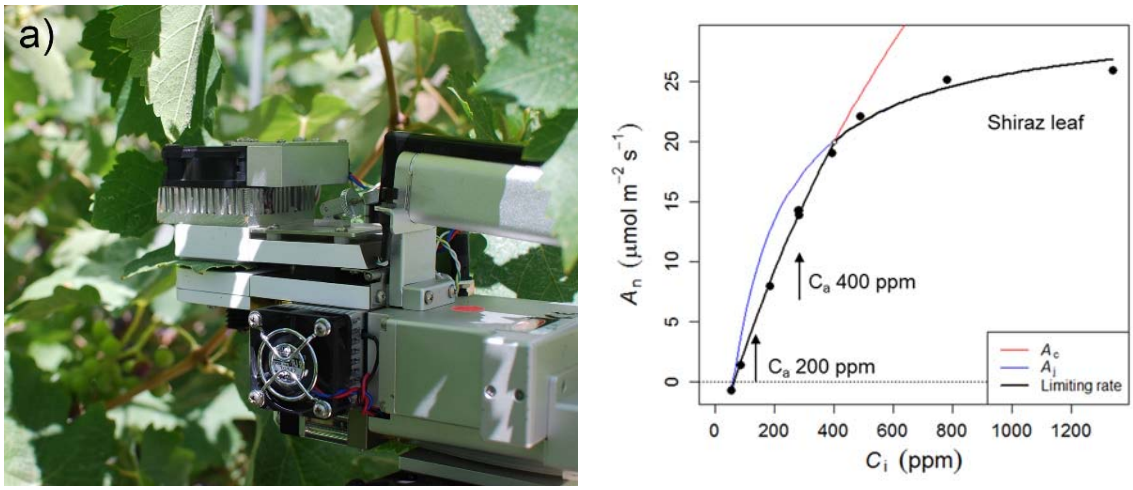


Figure 4.1 Measurement of leaf photosynthesis responses to CO_2 concentration with the Li-6400XT gas analyser (a), and (b), resulting A:Ci function fitted with Plantecophys (Duursma, 2015).

For CO_2 concentrations in the air below current atmospheric values of approximately 400 ppm, reducing CO_2 supply will cause a nearly proportional reduction in photosynthesis. In the present study, a target of 200 ppm reduction in CO_2 relative to ambient was chosen to provide an approximate halving of net canopy photosynthesis. This had the equivalent effect of doubling the fruit yield in relation to carbohydrate assimilation capacity for the period the vines were exposed to the low CO_2 treatments, but without the need to reduce canopy leaf area or change fruit exposure.

4.2.1.2 Location and planting material - The experiments described in this chapter were undertaken using two groups of 20 large mature pot-grown Shiraz vines in a bird-proof enclosure located at the National Wine and Grape Industry Centre, Charles Sturt University in Wagga Wagga (Figure 4.2). All of the vines were on own-roots, with one group of 20 (clone PT23) planted as one year old vines in 2008, and the second group of 20 (clone 1654) planted in 2009. The vines were grown in 52 L pots (500 mm wide x 380 mm high), and trained to single spur-pruned bilateral cordon on a fixed fruiting wire 80 cm from the ground. A second fixed foliage wire was positioned at 120 cm above the ground. For both groups of vines, the pots were arranged with 1 m spacing in four rows of five vines in an E-W alignment, and with a distance of 3 m between rows.

After pruning weights were collected each winter during the study period, bud numbers were adjusted to 36 per vine. However, apart from removing any shoots that arose from the trunk or would have prevented the sides of the whole vine chambers properly closing, no attempt was made to adjust the vines to specific shoot or bunch number. Average yield ranged between 3.6 and 5.5 kg vine^{-1} depending on experiment and season, of which approximately 1 kg was used for the small scale winemaking. Shoot number ranged from approximately 30 to 38 per vine, providing a sprawl canopy with similar structure and density to field grown grapevines at the Hilltops site. Longer shoots were also trimmed in early summer to ensure a subsequent fit of the canopy to the internal dimensions of the whole chambers described below.



Figure 4.2 Pictures showing group of 20 vines used for experiment in the 2013-14 season showing (a) full canopy on 23 December prior to the start of the first 'véraison' experiment, and (b) after leaf-fall on 30 May.

The pots were filled with a commercial bulk composted potting mix, and fertilised manually with diluted liquid fertiliser (Megamix Plus®, RUTEC, Tamworth) at approximately eight weekly intervals to provide a total of 25 g N per vine per season. Additional applications of magnesium sulfate were made at a rate of approximately 2 g per vine per season, and 10 g of gypsum was applied to the soil surface over winter to provide a source of calcium. For irrigation, a 2 L hr^{-1} pressure compensated emitter was installed on each side of the trunk to provide water to two sides of the root system. Irrigation was controlled by an automated timer, with the schedule and duration of 3 - 4 daily irrigations adjusted manually according to seasonal water requirements. This was based on visual observations at 2 - 3 day intervals, or daily while experiments were running, with the aim of ensuring that the pots were, on average across the time period, just returned to field capacity overnight. During rainfall or low demand days some drainage did occur, and on days with very high evaporative some additional manual watering was undertaken if needed.

4.2.1.3 Gas exchange chambers - For the measurement of whole vine photosynthesis and transpiration, and to provide an enclosed environment around the vine to allow CO_2 supply concentrations to be modified, six whole vine gas exchange chambers were constructed (Figure 4.3). These consisted of a transparent acrylic top with a lightweight aluminium frame and volume of 1.2 m^3, and base made from polyethylene plastic panels and galvanised steel frame. The chamber top and base were both designed in two halves that could be installed from each side of the trellis. Air intake ports, which were located under the fans of each chamber base half, were then connected to a common air intake chimney made from 100 mm poly vinyl chloride (PVC) pipe. Air was drawn through the intakes from a height of 3 m with six 24 VDC blower fans (San Ace B97, Sanyo Denki Co. Ltd, Tokyo, Japan) arranged in two groups of three, and angled to mix and distribute air in a horizontal plane across the base of the chamber. The air inside the chamber was then displaced vertically through the canopy and exited through a vent at the top of the chambers. Smoke tests confirmed uniform air distribution with good mixing and movement of air between the leaves and any remaining space outside the canopy. Removable side panels of the acrylic chamber tops allowed access to fruit on both side of the vine for berry sampling.



Figure 4.3 Pictures showing group of 20 vines used for experiment in the 2013-14 season showing (a) full canopy on 23 December prior to the start of the first 'Véraison' experiment, and (b) after leaf-fall on 30 May.

Fan speed was controlled by a pulse width modulation (PWM) signal from a micro-controller mounted in the base of the chambers, which during the day was set to a duty cycle of 25%. A manually operated switch was used to slow the PWM to duty cycle of 10% at night. For the day time speed, this equated to a flow rate of 2.6 m^3 per minute, or 2.2 chamber volumes per minute, and at night 1.6 m^3 per minute or 1.3 chamber volumes per minute. The exact flow rate response to the fan speed setting varied slightly according to the size of the vine in the chamber. The day time speed was selected based on results of earlier chamber studies to provide enough air flow to limit the temperature rise in the chamber to approximately 2^oC above ambient, but low enough to provide sufficient CO_2 and water vapour differential concentration across the inlet and outlet (Perez Pena and Tarara, 2004). The CO_2 concentration of incoming and outgoing air was monitored at 5 second intervals and the average saved at 30 second intervals over a period of 5 minutes with an infrared gas analyser (Li840A, LiCOR Biosciences, Lincoln, Nebraska, USA) connected to a data logger (CR1000, Campbell Scientific, Logan, Utah, USA) and multiplexed gas sampling system. During the measurement period for each chamber, air was sampled simultaneously from the chamber inlet and outlet at a rate of approximately 2 L min^{-1} via 10 m of 6 mm external diameter polyethylene compressed air tubing, and pumped into a 50 mL buffer volume that vented excess air to atmosphere. The outlet sample was collected from 20 cm below the chamber outlet vent at two points 20 cm with T-shaped connector the main sampling line. For the inlet measurement, air was sub-sampled from the buffer volume at a rate of ~ 800 mL min^{-1} for 2 minutes, and then from the outlet buffer volume for 3 minutes. Switching between air sampling lines was controlled by solenoid valves (and a relay controller: SDM-CD16AC, Campbell Scientific).

Chamber and external environmental parameters were recorded with a second data logger and multiplexer (CR1000 and AM25T, Campbell Scientific, Logan, Utah). Air inlet temperature was measured with T-type thermocouples (24 AWG) at a single point inside the inlet tube under the chamber base, and at two outlet points on each side of the canopy (wired in parallel for average value) from under the white plastic radiation shield attached to the fixed foliage wire. Air velocity was recorded in the centre of the inlet tube with a hot-film element sensor (EE576 or EE671, E+E Elektronik, Engerwitzdorf, Austria). Photosynthetically active radiation (PAR) outside the chamber was recorded with a Quantum sensor (LI-190R, LI-COR Biosciences), and temperature and relative humidity (HMP-50, Vaisala, Helsinki, Finland) inside the bird-proof enclosure was recorded at the same intervals as the chamber measurements.

4.2.1.4 CO_2 Scrubbers - Portable air scrubbers were constructed to reduce the CO_2 concentration of supply air to three chambers by approximately 200 ppm compared to ambient air supplied to three non-scrubbed control chambers (Figure 4.4a). The scrubbers could be attached to the chambers as required in place of the ambient air intake pipe.



Figure 4.4 Soda-lime based CO_2 scrubber tube (left) and whole scrubber connected to chamber in background with ambient supplied chamber in foreground (right).

Each scrubber contained 27 kg of soda-lime (Sofnolime®, Molecular Products Limited, Harlow, UK) divided between eight inlet tubes of 10.3 cm diameter and 48 cm deep. Air was pulled through the soda-lime beds, and then an open-cell foam filter with a single 24 V DC centrifugal fan (SanAce C175, Sanyo Denki Co. Ltd). A manually operated valve was used to allow some air to by-pass the scrubbing beds on the upstream side of the fan. The fans were also speed controlled by a PWM signal from a micro-controller providing a combination of ambient air by-pass and fan speed to vary the airflow rate and CO_2 concentration range.

With fresh soda lime, the incoming air was depleted to 0 ppm CO_2 within the first 10 cm depth of absorbent. However, to facilitate refiling, the pipe fittings that were used to construct the scrubbing beds were left as a push-on fit only. With the base on the scrubber body under vacuum, slight leakage therefore increased the minimal obtainable CO_2 concentration to approximately 15 ppm at 1.5 m^3 min^{-1}. At full bypass, but without blocking the inlet to the scrubbing beds, the scrubbers delivered approximately 300 ppm air at 3.5 m^3 min^{-1}. To achieve 200 ppm and match the intake air velocity of the ambient chambers, the scrubbers were run at approximately 50% bypass and full fan speed. During the Low CO_2 treatment periods for each experiment, the scrubbers were connected to the inlet of the chamber at first light, and then removed again at dusk. As the reaction of soda-lime with CO_2 is exothermic, a 15 L block of ice was placed on top of each scrubber and renewed several times a day in hot weather to provide some cooling to outgoing air. The scrubber assembly was also covered with reflective foil covering during normal operation. This reduced, although did not completely prevent, any slight temperature increase in the low CO_2 chambers during the day.

4.2.1.5 System performance and control of whole vine photosynthesis - For ambient supplied chambers, the CO_2 differential between incoming and outgoing air reached a maximum of approximately 30 ppm at the maximum canopy assimilation rate. For the low CO_2 supplied chambers this was reduced to approximately 15 ppm. Over the 30 minute cycle across the six chambers, the CO_2 reading required 1 minute to stabilise between chambers and approximately 30 seconds between inlet and outlet lines from the same chamber (Figure 4.5).

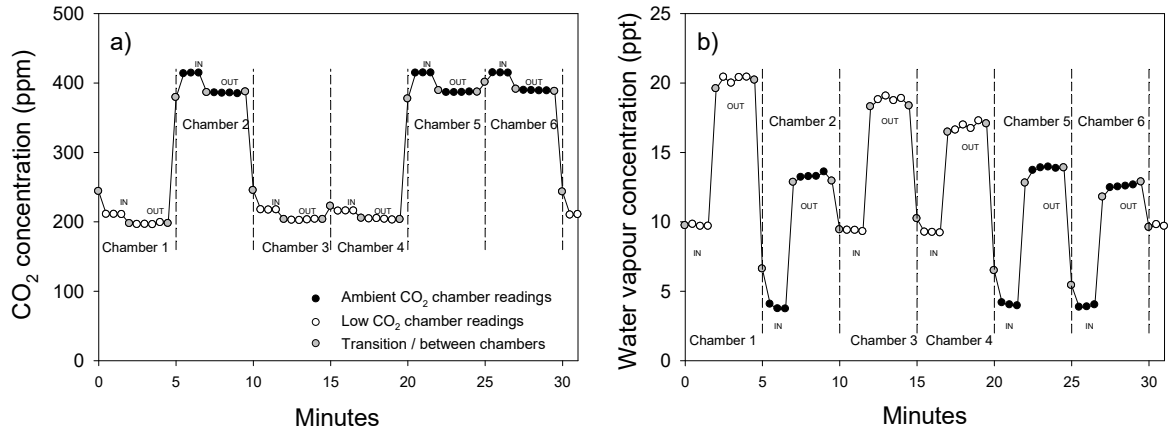


Figure 4.5 Sample data for a 30 minute measurement cycle of the six chambers showing CO_2 concentration (a) and water vapour concentration (b) of inlet and outlet lines.

Water vapour measurements stabilised in a similar manner, but the concentrations of air entering the low CO_2 chambers was increased by water evaporation from the soda lime (Figure 4.5b). This value varied according to ambient air conditions and the water content of the soda lime, but was typically in the range of 5 to 10 mmol H_2O higher than control. This would cause a slight reduction in transpiration, and thus partly offset the increase in stomatal conductance expected under reduced CO_2 supply.

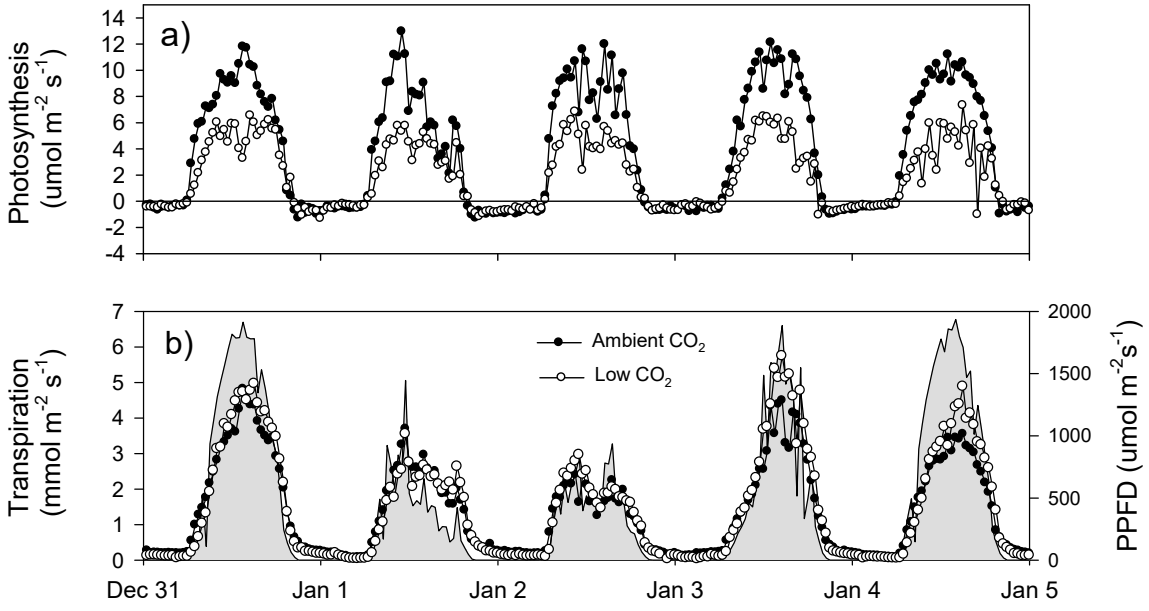


Figure 4.6 Example of gas exchange data collected during the véraison experiment showing effect of ambient and low CO_2 on the average whole canopy photosynthesis rate (a), and transpiration (b).

4.2.2 Experimental outline.

Five separate experiments were conducted over the first three seasons of the project to target different stages of berry development (Table 4.1). For each experiment, six previously untreated vines were selected, and an estimate of yield was made based on bunch number

and visual assessment of bunch size. The chambers were then installed over a period of one day, with the low and ambient treatments assigned in advance to pairs of vines with approximately matching yield. Depending on the experiment, the low CO_2 treatment was started on the first day of chambers being installed on the vines, or after several days of all chambers running at ambient CO_2. The period over which the chambers were installed on the vines, and the time within that period where the scrubbers were running, is indicated in Table 4.1. The experiments were conducted in an order determined largely by the initial chamber construction schedule, with the first experiment commencing at the onset of véraison in the 2013/14 season, and the last experiment at fruit-set in the 2015/16 season. Although there was some overlap in relation to the berry development stages across seasons, the five experiments will be referred to here according to the main target period for the low CO_2 treatment and presented in order of developmental stage rather than chronological order. Each of these experiments are described in further detail under subsequent sub-headings.

Table 4.1 Summary of whole vine chamber experiments

Experiment name	**Growth stage**	**Chambers installed**	**Duration (days)**	**Low CO_2 period**	**Duration (days)**
Fruit-set	EL19 - EL27	23/10/15 - 17/11/15	26	24/10/15 - 15/11/15	23
Pre-véraison	EL31 - EL34	03/12/14 - 22/12/14	20	07/12/14 - 20/12/14	14
Véraison	EL35	26/12/13 - 08/01/14	14	26/12/13 - 04/01/14	10
Ripening	EL35 - EL37	01/01/15 - 02/03/15	61	04/01/15 - 30/01/15	27
Late ripening	EL37 - EL38	24/01/14 - 08/02/14	16	26/01/14 - 04/02/14	10

4.2.2.1 Fruit-set experiment - The fruit-set experiment targeted the effects of carbohydrate supply in the period from just prior to the start of flowering to the end of fruit-set. It completed the last of the main berry development periods assessed with the chamber experiments, and allowed a more detailed investigation of the early defoliation treatment effects on wine tannins and colour from the field trials sites. The chambers were installed three days prior to the start of flowering, and the CO_2 scrubbers connected to three of the chambers on the day before flowering. The low CO_2 treatment then ran for a period of 23 days. An additional six vines from outside the chambers were included in the experiment, with three defoliated to node 8 on the day before the start of flowering. The study therefore included treatments that duplicated the T1 v T2 comparison from the field, with a reduction in leaf area and increase in fruit exposure, or through the use of low CO_2, targeted just the carbohydrate supply aspect of T2 during flowering and fruit-set.

4.2.2.2 Pre-véraison experiment - The pre-véraison experiment targeted the second half of the fruit-set to véraison period, with the low CO_2 treatment running for a period of 14 days that centred approximately on bunch closure. The chambers were installed on 3 December, and the low CO_2 treatment commenced four days later on 9 December. Berries were sampled the day prior to the start of the low CO_2 treatment, and then on five dates while the low CO_2 treatment was running.

4.2.2.3 Véraison experiment - The véraison experiment commenced with both ambient and low CO_2 chambers running just as the first berries had begun to change colour (Figure 4.7 a-b). The low CO_2 treatment was maintained for a period of 10 days, and concluded when approximately 70% of the berries on each bunch was fully coloured (Figure 4.7). Berry samples were collected on the morning of the first day of scrubbing, and on five dates in total during the scrubbing period. Additional berry samples were collected on two dates after the low CO_2 vines returned to ambient.

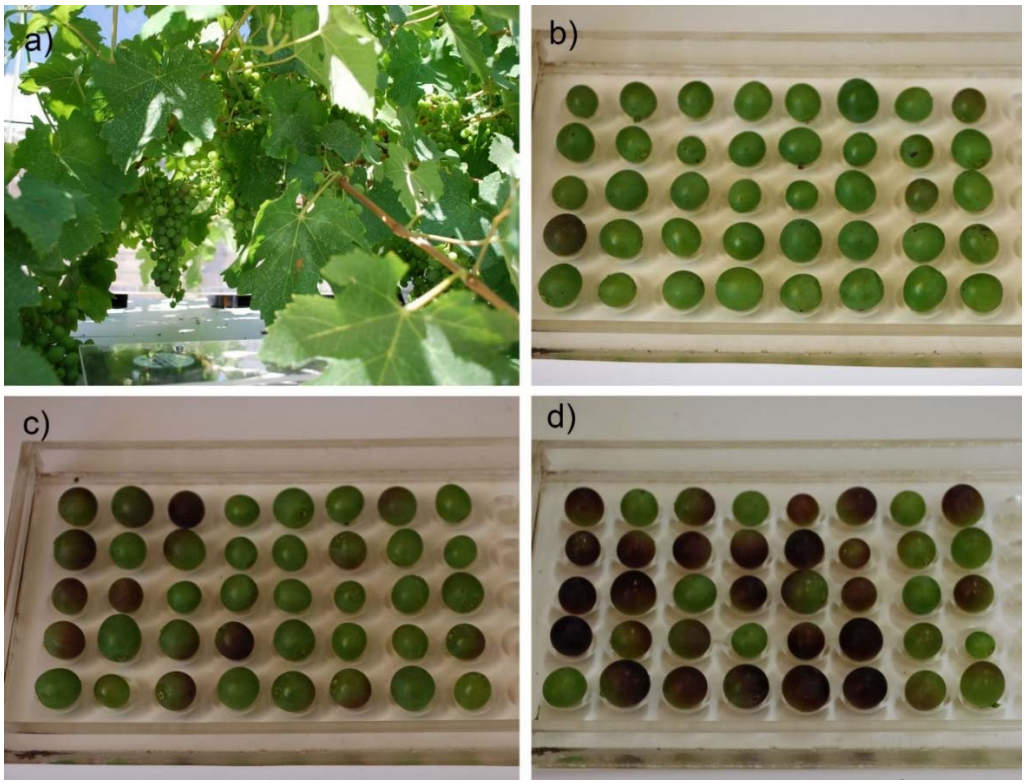


Figure 4.7 Photographs of bunches inside a chamber (a) and one set of berry samples (b) at the start of the Véraison experiment on 26 December. Subsequent berry samples are shown from day 5 of scrubbing (c), and following the full 10 days of the CO2 treatment.

4.2.2.4 Ripening experiment - The ripening experiment commenced with the chambers being installed at the onset of rapid sugar accumulation on 1 January, and when approximately 80% of berries on each bunch were partly or fully coloured. The low CO_2 treatment then commenced on 4 January from average soluble solid content of 11.6°Brix and then ran for a period of 27 days until control chambers reached an average of 19.2°Brix. The scrubbers where then removed from the low CO_2 chambers to allow return to ambient conditions, with all chambers remaining in place until the last vine was harvested 32 days later. Berry samples were collected on the morning of the first day of scrubbing, and on seven dates in total during the scrubbing period. Additional berry samples were collected on two dates after the low CO_2 vines returned to ambient.

4.2.2.5 Late ripening experiment – For the latest experiment conducted during the berry development period, the chambers were installed at the end of the rapid sugar accumulation period. This corresponded to an average of 19.2°Brix across all six chambers, with 10 days of

low CO_2 treatment commencing on 26 January. Berry samples were collected on three dates during the scrubbing period, and on one date during the four days chambers remained installed after the low CO_2 treatment.

4.2.3 Canopy measurements.

Total leaf area per vine was determined by measuring the length of every leaf and converting to area based on separate calibrations made on destructively sampled leaves from spare vines. Leaf area measurements were made before the chambers were installed for the Late Ripening experiment, and just after the chambers were removed in the other three experiments. Pruning weights were determined from the weight of fresh dormant canes removed at pruning.

4.2.4 Berry sampling and harvest

Berry samples were collected from each vine through removable access panels in the side of the chambers. For the first season (Véraison and Late Ripening experiments), 40 random berries were collected from both sides of the vine. For the second and third season (Fruit-set, Pre-véraison and Ripening experiments) 50 berries per vine were collected. The samples were immediately weighed, separated into skin and seeds over ice and then frozen in liquid nitrogen. The pulp was manually homogenised while cooled over ice, and juice separated and 1 mL sub-samples frozen in liquid nitrogen. Remaining juice was used to determine soluble solids. All samples were stored at -80°C prior to subsequent analysis.

4.2.5 Berry analysis

Juice samples were thawed, vortexed to mix and filtered to 0.22 µm and sugar and organic acids determined by HPLC-PDA-RI on a 300 mm × 7.8 mm Aminex HPXM87H ion exclusion column (BioMRad Laboratories, Berkeley, USA) using the method of Frayne (1986). Fructose and glucose were quantified by RI and malic and tartaric by PDA against respective standards. For anthocyanins, berry skins were ground under liquid nitrogen (A11 Basic Analytical Mill, IKA Works, USA) and anthocyanins extracted from a 100 ± 5 mg sample in 1 mL of 50% (v/v) aqueous methanol and analysed by UPLC as described in Chapter 3 for the field site berry and wine samples.

4.2.6 Winemaking and analysis.

The fruit from each vine from the Ripening experiment was harvested at a target of 24°Brix. Berries were hand-destemmed, crushed and transferred into 1.5 L coffee plungers, inoculated with a commercial yeast strain (Lalvin EC1118, Lallemand) and fermented. After seven days the wines were pressed and stored at 1°C for cold stabilisation for one week, racked and then bottled. After one week, wines were analysed for alcohol, pH and TA. Wine colour density and colour hue were determined.

4.3 Results and discussion.

4.3.1 Overall vegetative and yield parameters

By growing cordon trained vines in large pots, the objective was to obtain vines with yields and canopy sizes that were comparable to field grown vines. Across the three seasons of experiments, yields ranged between 3.61 and 5.85 kg per vine for vines inside the chambers (Table 4.2), and pruning weights ranged between 0.55 and 0.68 kg per vine. With a cordon length of 1 m compared to a range of 2 to 2.4 m at the field trial sites, fruit production per

unit of vine row was therefore between the higher yields observed at Langhorne Creek and the lower yielding season at the Murray Valley site. With pruning weight ratios ranging between 5.5 and 8.3, the vines would not be considered under or over cropped by the commonly cited ranges of 5 to 10 for pruning weight ratio (Bravdo et al., 1984).

Table 4.2 Summary of basic yield and vegetative growth parameters for the whole vine chamber experiments between 2013 and 2015. Data are means of three replicates ± standard error.

Experiment / CO_2 treatment	Yield (kg vine^{-1})	Bunch number	Leaf area (m^2 vine^{-1})	Pruning weight (kg vine^{-1})	Cane number	Pruning Weight ratio	Fruit to leaf area ratio (g cm^2)
Fruit-set							
T1	2.88±0.74	44±2.6	-	0.93±0.12	36±1.7	-	-
T2	1.34±0.20	41±9.2	-	0.87±0.11	38±5.1	-	-
Ambient	3.53±0.20	49±7.3	4.09±0.11	0.55±0.02	39±39	6.5±.0.60	8.63±0.51
Low	3.63±0.61	49±8.7	4.49±0.20	0.63±0.05	34±34	5.9±1.21	8.15±1.48
Pre-véraison							
Ambient	5.46±0.26	58±5.29	4.53±0.36	0.68±0.08	32±1.2	8.3±1.32	12.3±1.44
Low	5.02±0.28	54±2.31	5.16±0.33	0.62±0.08	32±0.9	8.3±0.92	9.7±0.08
Véraison							
Ambient	3.76±0.59	38±4.70	4.49±0.13	0.66±0.06	34±0.3	6.0±1.23	8.3±1.07
Low	3.70±0.29	41±2.03	4.26±0.13	0.61±0.06	34±1.3	6.2±0.85	8.7±0.94
Ripening							
Ambient	5.85±0.15	59±3.93	4.41±0.26	0.63±0.09	32±0.7	9.4±0.35	13.3±0.52
Low	4.54±0.34	61±3.84	4.38±0.07	0.63±0.07	30±1.7	7.2±0.13	10.3±0.62
Late ripening							
Ambient	3.61±0.34	41±2.08	4.36±0.26	0.65±0.06	37±1.2	5.5±0.18	8.2±0.32
Low	4.01±0.45	42±4.33	4.03±0.10	0.55±0.07	37±3.5	7.3±0.71	9.9±1.02

4.3.2 Fruit-set experiment

The fruit-set experiment targeted the effects of a CO_2-mediated reduction carbohydrate supply from flowering until the end of fruit-set, and compared these with vines outside the chambers which had either no treatment applied (equivalent to T1 from field trials) or basal leaves removed just prior to the start of flowering (equivalent to T2 from field trials). The aim of this experiment was to further investigate the mechanisms underlying the increased tannin production seen in response to T2, and in particular the response of key regulatory genes. These results are presented in Chapter 5.

Berry samples were collected on an additional eight dates between berry set and harvest to follow responses to the defoliation and 23 days of low CO_2 supply. However, a series of high temperature events through mid-November, December and in particular mid-January impacted on berry development to a greater extent than in previous seasons. Berry growth rates were initially greater for vines inside the chambers than out, and through the later part of the ripening period was higher for the T1 treatment than T2 (Figure 4.8a). This may reflect a difference in water status, with water use of vines outside the chambers higher, and also heat damage which was greater for the more exposed T2 fruit outside the chambers.

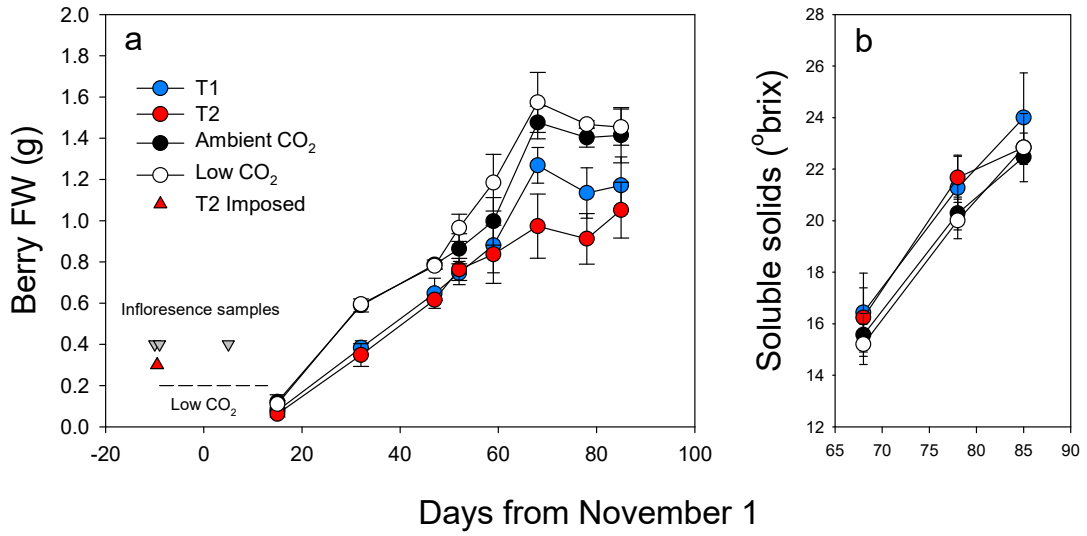


Figure 4.8 Summary of inflorescence sampling dates, berry growth (a) and berry sugar accumulation (b) following the removal of the eight basal leaves just prior to flowering (T2) and 23 days of low CO_2 supply through flowering and fruit-set. Error bars are SD.

4.3.3 Pre-véraison to late ripening experiments

4.3.3.1 Berry growth - Berry weight was recorded at intervals of 1 to 7 days during the scrubbing periods of the four experiments from pre-véraison to late ripening (Figure 4.9a). Additional samples were collected after the Low CO_2 treatment had returned to ambient conditions and/or at harvest. The Low CO_2 treatment had no significant effect on berry growth in any of the experiments. In the ripening experiment, which spanned the longest part of the berry development period, low CO_2 did not change the maximum weight or date at which maximum berry weight was reached.

4.3.3.2 Juice sugar accumulation - On a concentration basis, the rate of juice sugar accumulation was highest during the véraison experiment followed by the ripening and pre-véraison experiments. The increase in juice sugar concentration in the late ripening experiment could be fully explained by the loss of berry weight. For the individual experiments, the relative effect of the low CO_2 supply was greatest in the pre-véraison experiment with the average rate of sugar accumulation reduced from 0.38 g L^{-1} per day to 0.11 (Figure 4.10a inset). As the subsequent samples collected from the same vines after the chambers had been removed indicate, the pre-véraison experiment concluded very close to the onset of rapid sugar accumulation. This difference in sugar accumulation could therefore reflect a reduced rate of sugar transport, a slight developmental delay, or a combination of both factors.

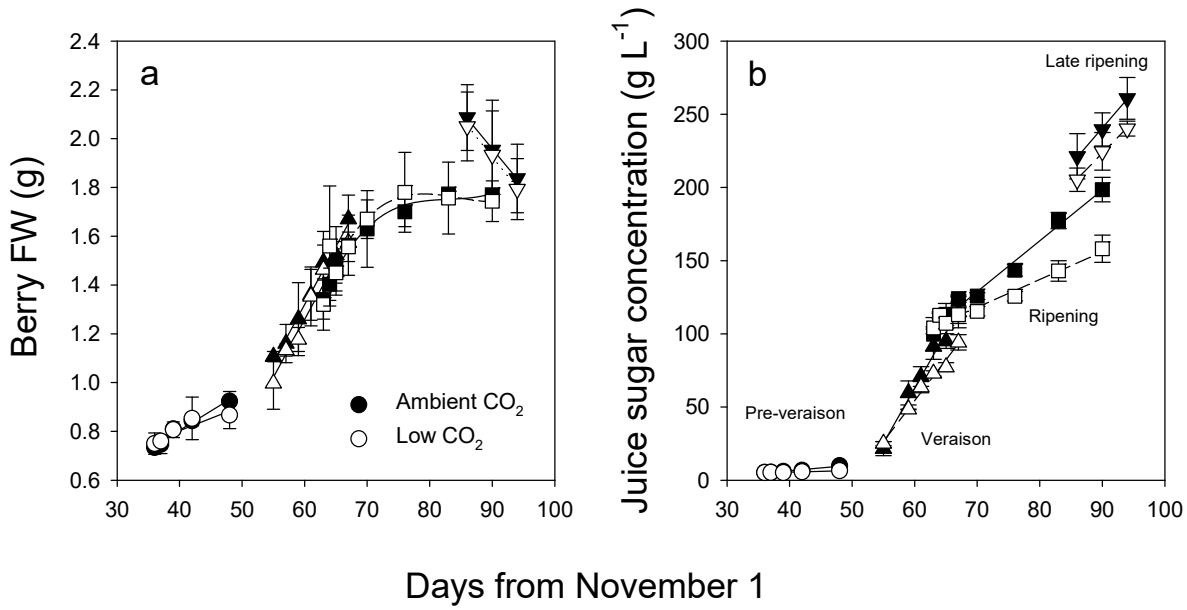


Figure 4.9 Summary of low CO_2 effects on berry growth (a) and total glucose and fructose juice sugar concentration (b) for the four experiments from pre-véraison until late ripening. Samples collected before or after the scrubbing periods are not shown. Error bars are SD

During the véraison experiment, juice sugar concentration increased at a rate of 7.5 g L^{-1} per day in the ambient treatment compared 5.6 g L^{-1} day in the low CO_2 treatment (Figure 4.10b). For the ripening periods the sugar accumulation rates for these treatments was 3.5 and 1.9 g L^{-1} per day respectively (Figure 4.10c). As the pre-véraison and ripening experiment were conducted in a different season to the véraison experiment, and also with a different population of vines, there are factors other than developmental stage that may have contributed to this variation in magnitude of CO_2 response. Crop load was also lower for the vines used in the véraison experiment than for the pre-véraison and ripening experiments (Table 4.1). However, irrespective of the comparison between berry developmental stages, the pre-véraison response to low CO_2 is important because it indicates an influence of carbohydrate supply even at a time when demand from the fruit is lower, and the canopy is reaching peak photosynthetic capacity.

The reduced, although still significant, response to low CO_2 as the berries went through véraison may suggest that the high sink strength of the fruit at this point may compensate for the overall reduction in carbohydrate availability. In the shorter term, mobilisation of sugar or starch reserves could also be expected to make some contribution to berry sink demand that may not be sustainable over a longer period of carbohydrate shortage (Rossouw et al., 2017). With both the pre-véraison and véraison experiment the small difference in sugar content were still evident in the later berry samples and at harvest. For the ripening period experiment, the rate of juice sugar accumulation was approximately halved from 3.5 to 1.8 g L^{-1} per day (Figure 4.10c). On a per berry basis this represented a difference of 6.1 compared to 3.4 mg sugar $berry^{-1}$ per day, indicating a reduction in berry sugar accumulation directly proportional to the reduction in photosynthesis. Analysis of carbohydrate reserves following destructive harvest of the vines at the end of the season (not presented) suggested some residual effect of the CO_2 treatment on stored starch. However, overall it appears that reserves did not compensate for the reduction in photosynthetic carbohydrate supply caused by halving the concentration of CO_2.

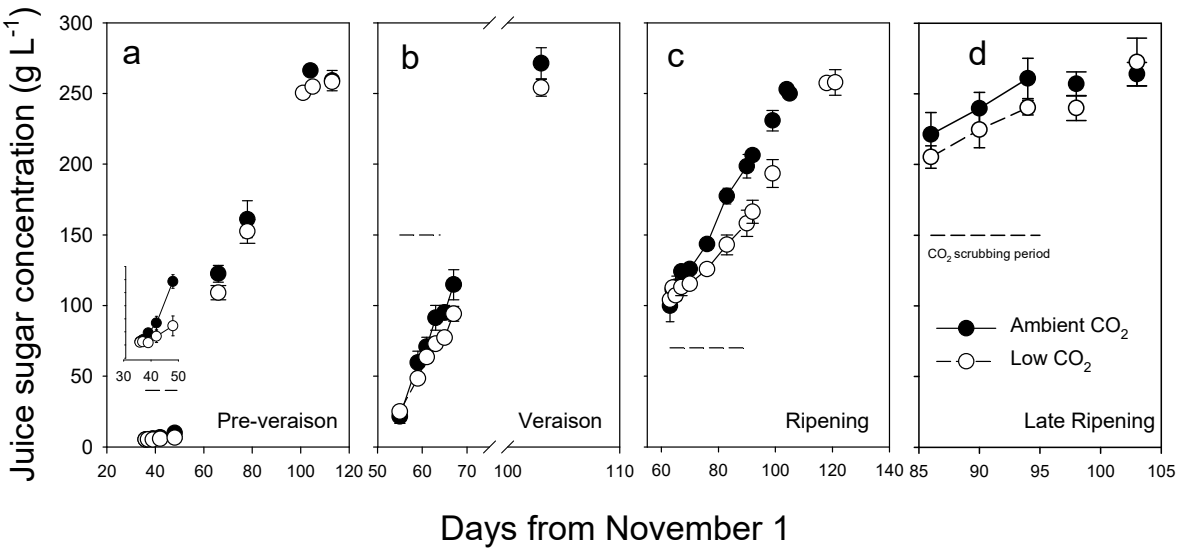


Figure 4.10 Total juice sugar (fructose + glucose) concentrations of berry samples collected during the Pre-véraison, Véraison, Ripening and Late Ripening chamber experiments. Error bars ± SD.

4.3.3.3 Organic acid - Tartaric and malic acids are produced between fruit-set and véraison, and then the concentration of both in declines through to harvest. This is in part due to dilution with ongoing berry expansion, and also because malic acid is catabolised during the berry maturation period. During the pre-véraison experiment there was increase in malic acid concentration indicating ongoing biosynthesis in both treatments, but this was slower in the low CO_2 treatment compared to ambient (Figure 4.11a). There was no effect of the low CO_2 treatment in the subsequent three experiments, suggesting that the production of malic acid may be sensitive to photosynthetic carbohydrate supply, but the rate of subsequent utilisation is not.

The accumulation of tartaric acid was completed by the commencement of the pre-véraison experiment and there was no effect of low CO_2 on juice concentrations in any of the treatments. The difference in the relative response of organic acids and sugars to low CO_2 supply during ripening meant that the fruit in low CO_2 treatment had lower sugar concentrations of sugar relative to acids at the end of the scrubbing period. However, as the low CO_2 treatment vines were returned to ambient and the decline in acidity slowed in relation to the increase in sugar concentration, this difference was lost by harvest.

4.3.4 Berry anthocyanins

4.3.4.1 Véraison and ripening experiment - Following 10 days of reduced CO_2 supply during the véraison period, total anthocyanins in the Low CO_2 treatment were significantly lower ($p=0.003$) than ambient CO_2 vines at 0.09 and 0.05 mg g^{-1} berry. At harvest 38 days later, during which time the low CO_2 treatment had returned to ambient conditions, total anthocyanins in the previously low and ambient treatments were 0.73 and 0.67 mg g^{-1} berry respectively. Although a small difference was maintained between the treatments, this effect was not significant.

After 27 days of CO_2 scrubbing in the ripening experiment, total anthocyanin concentrations on a berry weight basis were also significantly lower (p=0.025) than for ambient at 0.47 and 0.36 mg g^{-1} berry respectively. In contrast to the véraison experiment which was all harvested on the same day, harvest dates were adjusted in the longer ripening experiment to allow fruit on all vines to reach a common sugar content. This resulted in a 15 day delay between the average harvest date for the ambient and previously low CO_2 treatment, and an average of 35 days since the vines were returned to ambient conditions. Anthocyanin concentrations at this point did not differ significantly at 0.86 and 0.85 mg g^{-1} berry for the ambient and previously low CO_2 vines.

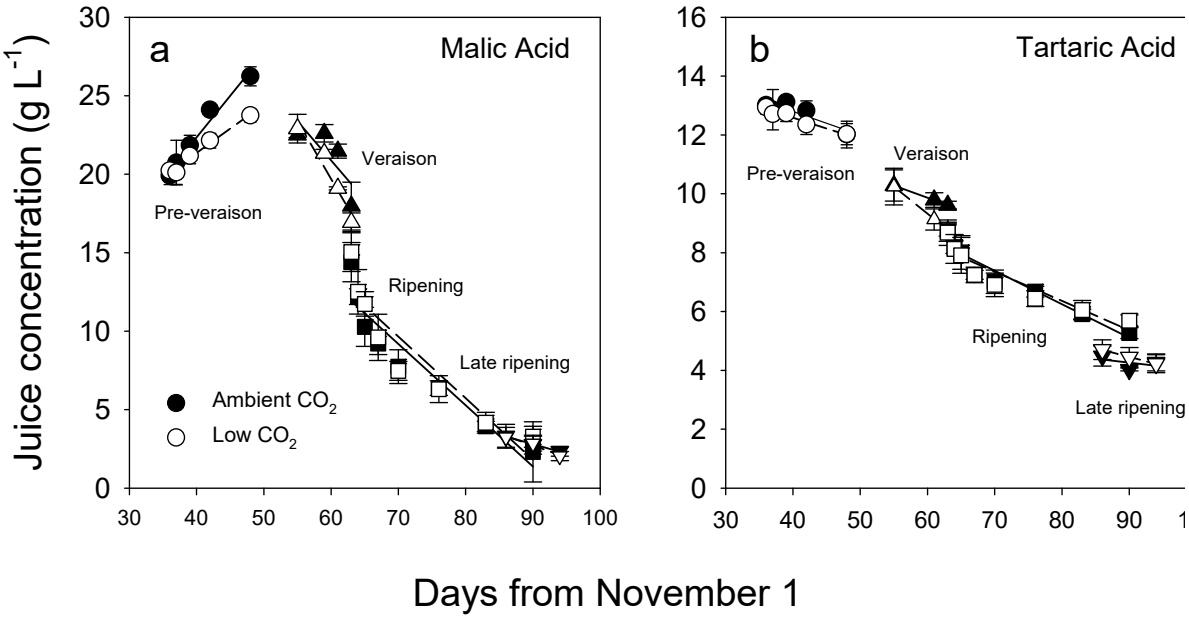


Figure 4.11 Juice malic and tartaric acid concentrations during the CO2 scrubbing period of the Pre-véraison, Véraison, Ripening and Late Ripening chamber experiments. Error bars ± SD.

Despite the decrease in total anthocyanins under reduced carbohydrate supply, the effect was proportional to the decrease in berry sugar accumulation. Therefore, the relative concentrations of total anthocyanins and juice sugars were not changed (Figure 4.10). The relationship was also maintained when vines were returned to ambient CO_2 and normal carbohydrate supply, indicating that carbohydrate supply did not modify or uncouple the relative concentrations of juice sugar and berry anthocyanins. From a winemaking perspective, it suggests that while high yield or crop load can reduce colour and berry sugar accumulation, carbohydrate availability alone does not appear to modify the relationship between the two. However, in the véraison experiment anthocyanin concentrations increased more rapidly relative to juice sugars than during the ripening experiment in the following season. The earlier statement could therefore be qualified by saying that carbohydrate supply may not have separated juice sugar and berry colour for a given group of vines, but other seasonal factors may have across these two experiments.

For the individual anthocyanins, the véraison experiment was the most impacted. The low CO_2 treatment caused a decrease in the proportion of monoglucosides, and a slight increase

in malvidin derivatives. While those changes were evident during the maturation period, there were no more differences at harvest.

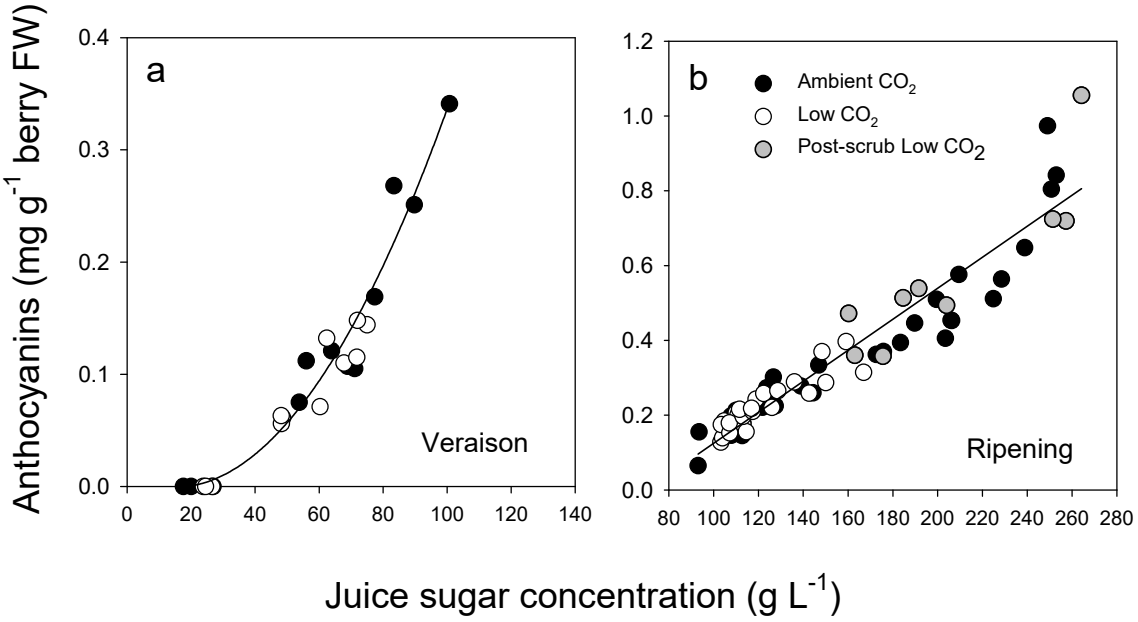


Figure 4.12 Relationship between total juice sugar (fructose+glucose) and skin anthocyanins for the CO_2 scrubbing periods of the Véraison and Ripening chamber experiments.

4.3.5 Wine composition

Small scale ferments were undertaken for each of the chamber experiments between flowering and the late ripening period, and in general these showed minimal effects of the low CO_2 treatment on wine composition (Table 4.3). As was apparent with the sugar anthocyanin relationship there was a seasonal difference in colour development with similar sugar concentrations reached at harvest, but wine colour significantly lower for the pre-véraison and ripening experiments in the second season of the project. The fruit-set and pre-véraison experiments were both conducted for shorter periods early in berry development with a more immediate focus on gene expression, and after returning to ambient conditions for the full ripening period, smaller effects on composition may have been difficult to detect by harvest. The véraison experiment did extend into the ripening period, but the apparent difference in wine colour reflected differences in maturation that were not allowed for with a common harvest date, and consequently high variability masked differences between treatments.

The ripening experiment, which ran for the longest period and had harvest dates based on maturity, did however support the limited responses seen to low CO_2 in the other experiments. In this case there were no effects on anthocyanin or tannin concentrations in the wine. The only significant difference ($p=0.037$) that was juice yeast assimilable nitrogen was reduced by the low CO_2 treatment and this could have had other quality and sensory implications for the wine produced from the fruit.

Table 4.3 Summary of basic must and wine parameters from small scale ferments.

	Must			Wine			
Experiment / CO_2 treatment	**Density (°Baume)**	**YAN (mg N/L)**	**TA (g/L)**	**pH**	**Wine colour density (AU)**	**Total red pigments (AU**	**Total tannins (g L-1 ep. Equiv.)**
Fruit-set							
Ambient	13.1±0.20	73±7	4.8±0.2	3.3±0.1	18.5±2.9	45±5.2	1.72±0.30
Low	13.0±0.7	85±10	5.2±0.1	3.3±0.1	15.9±2.7	43±5.8	1.48±0.34
Pre-véraison							
Ambient	13.1±0.3	102±13	3.7±0.3	3.5±0	7±2.5	32±7.1	0.93±0.93
Low	12.9±0.3	93±10	4.0±0.5	3.5±0.1	5.3±0.8	32±1.4	0.84±0.84
Véraison							
Ambient	14.2±0.5	135±21	-	3.7±0.1	18.8±8.3	60±8.9	1.61±0.32
Low	13.8±0.4	155±31	-	3.9±0.1	9.5±0.4	54±4.2	1.38±0.1
Ripening							
Ambient	12.8±0.2	85±14	3.8±0.2	3.4±0.1	3.5±0.3	26±6.1	0.67±0.07
Low	13.3±0.2	128±20	3.3±0.2	3.6±0	4.0±0.7	26±6.4	0.79±0.08
Late ripening							
Ambient	13.6±0.3	121±11	-	3.6±0.1	11.8±1.9	53±8.2	1.52±0.30
Low	14.3±1	146±32	-	3.7±0.1	16.6±10	55±13	1.62±0.52

4.4 Conclusions

This component of the project set out to develop a system for reducing carbohydrate supply to the fruit independently of changes in crop load or fruit exposure. The aim was to determine whether carbohydrate supply alone could modify the relationship between juice sugar accumulation and the production of other metabolites. It was found that reducing CO_2 supply from ambient to approximately 200 ppm halved the whole vine photosynthetic output in a manner consistent with single leaf photosynthesis responses to CO_2 concentration. What was not known was the extent to which non-structural carbohydrates might compensate for the reduced supply from photosynthesis, or photosynthesis may up-regulate in response to the low CO_2. With the variation in relative effects of low CO_2 compared to ambient across experiments, there is some indirect evidence that these factors may have partly offset the effects of halving of the original photosynthetic output of the canopy. However, when applied across the period of berry development from approximately 35 to 90 days after flowering, low CO_2 was found to be effective in slowing the rate of juice sugar accumulation. The relationship between juice sugar and anthocyanins per unit berry weight, which is the most direct comparison with the must during fermentation, was not changed by the low CO_2 treatment. In both cases accumulation was slowed, but negative effects on wine composition could be avoided from delaying harvest until the same juice sugar concentration was reached. There was indication of more subtle changes in the composition of anthocyanins, but further work is required to understand the implications of these for wine composition.

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Chapter 5

Investigating molecular markers using targeted gene expression patterns

5.1 Background

The treatments have led to some consistent changes in berry ripening and in some quality measures in berries such as tannins and anthocyanins. These quality differences were also detected in the wines by chemical analysis and sensory evaluation and difference testing. However, they were not consistent over all sites and seasons, but some trends such as increased flavonoids in the early defoliation treatment T2 are detectable. Nevertheless, in this chapter we sought to determine if changes in gene expression (a form of molecular marker) could be useful in predicting changes to wine characters as a result of treatments.

Quality measures for grapes arriving at the weighbridge include anthocyanins and total phenolics (which are mainly a measure of anthocyanins and condensed tannins (also called proanthocyanidins) added together). During fermentation and subsequent aging, pigmented polymers are formed providing colour and texture to the wine. The sensory experience of a wine is also reliant on the combination of many flavour and aroma compounds giving great complexity to the wine (Robinson et al., 2014a, 2014b). Many of these flavour and aroma molecules, present only in small quantities, can change significantly during fermentation and most are comparatively difficult to measure in the berry and in the wine. The pathways by which the precursor molecules are synthesised in berries have only been elucidated for a few compounds. However the levels of some compounds have been shown to change with crop manipulation (Bureau et al., 2000).

Genes involved in flavonoid synthesis have been identified by several groups (Sparvoli et al., 1994; Bogs et al., 2005; Bogs et al., 2006; Harris et al., 2013). The pathway illustrated in figure 5.1 leads to a number of different flavonoids; in grapes, this includes flavonols, condensed tannins and anthocyanins. There are several shared or core enzymes (e.g. chalcone synthase - CHS) that are essential to the synthesis of all the flavonoids, while four enzymes are specific to the first step to the synthesis of the particular compounds, described below.

Flavonols are synthesised very early even before flowering and then much later in the last few weeks of ripening. Ristic et al., 2007 and others have also demonstrated that flavonol content increases as a result of exposure of fruit to sunlight (Matus et al., 2009), possibly acting as a sunscreen. In wine quality, the role of flavonols is not known though Ristic et al., (2007) found that dark-grown fruit did not make wines that ranked as highly as that from control fruit. By measuring the expression of *flavonol synthase* (*FLS*) (Downey et al., 2003) in different treatment berry samples, a gauge of fruit exposure to sunlight is possible. Since flavonols were not measured in our samples, changes in gene expression can only be related back to the treatment.

Anthocyanins are the red colours in the skin of black/red grapes forming from véraison onwards. The major anthocyanins in Shiraz skins are trihydroxylated compounds: malvidin glucosides and derivatives. UDP-glucose:flavonoid glucosyltransferase (UFGT) is the enzyme which converts colourless anthocyanidins to anthocyanins by adding the glucose molecule, hence it is the definitive step for anthocyanin synthesis. The *UFGT* gene is expressed from véraison onwards (Boss et al., 1996) and its expression correlates well with anthocyanin synthesis.

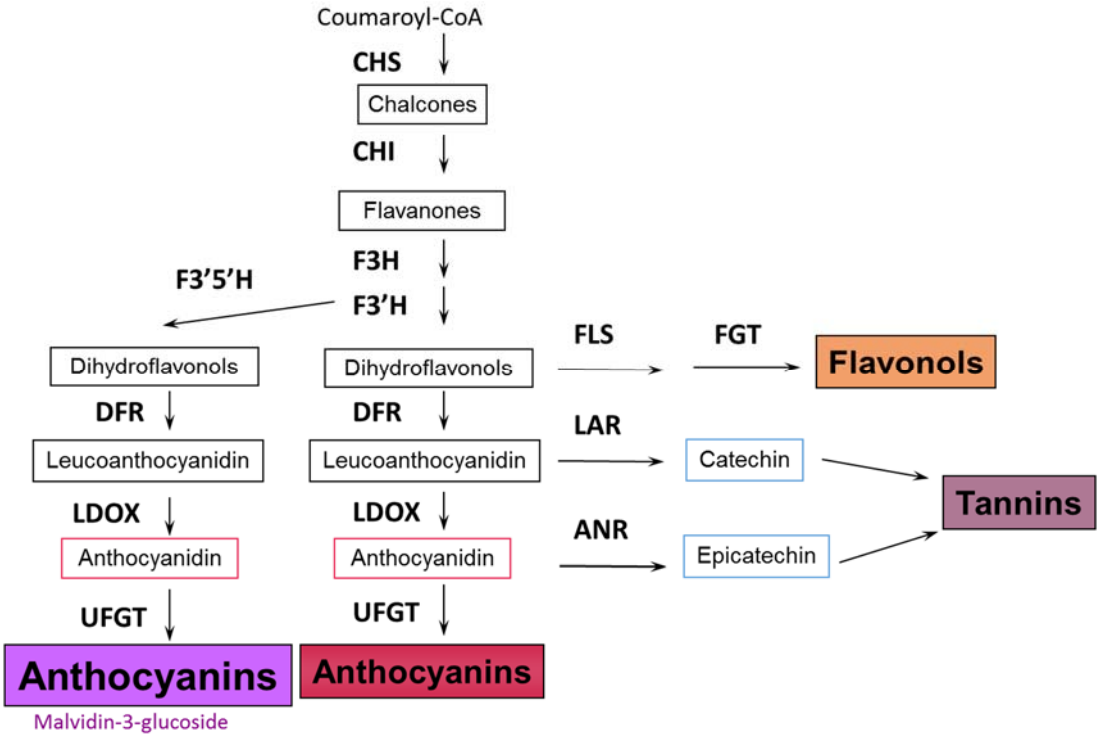


Figure 5.1 Flavonoid biosynthetic pathway. Three major groups of compounds are synthesised in grape berries by a number of enzymatic steps. Core pathway steps CHS- chalcone synthase, CHI- chalcone isomerase, F3H- flavanone 3 hydroxylase, DFR- dihydro 4-reductase, , LDOX- leucoanthocyanidin oxygenase; modifications F3'H- flavonoid 3' hydroxylase, F3'5'H- flavonoid 3'5' hydroxylase; flavonols: FLS- flavonol synthase, FGT- flavonol glycosyl transferase; tannins: LAR- leucoanthocyanidin reductase, ANR- anthocyanidin reductase; anthocyanins: UFGT- UDP-glucose:flavonoid glucosyltransferase.

Tannins contribute to texture and astringency of the wine, and to colour through the stabilisation of anthocyanins as pigmented polymers. The synthesis of tannins is not completely understood; the process of polymerisation has not been described as yet. However, the basic subunits are synthesised by two enzymes, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) in skins and seeds, the tissues where tannins are formed. LAR2 has sequence similarity to LAR1 and is expressed in a pattern that suggests a role in tannin synthesis though this is yet to be proven (Bogs et al., 2005). A new gene *TDS6* for *tannin deficient seed 6* contributes to tannin synthesis or storage in Arabidopsis seed (Abrahams et al., 2002) although the actual role for this gene has yet to be elucidated. The grapevine *TDS6* gene is expressed more highly around véraison indicating a role in flavonoid synthesis (Walker et al., unpublished).

MYB transcription factors regulate different branches of the pathway to control which flavonoids are synthesised in a particular tissue at a specific developmental point or in

response to environmental conditions. Genes encoding the MYB factors have also been isolated: MYBF1 regulates flavonol synthesis (Czemmel et al., 2009); MYBA1 and MYBA2 (Kobayashi et al., 2002; Kobayashi et al., 2004; Walker et al., 2007) regulate anthocyanin synthesis by controlling UFGT expression; MYBPA1, MYBPA2 and MYBPAR regulate tannin synthesis (Bogs et al., 2007; Terrier et al., 2009; Koyama et al., 2014) while the shared pathway is controlled by MYB5a and MYB5b (Deluc et al., 2006; Deluc et al., 2008).

As berries ripen, the relationship between colour and sugar accumulation is usually closely linked (Poni et al., 2013). Various plant manipulations can alter the phenology but usually at harvest, anthocyanins and sugars are similar to control fruit when the same sugar level is reached.

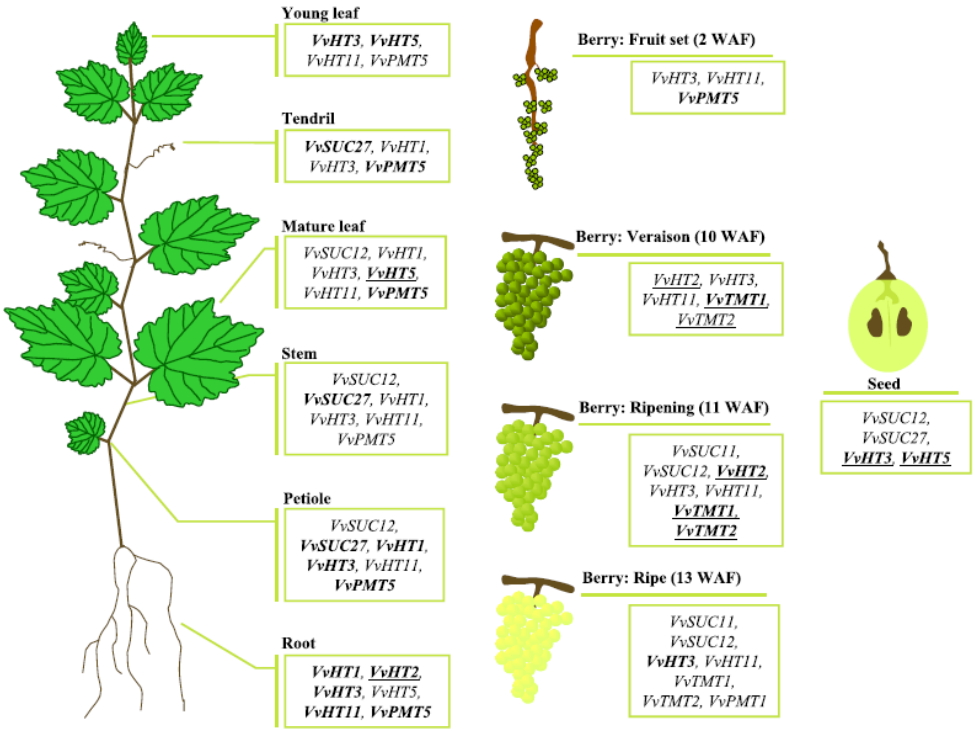


Figure 5.2 Preferential expression of sugar transporter genes in different parts of grapevine (after Afoufa-Bastein et al., 2010).

In grapes, sugars specifically sucrose are transported around the plant from the leaves to the berries and other sinks such as roots. Once the sucrose reaches the berry via the phloem it is unloaded into the apoplast where it may be cleaved by cell wall invertases and transported as either sucrose or hexose into the berry flesh cells or mesocarp. Transporters then transfer the sugar as sucrose or hexose into the vacuole where it is stored as glucose and fructose. Hexose transporters together with a cell wall invertase were identified and characterised (Hayes et al., 2007; Hayes et al., 2010). They demonstrated that *Hexose transporter 2* (*HT2*) was expressed in ripening berries while the expression of *HT5* and *Cell wall invertase* (*cwINV*) genes were both induced by fungal pathogens and other stresses such as the addition of abscisic acid. The sugar transporter gene families have been reviewed (Afoufa-Bastien et al., 2010; Lecourieux et al., 2013) and analysis of expression profiling led the authors to conclude that several genes probably played significant roles in the sugar filling of berries during and after véraison (figure 5.2). *Hexose transporter 2* (*HT2*), *Tonoplast monosaccharide transporter*

1 (*TMT1*) and *Sucrose transporter 12* (*SUC12*) were also appears to play major roles in sugar loading of the berry. These genes (*HT2*, *HT5*, *TMT1*, *SUC12* and *cwINV*) were selected for expression analysis as they were the best candidates to represent the processes of sugar accumulation in the berry.

The aim of the work described in this chapter was to identify molecular markers that at an early stage of development could predict the outcome in berries and more importantly wine. To achieve this, berry samples from the field and chamber experiments were collected, RNA extracted and the expression of a number of genes monitored at specific time points before and after treatments.

5.2 Methods and materials

5.2.1 Berry sampling

Berry samples from the field were collected as reported in Chapter 2 (section 2.2.4). Generally, samples were taken from four bunches from different vines and different parts of the canopy in each rep. Flowers, young berries and whole berry samples were frozen immediately in liquid nitrogen, while skins and seeds from 40 or 50 randomly selected berries were separated on ice, weighed and frozen immediately. All samples were stored at -80^{o}C.

For the chamber experiments berry sampling is described in section 4.2.4. Briefly, samples of 40 or 50 berries per vine were collected from both sides of the vine and after véraison separated on ice into skins and seeds before freezing in liquid nitrogen.

5.2.2 RNA extraction and cDNA synthesis

Flower, berry, seed or skin tissues, which had been snap frozen at the time of sampling, were ground under liquid nitrogen to a fine powder. 60-80 mg of ground tissue was used for RNA extraction with the Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich) with some minor modifications to the manufacturer's instructions. PVP-10 (Sigma-Aldrich) (8%w/v) was added to the lysis buffer to assist with the prevention of phenolic compounds and polysaccharides adhering to the nucleic acids. Binding buffer (300 µL) was added to the flowthrough prior to adding it to the binding column. On column DNase digest was performed following the manufacturer's optional instructions (DNase I, Sigma-Aldrich). RNA was eluted with 40 µL elution buffer and quantified by spectrophotometry. First strand cDNA was synthesised from 500 ng total RNA using the Transcriptor First Strand cDNA synthesis kit (Roche), utilising Oligo-d(T) primer. The resulting cDNA solutions were then diluted 1:20 in water and stored at -20C.

5.2.3 Measuring of gene expression

For the standard curve, PCR products of each gene using primers listed in table 5.1 were purified using the Qiagen PCR purification kit, quantified by spectrophotometry and diluted to a concentration of 1 ng/µL. This stock solution was used to create a dilution series (10^{-1} to 10^{-8}). Four different standard dilutions were included in each qPCR experiment (usually 10^{-4} to 10^{-7} or 10^{-5} to 10^{-8} depending on the expression levels of the target gene) to establish a standard curve.

Table 5.1 Primers used for qPCR experiments

Gene	Primers	Sequence
FLS	FLS36-01L	CAGGGCTTGCAGGTTTTTAG
	FLS36-01R	GGGTCTTCTCCTTGTTCACG
UFGT	UFGT3	TAACACATTCTGGATGGAACTGAT
	UFGT4	ACCTTCAATTCTCACTCCAATCTC
ANR	RT-BAN-FOR	CAATACCAGTGTTCCTGAGC
	RT-BAN-REV	AAACTGAACCCCTCTTTCAC
LAR1	LAR3F	ACTCTGCAATTGCCAACAC
	LAR4R	CCCATGTTACAACAAGGAGTAG
LAR2	LAR2-JWq1F	AATGGGAGGTCAAGCAGCAG
	LAR2-JWq1R	GGAGCTGGCCACATCATCAT
LDOX	VvLDOXF1	ACCTTCATCCTCCACAACAT
	VvLDOXR2	AGTAGAGCCTCCTGGGTCTT
TDS6	VvTDS6P1	TGAAGAAGAGGAGGAGGAAG
	VvTDS6P2R	CGAGATGGTTGTAGGAGACA
MYBA	MybrealF	GAGGGTGATTTTCCATTTGAT
	MybreaR	CAAGAACAACTTTTGAACTTAAACAT
MYBF2	RTMybF2.fwd1	TTGAGTGTGTAGGTTGGGAGTG
	RTMybF2.rev1	CTAATCCTAGCGCCTTGCAC
MYBPA1	VvMybPA1F	TTGACGGGGTTGACTTCTTC
	VvMybPA1R	GAGTAGTGATTCGGCGAAGG
MYBPA2	VvMybPA2F	GACATTGGCGACCTCCTTAC
	VvMybPA2R	CGAGAAAATCGGAACAAGGA
HT2	VvHT2f	GCCCAGACATTCTTGACCATG
	VvHT2r	GGGACTTGAGAAGGAATTTAGC
HT5	VvHT5f	AGAGATGGGTACGGCAATGG
	VvHT5r	GGCACAAGAACACATATAC
TMT1	VvTMT1f	CCGAGACAAAAGGCATGCCAC
	VvTMT1r	CGACCATCAAAATATCCACAC
cwINV	VvcwINVf	CATAACAGCTAGGGTTTATCC
	VvcwINVr	ATAAATTTGCAAAAGCCTGGC
SUC12	VvSUC12 F	CCTAGCAGCTGGTGTTATTGC
	VvSUC12 R	CGTACGGATGATAGTAGAACCAC
Ubiquitin	Vv Ubiq F1	AACCTCCAATCCAGTCATCTAC
	VvUbiq 1R	GTGGTATTATTGAGCCATCCTT
Actin	VvActinF1	TCCTTCGTCTTGACCTTGCT
	VvActinR1	AACGGAATCTCTCAGCTCCA
EF1	RT EF1 alpha.Fwd	GAACTGGGTGCTTGATAGGC
	RT EF1 alpha.Rev	AACCAAAATATCCGGAGTAAAAGA
GAPDH	RT GAPDH.Fwd	TTCTCGTTGAGGGCTATTCCA
	RT GAPDH.Rev	CCACAGACTTCATCGGTGACA

Quantitative PCR (qPCR) was performed using two technical reps with a LightCycler 480 (Roche) in a reaction volume of 7.5 µL containing 3.75 µL SYBR Green (Roche), 0.5 µM of each primer and 2.5 µL cDNA from each of the four biological reps. The PCR cycle profile was as follows: 1 cycle of 5 min at 95°C, 45 cycles of 20 sec at 95°C, 20 sec at 58°C, 20 sec at 72°C, and a final cycle of 5 min at 72°C. Melting curves were performed by elevating the temperature to 95°C for 15 sec, then cooling to 50°C for 45 sec and elevating again to 95°C at a rate of 0.11°C/sec.

Reference genes (housekeeping genes – *HKG* including *Ubiquitin*, *Elongation Factor1* (*EF1*), *Actin*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)) were used to determine the relative expression of the genes of interest. Where multiple reference genes were used, the geometric mean of the concentration of each housekeeping gene was determined. The concentration of the target gene was then divided by the geometric mean of the housekeeping genes for each individual sample.

5.3 Results

Samples were collected every two weeks from flowering through to harvest at all sites. Based on results from the berry analysis and subsequent wine sensory results where differences were consistently observed, pertinent samples, stored at -80^{o}C, were selected for analysis. Chamber samples from experiments corresponding to some of the more interesting results were also analysed. In most cases no consistent differences in gene expression were observed and while this is a large body of work, only some results are shown where small differences were observed.

5.3.1 Early defoliation treatment T2

In some wine samples from T2 treatments there were increases in the tannins which translated as texture in the sensory analysis, resulting in a preference for the wines made from T2 fruit (Figure 3.9). To investigate whether a molecular marker could be identified flagging these changes, flower and very early berry samples from the early defoliation treatments (T2) were subjected to RNA extraction, cDNA synthesis and qPCR to determine changes in the expression of key genes. In season 3, a pre-flowering CO_2 scrubbing chamber experiment was conducted to emulate T2 treatments without stripping the leaves off the canes. A simultaneous T2 treatment was conducted in potted vines alongside the chambers. Gene expression was also studied in these chamber samples.

5.3.1.1 *FLS* expression - The transcript levels of the *FLS* gene were determined in flowers and early berries and shown in Figure 5.3 (season 1). In most cases there is a small increase in expression of *FLS* after the defoliation compared to the control, especially in the Hilltops samples after seven days, and this trend was maintained over several weeks as *FLS* expression gradually goes down as the berries develop.

In season 2, flower samples were taken 8-14 days after treatment, though Murray Valley was the only site where *FLS* expression was higher in the T2 samples (Figure 5.4).

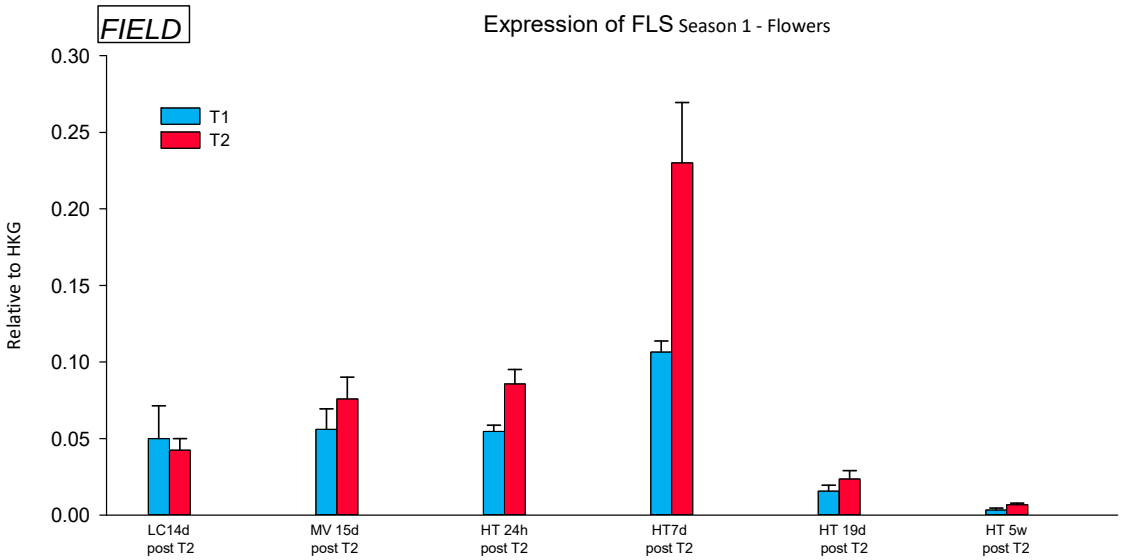


Figure 5.3 *FLS* expression in very young fruit two weeks after T2 treatment was applied at Langhorne Creek (LC) and Murray Valley (MV) and in a developmental series from Hilltops (HT) from one day after treatment (flower buds) to five weeks later (small berries). Control T1 is in blue, T2 is red.

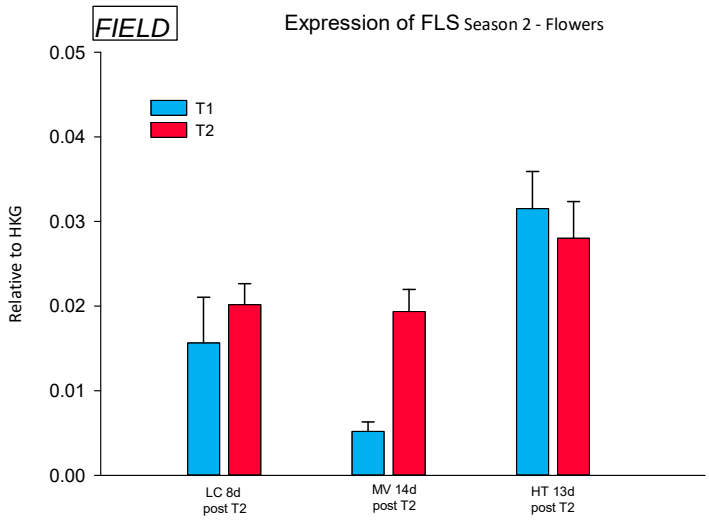


Figure 5.4 Transcript levels of *FLS* in flower samples from all three sites in season 2. Samples were taken 8-14 days after the application of the treatment.

Figure 5.5 shows FLS expression in flower samples from all three sites. After treatment, at all three sites there is an increase in FLS expression compared to the day before. The control samples post treatment were not taken for Langhorne Creek or Murray Valley. This higher trend of gene expression is maintained in subsequent samples 11-15 days later.

In season 3, the chamber experiment (Fruit set) included a 'T2 treatment' on potted vines carried out at the same time as the ambient and low CO_2 chambered vine experiment began. *FLS* expression increased only in the vines that were subjected to the defoliation and there was no difference in the chambers (figure 5.6). Twelve days after the experiment start, *FLS* expression had decreased in all samples; however, the chamber samples were lower suggesting that the plastic of the chamber reduced light to some extent.

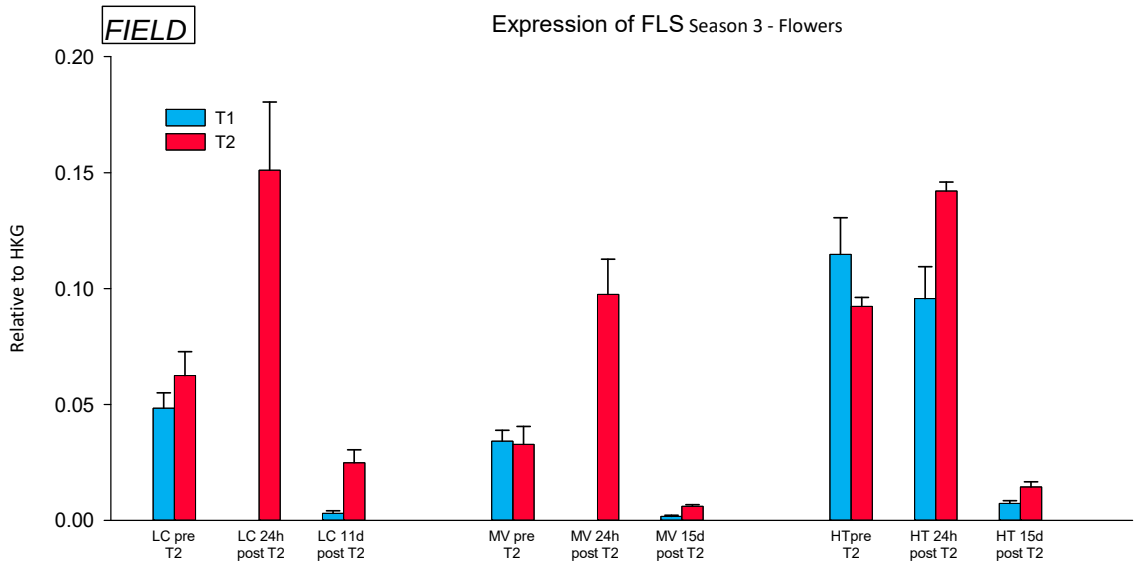


Figure 5.5 Transcript levels of *FLS* in flower samples from all three sites in season 3. Samples were taken before the treatment, 24 hours later (no controls were collected for Langhorne Creek or Murray Valley), then 11-15 days later.

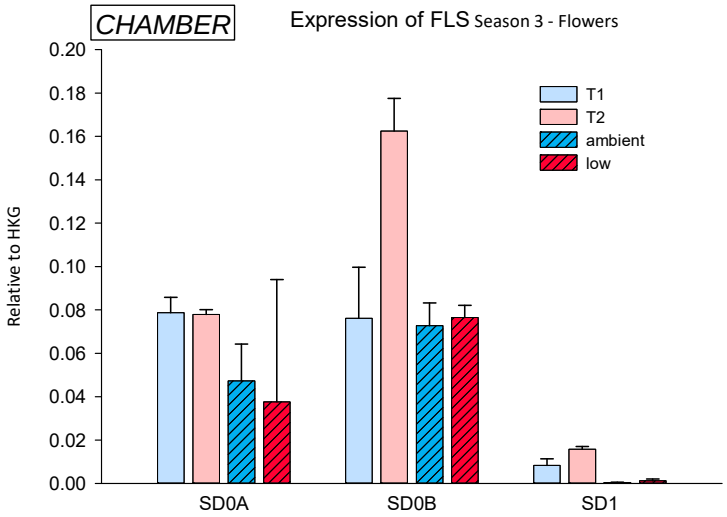


Figure 5.6 *FLS* gene expression in fruit set chamber experiment fruit. T1 and T2 – pot grown vines. SD0A samples were taken the day before scrubbing started, SD0B two days into the experiment and SD1 twelve days after the start of the CO2 scrubbing.

5.3.1.2 *ANR* expression - As there was an increase in tannins particularly in season 3 Langhorne Creek wines, the expression of *ANR* (Figure 5.7) was measured. There were no consistent differences observed as although there was a small increase in *ANR* expression in Murray Valley and Hilltops samples about two weeks after the treatment was applied, expression was lower in the equivalent Langhorne Creek samples.

The Fruit set chamber samples were analysed for *ANR* expression (Figure 5.8) which confirmed that there were no changes in gene expression consistent with the treatment.

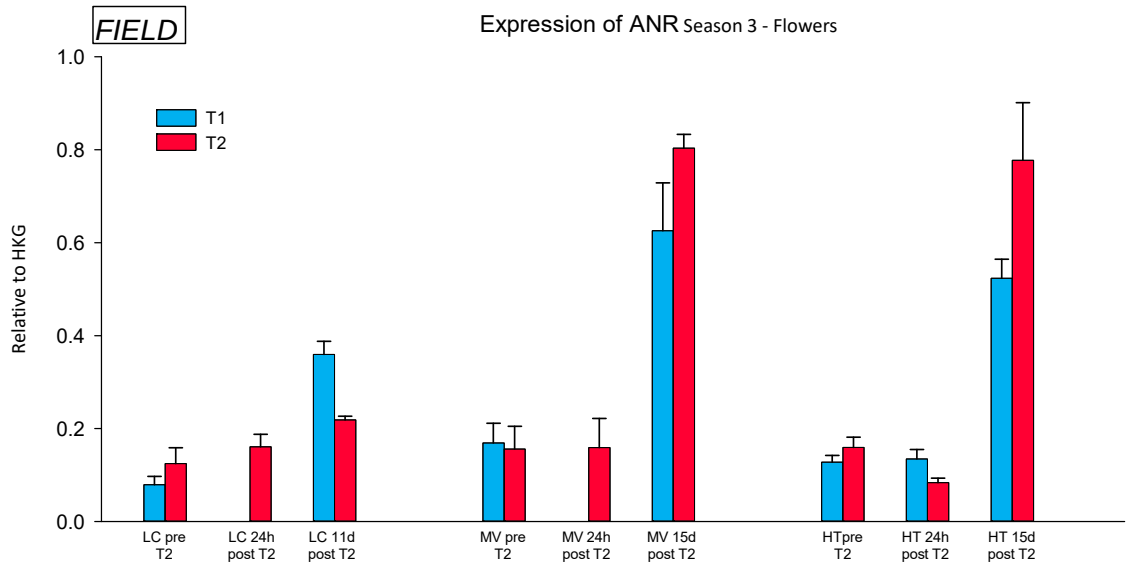


Figure 5.7 Expression levels of *ANR* in flowers in season 3 from all three field sites.

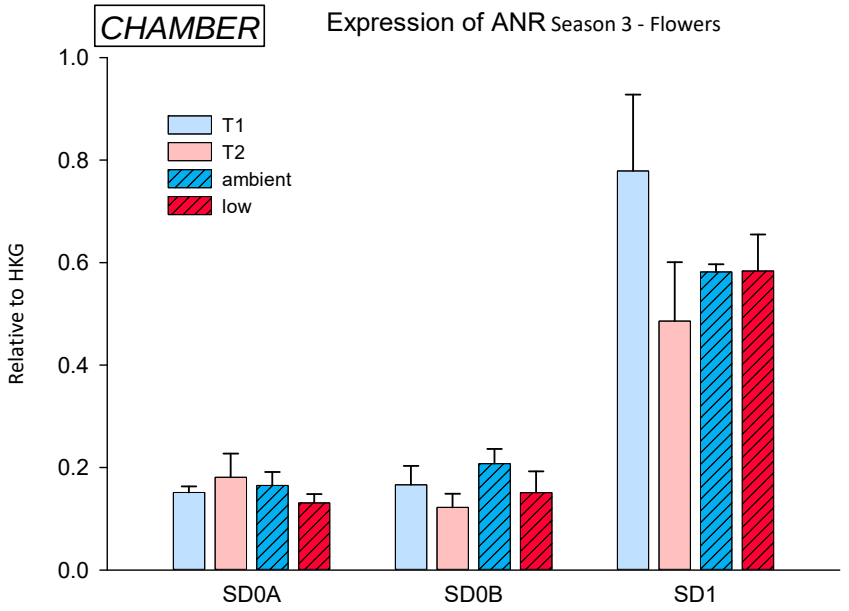


Figure 5.8 Levels of *ANR* expression in the chamber Fruit set experiment. T1 and T2 – pot grown vines. SD0A samples were taken the day before scrubbing started, SD0B two days into the experiment and SD1 twelve days after the start of the CO_2 scrubbing.

5.3.1.3 *MYBA* expression - The expression of *MYBA* is strongly linked to véraison and shortly afterwards as it controls UFGT expression, encoding the first step in the anthocyanin synthesis pathway. Though the expression levels of *MYBA* are low in flowers and early berries, there is a suggestion from our experiments that *MYBA* expression might be higher in T2 samples compared to the controls (Figure 5.9). However, this was not shown in the chamber experiment, where expression of MYBA was about 1000-fold less than at véraison and not very different between ambient and CO_2 samples (figure 5.10).

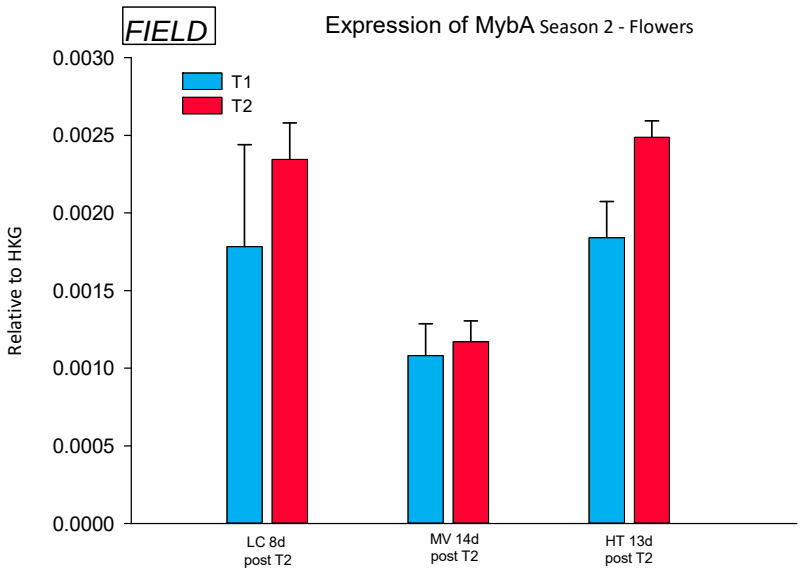


Figure 5.9 Expression levels of the anthocyanin regulator gene *MYBA* in samples from field experiments in season 2. Samples were taken 1-2 weeks after the T2 treatment was applied.

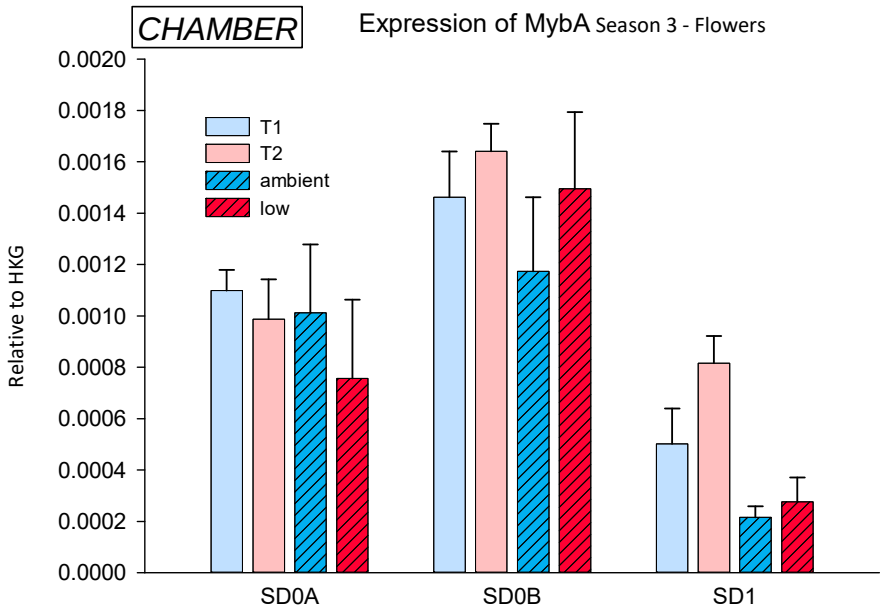


Figure 5.10 Expression of *MYBA* in Fruit set chamber experiment. T1 and T2 – pot grown vines. SD0A samples were taken the day before scrubbing started, SD0B two days into the experiment and SD1 twelve days after the start of the CO_2 scrubbing.

5.3.1.4 Conclusion – T2 treatment - In season 1, the expression of a large number of genes was tested including those shown above plus *LAR1* and *LAR2*, *MYBPA1* and *MYBPA2*, *LDOX*, *TDS6*, and *MYBF2* in samples taken about seven days after T2 was applied (data not shown). *MYBA* expression was higher but only at one site and is generally much lower than at véraison by about 100-fold. *MYBPA1* and *MYBF2* expression were both very low compared to the housekeeping genes. While the other genes were expressed at levels similar to the housekeeping genes, there was no consistent pattern across the sites. Of the genes studied, *FLS* provides the best opportunity to develop a molecular marker as the expression of this

gene was generally higher in samples after T2 and this trend was maintained even after several weeks.

5.3.2 Late defoliation treatment T4

Applied just before véraison, this treatment involved hedging the vines removing shoots and exposing fruit to more sunlight than the control. Harvest was delayed by at least several days indicating a change in phenology. In some T4 wine samples, anthocyanin levels were lower than the control, despite the harvest being at similar sugar levels.

5.3.2.1 Expression of sugar genes - Gene expression studies were carried out to try to identify particular differences that could relate to the wine data. The expression of four genes involved in sugar transport in berries was measured in whole berry samples collected two weeks after the treatment was applied and four weeks later in season 1 at all three sites. *HT5* and *TMT1* levels were higher in the later samples while *cwINV* was lower in the later samples; however, there was no consistent difference between the control and T4 samples (data not shown). The only gene that had a consistent pattern was *HT2* which was a little higher in the later T4 samples (figure 5.11).

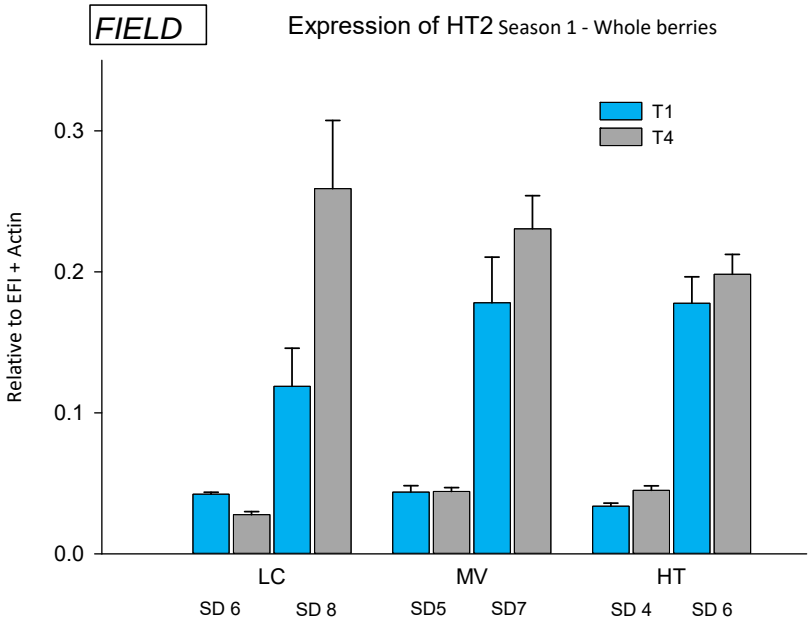


Figure 5.11 Expression of HT2 in whole berry samples from season 1 at all three sites.

By comparison in a similar chamber experiment, a reduction in *HT2* expression is indicated particularly at SD3 (figure 5.12).

5.3.2.2 Expression of anthocyanin genes - Genes involved in anthocyanin synthesis were also measured in a series of skin samples during ripening from Murray Valley collected in season 3. LDOX is the step before UFGT and can contribute to tannin synthesis. In accordance with this, expression of *LDOX* was measurable before véraison (figure 5.13), compared to *UFGT* (figure 5.14). The most interesting result was the reduced expression of *UFGT* particularly in the last sample. This could account for the lower levels of anthocyanins found in T4 harvest berry and wine samples.

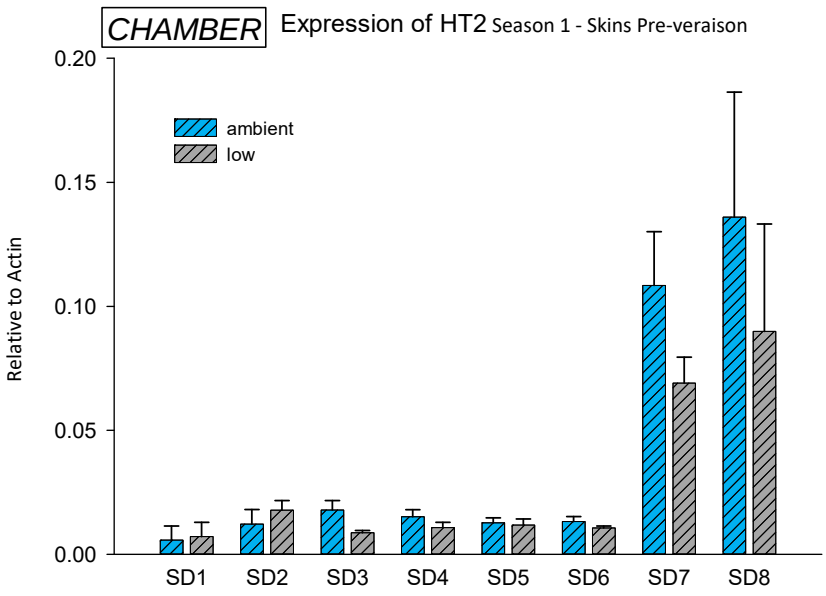


Figure 5.12 Expression of HT2 in skin samples Pre-véraison experiment from season 1. Samples were taken from the beginning of scrubbing at two day intervals except SD8 which was five days later. Scrubbing started at SD1 and finished at SD5.

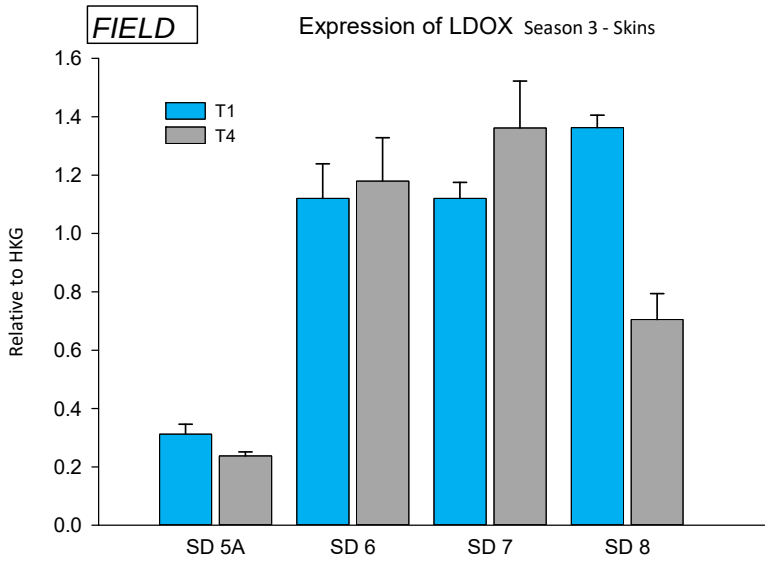


Figure 5.13 Expression of *LDOX* in skins season 3 from Murray Valley. Treatment was applied at SD5A, sampled every two weeks and harvested just after SD8.

Expression of *MYBA* was measured in the same samples (Figure 5.15). While expression was lower earlier in the T4 ripening samples, in the final samples expression levels were very similar.

5.3.2.4 Conclusion – T4 treatment - Expression pattern of *UFGT* was consistent with the reduction of anthocyanin observed in some T4 harvest samples; however, the only sugar gene with consistent changes showed a higher expression level. Its expression might be upregulated by the reduction of available sugars following the treatment. This result provides a basis for future experiments.

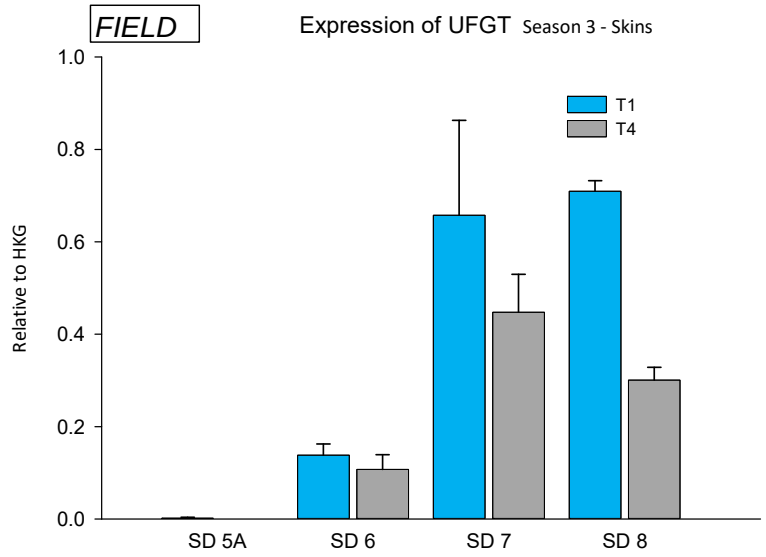


Figure 5.14 Expression of UFGT in skins season 3 from Murray Valley. Treatment was applied at SD5A, sampled every two weeks and harvested just after SD8.

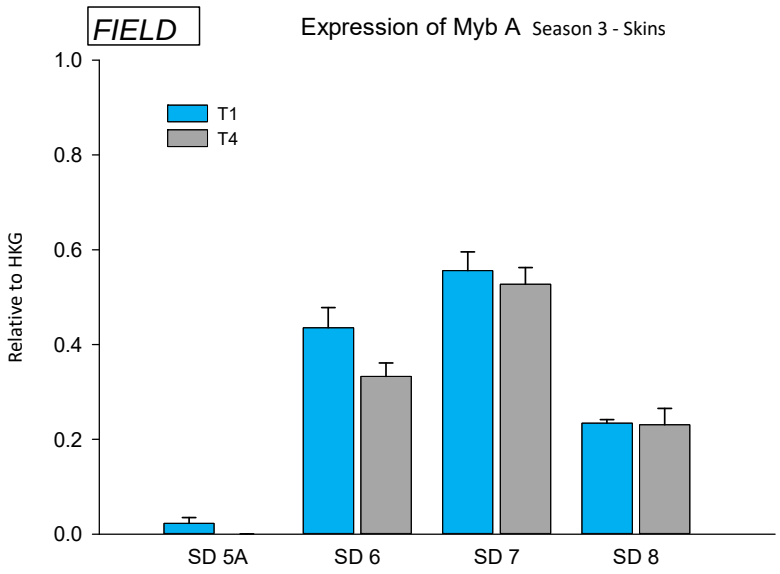


Figure 5.15 Expression of *MYBA* in skins season 3 from Murray Valley. T4 Treatment was applied at SD5A, sampled every two weeks and harvested just after SD8.

5.3.3 Fruit removal treatment T3

This treatment did not have a big effect on berry flavonoids despite reducing yield very significantly and altering harvest dates (Table 2.5). RNA was extracted from samples of whole berries, skins and seeds from Murray Valley and analysed for expression of flavonoid genes. After véraison, *MYBA* and *UFGT* levels were both a little higher in the T3 samples than the control consistent with an acceleration of ripening (data not shown).

5.4 Discussion

Over many years there has been a number of studies where leaves or fruit have been removed or manipulated. In some cases anthocyanins (Petrie and Clingeleffer, 2006) and other flavonoids in berries were measured as an indication of quality (Pastore et al., 2017) and sometimes wines have also been made and analysed for flavonoid content (King et al., 2012).

The early defoliation T2 treatment exposed the fruit to light early in the development of the berry leading to improved wine structure by altering tannin concentration. Although genes for tannin synthesis did not alter much in the samples we analysed, this does not preclude higher levels of transcripts for ANR and LAR in the treatment berries at times when they were not sampled, for example during the night or 2-7 days after treatment. It is also possible that tannin extractability is altered rather than the total amount of tannin (Sivilotti et al., 2016).

However flavonol synthesis is well-known to be induced by light via the upregulation of the gene encoding the enzyme flavonol synthase (Czemmel et al., 2009). While the role of flavonols in wine quality is still unclear, Ristic et al., 2007, demonstrated that there was a good correlation with the flavonol content of the berry and wine quality. In our experiments *FLS* gene expression was generally higher in T2 samples and this difference, while not large, was maintained for several weeks. Other groups have also found similar results for *FLS* (Pastore et al., 2013). In this study Pastore et al., 2013, performed transcriptomic microarray studies and discovered that from pre-bloom leaf removal samples collected at the beginning and end of véraison and at harvest, *FLS* expression was higher in at all three time points compared to controls. In a later publication (Zenoni et al., 2017), they identified three genes that had the potential to be molecular markers for the effect of the early defoliation, which included *FLS* and two genes involved in stress responses. If developing this marker further was considered important, a very thorough sampling regime in field samples would need to be implemented to identify the best times of day and timing after the treatment to maximise the small differences seen in our experiments and that of others (Zenoni et al., 2017).

In the pre-flowering chamber experiment, *FLS* expression did not seem to alter, suggesting that *FLS* expression changes seen in the field experiment are the result of sun exposure and not source-sink relationship differences.

Other components that provide flavour and aroma that can also be light regulated include linalool synthesis and the transcription of genes involved in this pathway have been studied in shaded fruit (Erpeng et al., 2017) providing another possibility for developing molecular markers for quality. However, until flavour differences are described and identified in treatments, this area of research would be difficult to carry out effectively.

Exposing fruit around véraison to sunlight by leaf removal or manipulation can have different effects. Although *FLS* and *UFGT* expression increased after leaf removal post-véraison, the final concentration of flavonols and anthocyanins was lower in ripe samples (Matus et al., 2009). One possibility is that exposed fruit are subject to higher temperature which could lead to a reduction in flavonoids (Mori et al., 2007). In our experiments, T4 treated samples generally had a reduction in anthocyanins at harvest and in the wines, suggesting an uncoupling of the anthocyanin:sugar connection also seen by others (Zhuang et al., 2014).

While they did not study gene expression in their samples, our results suggest that *UFGT* expression is decreased in T4 leading to the reduction in anthocyanins.

Since T3 was often harvested several days earlier than the control, some differences related to phenology of the berry could be expected. However, no consistent differences were detected in gene expression patterns. Others have also reported that quality was not greatly altered with crop thinning but it was damaging economically.

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Chapter 6
Conclusions and recommendations

6.1 The role of vine balance in determining berry and wine composition

The central theme of this project was to attempt to determine whether vine balance directly affects fruit composition and whether this is reflected in wine composition. The difficulty in making that determination robustly is demonstrated in the large literature that has examined the relationship between various vine vigour properties and their effect on yield and fruit composition over many decades, albeit rarely with this precise intention.

Our approach to clarify the vine balance concept has been three-fold. Firstly, we have utilised three sites, with different climates and production aims, resulting in different vine balance, but the same variety and similar trellis/management. As with much previous work in this area, this approach (taken in isolation) is confounded by those climate differences (Chapter 2). Secondly, we have applied vine management strategies at those vineyards that aimed to alter vine balance and applied the same strategies in the same way across all three vineyards. This allowed us to combine similar management at different vine balance values (comparing sites) and different vine balance values (comparing management strategies) at the same site (Chapter 2). In addition, we have taken the effect of a particular change in vine balance (decreased canopy, unchanged yield) and simulated it artificially without altering the bunch environment. This was achieved through the reduced CO_2 chamber experiments (Chapter 4). Finally, in all cases the fruit from these experiments was used for winemaking and the wines assessed chemically and, for the field experiments, through sensory analysis.

Anthocyanin content was perhaps the berry component most responsive to site and treatment differences. Berry colour is also a key commercial quality parameter for fruit and of importance to wine quality. Comparing berry anthocyanin (Figure 2.13) or wine colour density (Figure 3.12) to vine balance suggests a superficial relationship in both cases. However, drilling down through the data set demonstrates, in both cases that the major driver of this relationship is vineyard site (Figures 2.14 & 3.13). It is arguable whether vine balance drives the site differences in fruit composition, or whether it is itself a consequence of the site environment. Irrespective, the vineyard manager is unable to change much of the vineyard site environment at will and there are many factors governing choice of site for a vineyard that are outside of the consideration of this project. The important question is whether a vineyard manager can produce a commercially beneficial change to the fruit composition within a site. Across the three sites and three seasons, the shifts to vine balance generated by the treatments imposed were fairly consistent, irrespective of the vine balance present at the site (T1 vines). Reducing vine balance (lower crop load to canopy size; T3) had little or no effect on fruit or wine composition. Increasing vine balance (higher crop load to canopy size; T4) resulted in a reduction in anthocyanin content.

The direct manipulation of carbohydrate availability to the fruit using the chamber experiment found that increasing vine balance during the maturation period (higher crop load to carbohydrate availability) had no effect on anthocyanin content for a given fruit sugar concentration. Considered together with field data, the results from this project suggest that

where the environment is adequate for the vine to ripen the fruit within a growing season, there is no impact of vine balance on fruit anthocyanin, phenolic or tannin concentration.

It is also notable that most of our vines had a yield to canopy ratio outside of the common recommendations made in other viticultural regions of the world (e.g. Kliewer and Dokoozlian, 2005), yet were always able to bring the fruit to commercial maturity (24°Brix) and did not show a reduction (T4 excepted) in the concentration of key compounds. Further to this, we observed significant differences in the effects of treatments (T2 and T4) in extracting anthocyanins.

It is possible that the failure to find a within-site link between vine balance and fruit composition was due to an inappropriate definition of vine balance. As already stated, canopy size (or leaf area) is used as a surrogate for potential carbon fixation, which is, in turn, a function of canopy size, canopy structure, solar radiation, temperature and a number of other variables. An alternative would be to directly measure seasonal photosynthetic carbon gain and use yield per unit carbon uptake for a definition of vine balance. Such an approach would also need to consider the source-sink relationship within vines, particularly during maturation. However, this is not practical for a scientific study, let alone a commercial tool.

6.2 Evaluation of crop and canopy management strategies and their potential for advancing colour relative to sugar

A secondary aim of the work was to evaluate vineyard management practices that might advance fruit colour relative to sugar at commercial maturity. Furthermore, to use manipulations that are being used or trialed elsewhere in the world but are not in use in Australian vineyards with a 'sprawl' canopy.

Crop removal (T3) is in relatively common use in Australia, typically with the aim of improving fruit composition, particularly fruit colour. Where vine balance is so extremely high that a crop cannot be adequately ripened it would be expected that this would indeed be the case, however, this extreme was not encountered in any of the sites or seasons examined here. Conversely, the crop removal treatments typically resulted in a vine balance below common recommendations, leading to a rapid maturation rate due to the higher availability of assimilates for the remaining grapes. This is a situation that, from a climate warming perspective, is anecdotally associated with reduced fruit colour and quality. In the current work, there was no statistically significant impact of crop removal on the anthocyanin to sugar ratio at harvest, suggesting that in the vineyards where the work was undertaken (representative of the majority of Australian viticulture), there was no commercial benefit to this practice. It should be noted, however, that changing maturation rate may have logistical benefits to an enterprise, by spreading the harvest period, and that the crop removal undertaken in this project was pre-véraison and not selective. Selective bunch thinning, where the removed bunches are selected as being anomalous, highly variable or similar, may have impacts on the composition of harvested fruit, but these would be due to the selective nature of the fruit removal and not due to an impact on vine balance.

Late defoliation (T4), also known as severe summer pruning, is a practice being trialed in Italy and Germany on both warm climate red fruit and cool climate white fruit to lengthen maturation periods that are otherwise being shortened by a changing climate. This again may

have logistical benefits, but is also expected to result in a more commercially attractive fruit composition due to the longer maturation period. In particular, it might be expected that the longer maturation period would lead to improved anthocyanin content. Conversely, in all cases here, the anthocyanin to sugar ratio at harvest was reduced in the late defoliation treatment fruit, with a similar impact on the wine. It is likely, given the chamber results, that this is a result of the change in bunch environment, particularly sun exposure, but there is no evidence from this project that late-defoliation provides any beneficial effect on fruit composition. The chamber experiments suggest that extending maturation rate *per se*, does not alter anthocyanin or other phenolic content of the fruit either.

Perhaps the most interesting treatment was the early-defoliation (T2), which is being extensively investigated in Italy as a means of controlling yield by reducing fruit set. Implemented in 'sprawl' vineyards, it appears to have no consistent effect on vine balance, but did consistently improve the anthocyanin to sugar ratio and tannin content. The current method of implementation, hand leaf plucking, is impractical commercially, in the vineyards used, but the strategy of early-defoliation for not only limiting yield slightly, but also improving fruit quality appears sound, based on the three sites and seasons for which data are presented here. Increased anthocyanin and tannin concentrations were reflected in T2 wines, which were the preferred wines upon sensory analysis.

6.3 Molecular mechanisms and potential for early season indicators

A further aim of the project was to examine the potential for molecular or physiological markers that would indicate situations where crop or canopy manipulation would be commercially beneficial. This aim was based on anecdotal impressions that crop removal was successful in some seasons and not others. In the results here, (non-selective) crop removal was rarely if at all beneficial to fruit composition and certainly not commercially viable. Consequently, no such indicator could be assessed. However, there were changes in gene expression for the T4 treatment, with *UFGT* expression reduced while *HT2* expression increased during ripening, suggesting that expression of these two genes may potentially be useful as an indicator of future anthocyanin content compared to sugar accumulation. However, as anthocyanin synthesis continues throughout ripening (driven by the enzyme encoded by *UFGT*) while sugars are continuing to accumulate, it is not easy to determine how expression of these genes could be utilised. For early defoliation FLS expression, probably driven by greater light exposure in flowers and young fruit, has the potential to be an indirect marker for increased tannins and anthocyanins.

6.4 Recommendations

The results of this project are fairly unequivocal with regard to the role of vine balance in directly driving berry composition, though the detailed impact of vine balance and vineyard management of it in situations where climate may not allow a crop to ripen, were not addressed. However, the field sites and situations examined were representative of a large part of the Australian industry, where fruit can be ripened with little or no management of canopy to crop ratio.

The interaction between selective bunch thinning (e.g. immediate post-veraison removal of bunches with a high proportion of green berries) and vine balance was also not examined, though it is likely this interaction is minimal. Similarly, shoot thinning implemented in spring

has not been investigated in this work, which would be likely to have more of an influence on bunch exposure than vine balance, depending on the fruitfulness of the shoots removed.

More likely to be of wide impact in the industry is further examination of the role of bunch environment on fruit composition, in particular its management in high production 'sprawl' vineyards. The positive effect of the T2 early defoliation treatment (in high and low production vineyards) demonstrates the potential for improved management options of this type and there is a possibility of developing a molecular marker that could be applied early to indicate the potential increase in berry flavonoids. In particular, an interesting area of practical research may be to adapt and test air jet leaf removal machinery to a sprawl canopy. However, the input cost of any approach to alter vine balance and/or bunch exposure would have to be carefully assessed against the benefits of an increased wine value.

Appendix 1

Communication

Communication to industry and the scientific community has been an integral part of this project. Results have been presented at a range of industry and scientific meetings throughout the life of the project.

Industry Publications

2014 E.J. Edwards, A.R. Walker, P.R. Clingeleffer, J. Smith, B. Holzapfel, C. Barril, et al., Collaborative research projects to determine the role of vine balance in driving fruit composition and develop a 'toolbox' for industry. *Grapegrower & Winemaker* Feb 2014.

2015 E.J. Edwards, P.R. Clingeleffer, A.R. Walker, J. Smith, B. Holzapfel, C. Barril. Understanding the influence of vine balance on berry composition: 2013-14 season project update *Wine and Viticulture Journal* **3**:43-48.

Published Conference Proceedings

2016 E.J. Edwards, J. Smith, A. Walker, C. Barril, A. Boettcher, D. Foster, J. Gouot, P.R. Clingeleffer, B. Holzapfel. Targeted manipulation of vine balance: does vine balance directly affect fruit composition? *Proceedings of the Sixteenth Australian Wine Industry Technical Conference* 96-100.

Scientific Conferences

2015 U. Knauer, E.J. Edwards, A. McGrath, W. Lief, J.M. Hacker, U. Seiffert. Towards grape-vine management based on mapping of airborne hyperspectral images. The 9th International Symposium on Mobile Mapping Technology, Sydney, December 2015.

2017 J. Gouot, E.J. Edwards, A. Walker, J. Smith, B. Holzapfel, C. Barril. Investigation of the effect of vine balance on grape and wine phenolic composition: How to overcome vineyard variability with chemometrics. Topics in Chemometrics, Newcastle, April 2017.

2017 J. Smith, E.J. Edwards, J. Gouot, C. Barril, A. Walker, D. Foster and B. Holzapfel. Manipulating source-sink relations with low CO_2 slows ripening of Shiraz grape berries but does not uncouple anthocyanin production from sugar import. COMBIO, Adelaide, November 2017.

2017 E.J. Edwards, J. Smith, A. Walker, C. Barril, A. Boettcher, D. Foster, J. Gouot, B.Holzapfel. To what extent does vine balance actually drive fruit composition? Crush 2017, Adelaide, November 2017.

2018 B. Holzapfel, J. Smith, J. Gouot, C. Barril, A. Walker, E.J. Edwards. Effect of severe summer pruning, leaf and bunch removal on nitrogenous compounds in Shiraz must in Australia. International Symposium on Viticulture: Primary Production and Processing, Istanbul, August 2018 (oral presentation accepted).

Industry workshops and presentations

2013 E.J. Edwards. Assessing and understanding vine balance – taking new research directions at Workshop W41 Vine Balance. 15th Australian Wine Industry Technical Conference, Sydney, July 2013.

2016 E.J. Edwards, J. Smith, A. Walker, C. Barril, A. Boettcher, D. Foster, J. Gouot, B. Holzapfel. Targeted manipulation of vine balance 1. Does vine balance directly affect fruit composition? 16th Australian Wine Industry Technical Conference, Adelaide, July 2016.

2016 J. Smith, E.J. Edwards, J. Gouot, C. Barril, A. Walker, B. Holzapfel. Targeted manipulation of vine balance 2. A whole-vine chamber system for controlling berry sugar accumulation. 16th Australian Wine Industry Technical Conference, Adelaide, July 2016.

2016 J. Gouot, C. Barri, B. Holzapfel, E.J. Edwards, A. Walker, J. Smith. Targeted manipulation of vine balance 3. Effect of carbohydrate supply on berry sugar/anthocyanin accumulation. 16th Australian Wine Industry Technical Conference, Adelaide, July 2016.

2016 E.J. Edwards, J. Smith, A. Walker, C. Barril, A. Boettcher, D. Foster, J. Gouot, P.R. Clingeleffer, B. Holzapfel. Targeted manipulation of vine balance: does vine balance directly affect fruit composition? AWRI Webinar, November 2016.

2017 Vine balance: what it is, what it does and how to measure it. Workshops held in the Barossa, 30th November 2017 and Langhorne Creek, 1st December 2017. E.J. Edwards, B. Holzapfel, C. Barril presenters.

Appendix 2

Intellectual property

The information generated by this project will enter the public domain and will be accessible for future reference.

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Appendix 3

References

Abrahams S, Tanner GJ, Larkin PJ, Ashton AR (2002) Identification and biochemical characterization of mutants in the proanthocyanidin pathway in Arabidopsis. Plant Physiology **130**: 561-576

Adams DO (2006) Phenolics and ripening in grape berries. American Journal of Enology and Viticulture **57:** 249-256

Afoufa-Bastien D, Medici A, Jeauffre J, Coutos-Thevenot P, Lemoine R, Atanassova R, Laloi M (2010) The Vitis vinifera sugar transporter gene family: phylogenetic overview and macroarray expression profiling. BMC Plant Biology **10:** 245

Baiano A, De Gianni A, Previtali MA, Del Nobile MA, Novello V, de Palma L (2015) Effects of defoliation on quality attributes of Nero di Troia (Vitis vinifera L.) grape and wine. Food Research International **75:** 260-269

Barril C, Clark AC, Scollary GR (2012) Chemistry of ascorbic acid and sulfur dioxide as an antioxidant system relevant to white wine. Analytica Chimica Acta **732:** 186-193

Bogs J, Downey MO, Harvey JS, Ashton AR, Tanner GJ, Robinson SP (2005) Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. Plant Physiology **139:** 652-663

Bogs J, Ebadi A, McDavid D, Robinson SP (2006) Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. Plant Physiology **140:** 279-291

Bogs J, Jaffe FW, Takos AM, Walker AR, Robinson SP (2007) The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. Plant Physiology **143:** 1347-1361

Boss PK, Davies C, Robinson SP (1996) Expression of anthocyanin biosynthesis pathway genes in red and white grapes. Plant Molecular Biology **32:** 565-569

Bouveresse DJ-R, Pinto RC, Schmidtke L, Locquet N, Rutledge DN (2011) Identification of significant factors by an extension of ANOVA–PCA based on multi-block analysis. Chemometrics and Intelligent Laboratory Systems **106:** 173-182

Bubola M, Sivilotti P, Janjanin D, Poni S (2017) Early Leaf Removal Has Larger Effect than Cluster Thinning on cv. Teran Grape Phenolic Composition. American Journal of Enology and Viticulture**:** ajev. 2016.16071

Bureau SM, Baumes RL, Razungles AJ (2000) Effects of vine or bunch shading on the glycosylated flavor precursors in grapes of Vitis vinifera L. Cv. Syrah. Journal of Agricultural and Food Chemistry **48:** 1290-1297

Cadot Y, Chevalier M, Barbeau G (2011) Evolution of the localisation and composition of phenolics in grape skin between veraison and maturity in relation to water availability and some climatic conditions. Journal of the Science of Food and Agriculture **91:** 1963-1976

Castañeda-Ovando A, Pacheco-Hernández MdL, Páez-Hernández ME, Rodríguez JA, Galán-Vidal CA (2009) Chemical studies of anthocyanins: A review. Food Chemistry **113:** 859-871

Castellarin S, Bavaresco L, Falginella L, Gonçalves M, Di Gaspero G (2012) Phenolics in grape berry and key antioxidants. *In* The Biochemistry of the Grape Berry. Bentham Science Publishers Ltd, pp 89-110

Clingeleffer PR (2010) Plant management research: status and what it can offer to address challenges and limitations. Australian Journal of Grape and Wine Research **16:** 25-32

Clingeleffer PR, Petrie P, Dunn G, Martin S, Krstic M, Welsh M (2005) Crop control for consistent supply of quality winegrapes. *In*, Final report to GWRDC

Coombe BG (1995) Growth stages of the grapevine: adoption of a system for identifying grapevine growth stages. Australian Journal of Grape and Wine Research **1:** 104-110

Czemmel S, Stracke R, Weisshaar B, Cordon N, Harris NN, Walker AR, Robinson SP, Bogs J (2009) The Grapevine R2R3-MYB Transcription Factor VvMYBF1 Regulates Flavonol Synthesis in Developing Grape Berries. Plant Physiology **151:** 1513-1530

Dai ZW, Meddar M, Renaud C, Merlin I, Hilbert G, Delrot S, Gomes E (2014) Long-term in vitro culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. Journal of Experimental Botany **65:** 4665-4677

Davis CR, Wibowo D, Eschenbruch R, Lee TH, Fleet GH (1985) Practical implications of malolactic fermentation: A review. American Journal of Enology and Viticulture **36:** 290-301

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Merillon JM, Hamdi S (2006) Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiology **140:** 499-511

Deluc L, Bogs J, Walker AR, Ferrier T, Decendit A, Merillon JM, Robinson SP, Barrieu F (2008) The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiology **147:** 2041-2053

Di Ferdinando M, Brunetti C, Fini A, Tattini M (2012) Flavonoids as antioxidants in plants under abiotic stresses. *In* Abiotic Stress Responses in Plants. Springer, pp 159-179

Di Profio F, Reynolds AG, Kasimos A (2011) Canopy management and enzyme impacts on Merlot, Cabernet franc, and Cabernet Sauvignon. I. Yield and berry composition. American journal of enology and viticulture**:** ajev. 2010.10024

Dokoozlian N, Ebisuda, N and Cleary, M (2011) Some new perspectives on the impact of vine balance on grape and wine flavour development. 17th GiESCO Symposium, Asti, Italy, August 29th-Sept. 2nd.

Downey MO, Dokoozlian NK, Krstic MP (2006) Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: a review of recent research. American Journal of Enology and Viticulture **57:** 257-268

Downey MO, Harvey JS, Robinson SP (2003) Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (Vitis vinifera L.). Australian Journal of Grape and Wine Research **9:** 110-121

Downey MO, Rochfort S (2008) Simultaneous separation by reversed-phase high-performance liquid chromatography and mass spectral identification of anthocyanins and flavonols in Shiraz grape skin. Journal of Chromatography A **1201:** 43-47

Drappier J, Thibon C, Rabot A, Geny-Denis L (2017) Relationship between wine composition and temperature: Impact on Bordeaux wine typicity in the context of global warming. Critical Reviews in Food Science and Nutrition**:** 1-17

Erpeng ZP, Chai FM, Zhang HH, Li SH, Liang ZC, Fan PG (2017) Effects of sunlight exclusion on the profiles of monoterpene biosynthesis and accumulation in grape exocarp and mesocarp. Food Chemistry **237:** 379-389

Harrington PdB, Vieira NE, Espinoza J, Nien JK, Romero R, Yergey AL (2005) Analysis of variance–principal component analysis: A soft tool for proteomic discovery. Analytica chimica acta **544:** 118-127

Harris JM, Kriedemann PE, Possingham JV (1968) Anatomical aspects of grape berry development. . Vitis **7:** 106-119

Harris NN, Luczo JM, Robinson SP, Walker AR (2013) Transcriptional regulation of the three grapevine chalcone synthase genes and their role in flavonoid synthesis in Shiraz. Australian Journal of Grape and Wine Research **19:** 221-229

Hayes MA, Davies C, Dry IB (2007) Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: differential roles in sink and source tissues. Journal of Experimental Botany **58:** 1985-1997

Hayes MA, Feechan A, Dry IB (2010) Involvement of Abscisic Acid in the Coordinated Regulation of a Stress-Inducible Hexose Transporter (VvHT5) and a Cell Wall Invertase in Grapevine in Response to Biotrophic Fungal Infection. Plant Physiology **153:** 211-221

Holzapfel BP, Smith JP, Mandel RM, Keller M (2006) Manipulating the postharvest period and its impact on vine productivity of Semillon grapevines. American Journal of Enology and Viticulture **57:** 148-157

Iland P, Ewart A, Sitters J, Markides A, Bruer N (2000) Techniques for chemical analysis and quality monitoring during winemaking (Patrick Iland Wine promotions, Campbelltown, SA, Australia). *In*. Australia

Intrieri C, Filippetti I, Allegro G, Centinari M, Poni S (2008) Early defoliation (hand vs mechanical) for improved crop control and grape composition in Sangiovese (Vitis vinifera L.). Australian Journal of Grape and Wine Research **14:** 25-32

Intrieri C, Poni S, Rebucci B, Magnanini E (1997) Effects of canopy manipulations on whole-vine photosynthesis: Results from pot and field experiments. Vitis **36:** 167-173

Izquierdo-Cañas PM, García-Romero E, Mena-Morales A, Gómez-Alonso S (2016) Effects of malolactic fermentation on colour stability and phenolic composition of Petit Verdot red wines. Wine Studies **5:** 1-6

Kennedy JA, Saucier C, Glories Y (2006) Grape and wine phenolics: history and perspective. American Journal of Enology and Viticulture **57:** 239-248

King PD, McClellan DJ, Smart RE (2012) Effect of Severity of Leaf and Crop Removal on Grape and Wine Composition of Merlot Vines in Hawke's Bay Vineyards. American Journal of Enology and Viticulture **63:** 500-507

King PD, McClellan DJ, Smart RE (2012) Effect of severity of leaf and crop removal on grape and wine composition of Merlot vines in Hawke's Bay vineyards. American journal of enology and viticulture**:** ajev. 2012.12020

Kliewer WM, Dokoozlian NK (2005) Leaf area/crop weight ratios of grapevines: Influence on fruit composition and wine quality. American Journal of Enology and Viticulture **56:** 170-181

Kliewer WM, Weaver RJ (1971) Effect of crop level and leaf area on growth, composition, and coloration of tokay grapes. American Journal of Enology and Viticulture **22:** 172-&

Kobayashi S, Goto-Yamamoto N, Hirochika H (2004) Retrotransposon-induced mutations in grape skin color. Science **304:** 982-982

Kobayashi S, Ishimaru M, Hiraoka K, Honda C (2002) Myb-related genes of the Kyoho grape (Vitis labruscana) regulate anthocyanin biosynthesis. Planta **215:** 924-933

Koyama K, Numata M, Nakajima I, Goto-Yamamoto N, Matsumura H, Tanaka N (2014) Functional characterization of a new grapevine MYB transcription factor and regulation of proanthocyanidin biosynthesis in grapes. Journal of Experimental Botany **65:** 4433-4449

Kriedemann PE, Kliewer WM, Harris JM (1970) Leaf age and photosynthesis in Vitis vinifera L. Vitis **9:** 97-104

Lanari V, Lattanzi T, Borghesi L, Silvestroni O, Palliotti A (2012) Post-veraison mechanical leaf removal delays berry ripening on'Sangiovese'and'Montepulciano'grapevines. *In* I International Workshop on Vineyard Mechanization and Grape and Wine Quality 978, pp 327-333

Larronde F, Krisa S, Decendit A, Cheze C, Merillon JM (1998) Regulation of polyphenol production in Vitis vinifera cell suspension cultures by sugars. Plant Cell Reports **17:** 946-950

Lecourieux F, Kappel C, Lecourieux D, Serrano A, Torres E, Arce-Johnson P, Delrot S (2013) An update on sugar transport and signalling in grapevine. Journal of Experimental Botany **65:** 821-832

Matthews MA (2016) Terroir and other myths of Wine. University of California Press

Matus JT, Loyola R, Vega A, Pena-Neira A, Bordeu E, Arce-Johnson P, Alcalde JA (2009) Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of Vitis vinifera. Journal of Experimental Botany **60:** 853-867

Mercurio MD, Dambergs RG, Herderich MJ, Smith PA (2007) High throughput analysis of red wine and grape phenolics - Adaptation and validation of methyl cellulose precipitable tannin assay and modified Somers color assay to a rapid 96 well plate format. Journal of Agricultural and Food Chemistry **55:** 4651-4657

Mori K, Goto-Yamamoto N, Kitayama M, Hashizume K (2007) Loss of anthocyanins in red-wine grape under high temperature. Journal of Experimental Botany **58:** 1935-1945

Nuzzo, V., & Matthews, M. A. (2006). Response of fruit growth and ripening to crop level in dry-farmed Cabernet Sauvignon on four rootstocks. American Journal of Enology and Viticulture, **57**, 314–324.

Osrečak M, Karoglan M, Kozina B (2016) Influence of leaf removal and reflective mulch on phenolic composition and antioxidant activity of Merlot, Teran and Plavac mali wines (Vitis vinifera L.). Scientia horticulturae **209:** 261-269

Pastore C, Allegro G, Valentini G, Muzzi E, Filippetti I (2017) Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese Vitis vinifera L. cultivars. Scientia Horticulturae **218:** 147-155

Pastore C, Allegro G, Valentini G, Muzzi E, Filippetti I (2017) Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese Vitis vinifera L. cultivars. Scientia Horticulturae **218:** 147-155

Pastore C, Zenoni S, Fasoli M, Pezzotti M, Tornielli GB, Filippetti I (2013) Selective defoliation affects plant growth, fruit transcriptional ripening program and flavonoid metabolism in grapevine. Bmc Plant Biology **13**

Petrie PR, Clingeleffer PR (2006) Crop thinning (hand versus mechanical), grape maturity and anthocyanin concentration: outcomes from irrigated Cabernet Sauvignon (Vitis vinifera L.) in a warm climate. Australian Journal of Grape and Wine Research **12:** 21-29

Petrussa E, Braidot E, Zancani M, Peresson C, Bertolini A, Patui S, Vianello A (2013) Plant flavonoids—biosynthesis, transport and involvement in stress responses. International journal of molecular sciences **14:** 14950-14973

Pirie A, Mullins MG (1977) Interrelationships of sugars, anthocyanins total phenols and dry-weight in skin of grape berries during ripening. American Journal of Enology and Viticulture **28:** 204-209

Poni S, Gatti M, Bernizzoni F, Civardi S, Bobeica N, Magnanini E, Palliotti A (2013) Late leaf removal aimed at delaying ripening in cv. Sangiovese: physiological assessment and vine performance. Australian Journal of Grape and Wine Research **19:** 378-387

Pourcel L, Routaboul J-M, Cheynier V, Lepiniec L, Debeaujon I (2007) Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends in plant science **12:** 29-36

Ravaz L (1930) Influence de la taille sur la vigeur et la production de la vigne. Prog. Agr. Vitic. **2:** 537

Reynolds AG, Price SF, Wardle DA, Watson BT (1994) FRUIT ENVIRONMENT AND CROP LEVEL EFFECTS ON PINOT-NOIR .1. VINE PERFORMANCE AND FRUIT COMPOSITION IN BRITISH-COLUMBIA. American Journal of Enology and Viticulture **45:** 452-459

Ristic R, Downey MO, Iland PG, Bindon K, Francis IL, Herderich M, Robinson SP (2007) Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. Australian Journal of Grape and Wine Research **13:** 53-65

Sarneckis CJ, Dambergs RG, Jones P, Mercurio M, Herderich MJ, Smith PA (2006) Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Australian Journal of Grape and Wine Research **12:** 39-49

Sivilotti P, Herrera JC, Lisjak K, Baša Česnik H, Sabbatini P, Peterlunger E, Castellarin SD (2016) Impact of leaf removal, applied before and after flowering, on anthocyanin, tannin, and methoxypyrazine concentrations in 'Merlot'(Vitis vinifera L.) grapes and wines. Journal of agricultural and food chemistry **64:** 4487-4496

Sivilotti P, Herrera JC, Lisjak K, Cesnik HB, Sabbatini P, Peterlunger E, Castellarin SD (2016) Impact of Leaf Removal, Applied Before and After Flowering, on Anthocyanin, Tannin, and Methoxypyrazine Concentrations in 'Merlot' (Vitis vinifera L.) Grapes and Wines. Journal of Agricultural and Food Chemistry **64:** 4487-4496

Smart RE, Robinson M (1991) A Handbook for Winegrape Canopy Management. Winefiles, Adelaide Australia

Smith JP, Holzapfel BP (2009) Cumulative Responses of Semillon Grapevines to Late Season Perturbation of Carbohydrate Reserve Status. American Journal of Enology and Viticulture **60:** 461-470

Smith PA, Mercurio MD, Dambergs RG, Francis IL, Herderich MJ (2008) Grape and wine tannin - Are there relationships between tannin concentration and variety, quality and consumer preference? Proceedings of the 13th Australian Wine Industry Technical Conference**:** 189-192

Somers CT, Evans ME (1977) Spectral evaluation of young red wines: Anthocyanin equilibria, total phenolics, free and molecular SO2, “chemical age”. Journal of the Science of Food and Agriculture **28:** 279-287

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) CLONING AND MOLECULAR ANALYSIS OF STRUCTURAL GENES INVOLVED IN FLAVONOID AND STILBENE BIOSYNTHESIS IN GRAPE (VITIS-VINIFERA L). Plant Molecular Biology **24:** 743-755

Stoll M, Bischoff-Schaefer M, Lafontaine M, Tittmann S, Henschke J (2013) Impact of Various Leaf Area Modifications on Berry Maturation in Vitis vinifera L. 'Riesling'. *In* S Poni, ed, I International Workshop on Vineyard Mechanization and Grape and Wine Quality, Vol 978

Tardaguila J, de Toda FM, Poni S, Diago MP (2010) Impact of Early Leaf Removal on Yield and Fruit and Wine Composition of Vitis vinifera L. Graciano and Carignan. American Journal of Enology and Viticulture **61:** 372-381

Terrier N, Ollé D, Verriès C, Cheynier V (2009) Biochemical & molecular aspects of flavan-3-ol synthesis during berry development. *In* Grapevine Molecular Physiology & Biotechnology. Springer, pp 365-388

Terrier N, Torregrosa L, Ageorges A, Vialet S, Verries C, Cheynier V, Romieu C (2009) Ectopic Expression of VvMybPA2 Promotes Proanthocyanidin Biosynthesis in Grapevine and Suggests Additional Targets in the Pathway. Plant Physiology **149:** 1028-1041

Walker AR, Lee E, Bogs J, McDavid DAJ, Thomas MR, Robinson SP (2007) White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant Journal **49:** 772-785

Zenoni S, Dal Santo S, Tornielli GB, D'Inca E, Filippetti I, Pastore C, Allegro G, Silvestroni O, Lanari V, Pisciotta A, Di Lorenzo R, Palliotti A, Tombesi S, Gatti M, Poni S (2017) Transcriptional Responses to Pre-flowering Leaf Defoliation in Grapevine Berry from Different Growing Sites, Years, and Genotypes. Frontiers in Plant Science **8**

Zhang P, Wu X, Needs S, Liu D, Fuentes S, Howell K (2017) The Influence of Apical and Basal Defoliation on the Canopy Structure and Biochemical Composition of Vitis vinifera cv. Shiraz Grapes and Wine. Frontiers in chemistry **5:** 48

Zhuang SJ, Tozzini L, Green A, Acimovic D, Howell GS, Castellarin SD, Sabbatini P (2014) Impact of Cluster Thinning and Basal Leaf Removal on Fruit Quality of Cabernet Franc (Vitis vinifera L.) Grapevines Grown in Cool Climate Conditions. Hortscience **49:** 750-756

Appendix 4

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Appendix 5

Other material

Appendix 5.1: Field Winemaking Protocol

Around 50 kg of grapes (Picture 1) were separately harvested for each treatment and replicate. Fruit was transported to the NWGIC experimental winery (Wagga Wagga) and kept at 4 °C until processing.

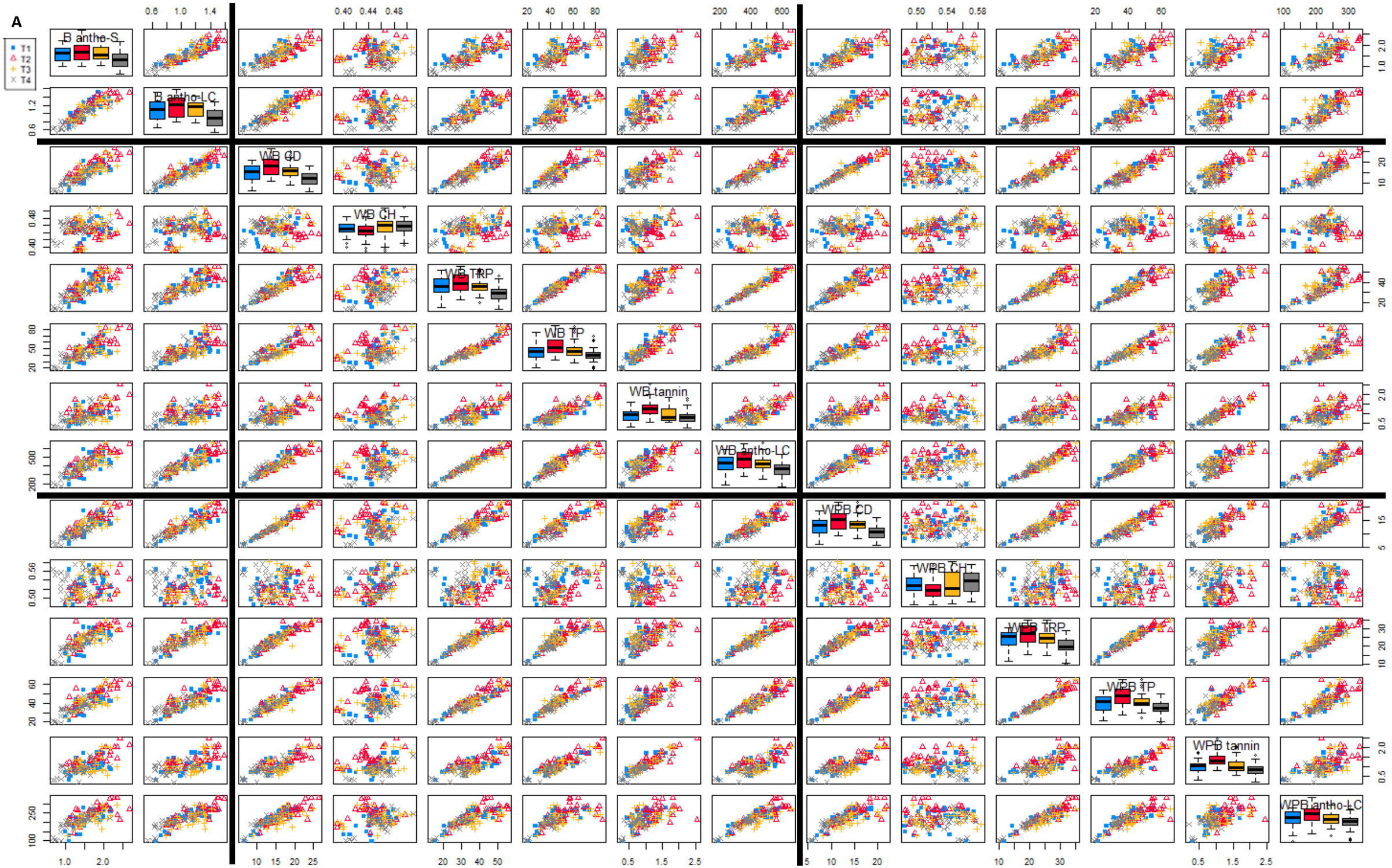
1. Bunches were destemmed/crushed (Picture 2) and the juice and the berries put together in a 100 L stainless steel tank for maceration (Picture 3).
2. Must was analysed for pH, TA and °Baumé.
3. pH was adjusted to about 3.6 by tartaric acid additions, while keeping TA below 7.5 g/L.
4. Sulfur dioxide (60 mg/L) was added in the form of sodium metabisulfite.
5. Must was warmed to 20 °C and inoculated with 30 g/hL of yeast (EC1118, Lallemand) and 30 g/hL of GO-FERM© Protect© (Lallemand).
6. Ferments were plunged, and temperature and °Baumé recorded, twice a day (8 am and 4 pm).
7. After a drop in 3 °Baumé, YAN was adjusted to 200 mg/L with Fermaid K (Lallemand 13% N), and then with diammonium phosphate to 220 mg/L (for 12-13 °Baumé must) or 250 mg/L (for 13-15 °Baumé must).
8. Ferments were pressed at -1 °Baumé at 1 bar.
9. Ferments were moved to 22 °C and °Baumé was monitored until the glucose plus the fructose concentration was below 0.5 g/L.
10. Lees were racked off and sulfur dioxide (80 mg/L) added.
11. Wines were moved to -4 °C for 3 weeks to cold stabilise (racked twice more during cold stabilisation).
12. Heat stability was tested as per Iland, Ewart, Sitters, Markides, & Bruer (2000).
13. SO_2 levels were adjusted to 30 mg/L free SO_2 to achieve 0.5-0.8 mg/L molecular SO_2.
14. Wines were bottled once cold tested, and pre-bottling analyses were preformed (pH, TA, % alcohol, free and total sulfur dioxide).



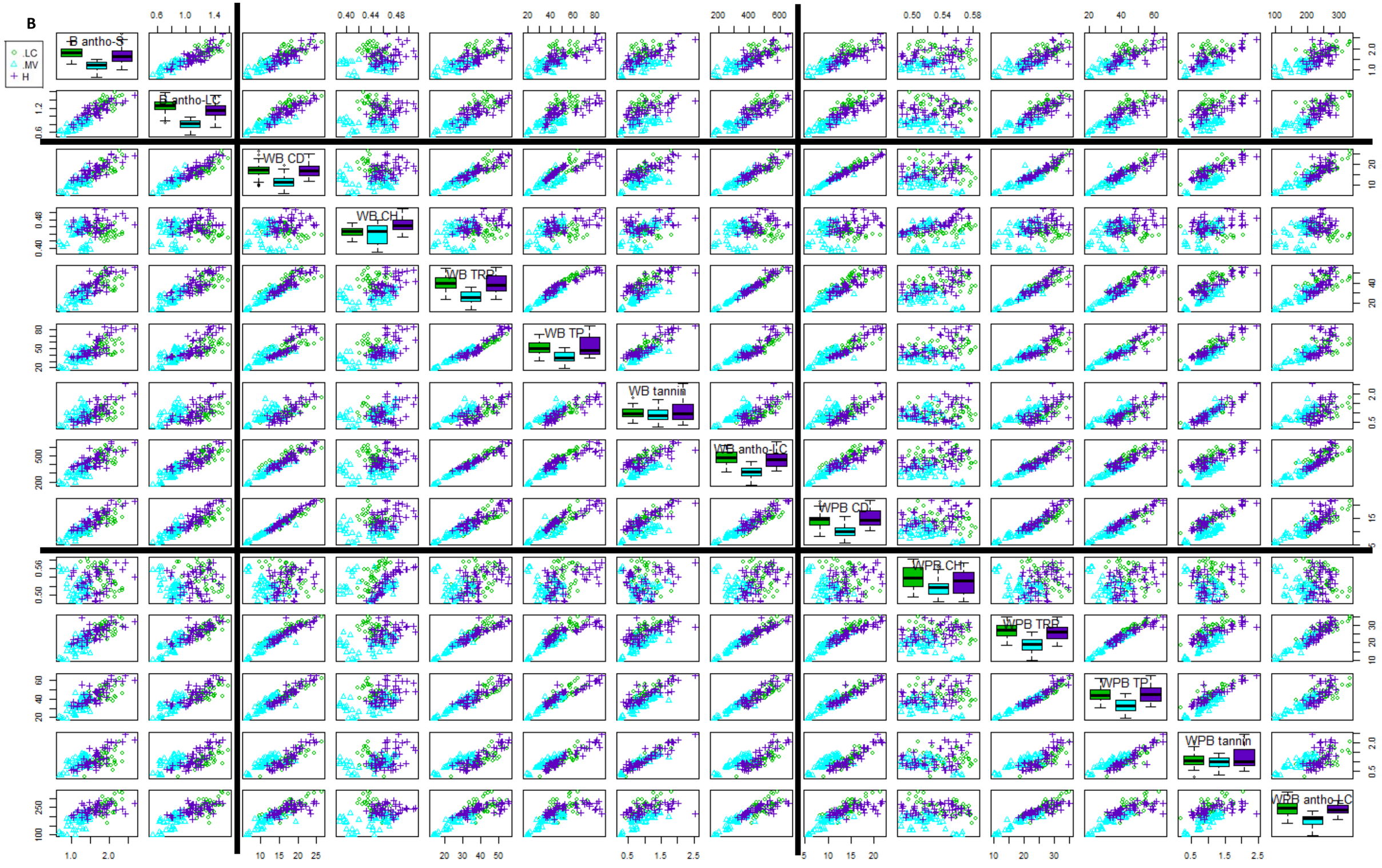




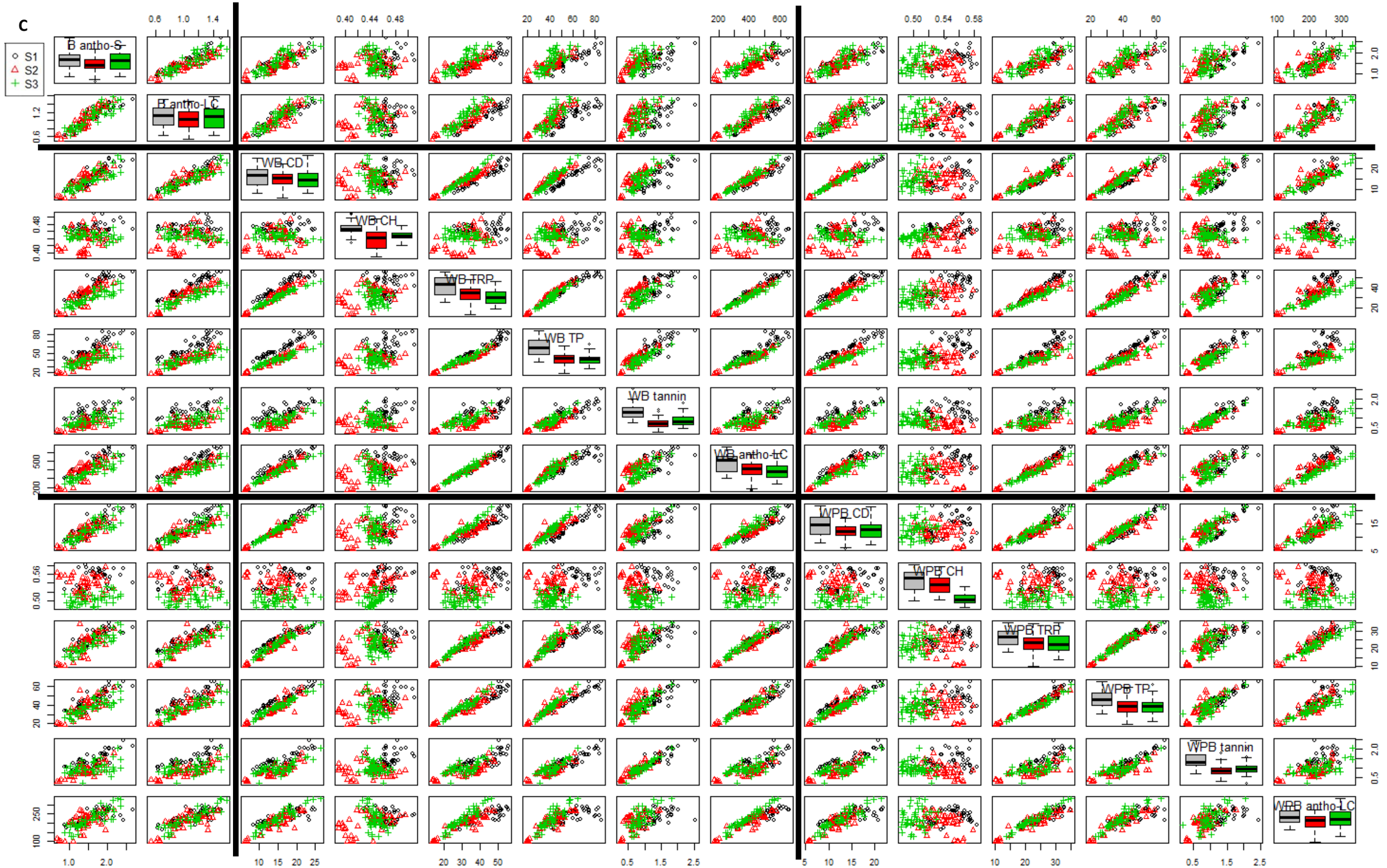
Appendix 5.2: Scatterplot matrices and boxplots (diagonal) for berry anthocyanins by spectrophotometry (B antho S) and by UPLC-PDA (B antho LC), wine at bottling (WB), and 10 months post-bottling (WPB) colour density (CD), colour hue (CH), total red pigment (TRP), total phenolics (TP), tannins and UPLC-PDA (antho LC) per treatments (A), per sites (B), per seasons (C).



B


.LC
.MV
H
B antho-S
B antho-LC
WB CD
WB CH
WB TRB
WB TP
WB tannin
WB antho-LC
WPB CD
WPB CH
WPB TRB
WPB TP
WPB tannin
WPB antho-LC

C


S1
S2
S3
B antho-S
B antho-LC
WB CD
WB CH
WB TRP
WB TP
WB tannin
WB antho-LC
WPB CD
WPB CH
WPB TRP
WPB TP
WPB tannin
WPB antho-LC

Appendix 5.3: Planned Project Outputs

Outputs and activities from the original project application are in black, changes to these and additional outputs from the project variation/extension approved in June 2016 are marked in red.

Outputs and Activities 2012–13

Year 1	Output	Target Date	Activities
a	A detailed and finalised project operational and management plan. A confirmed and committed industry reference group.	31/07/2013	In person group meeting held between CSIRO and NWGIC to coordinate research activities. Collaborate with UoA project (UA1207) to identify Shiraz trial vineyards in Young, NSW, Mildura, Vic and Langhorne Creek, SA. Industry reference group developed in conjunction with UA1207. Meetings with University of Adelaide (UoA) and GWRDC to inform project activities.
b	Operational environmental monitoring systems available to collect climate data for comparison between sites and seasons.	30/06/2013	At least five time domain reflectometer (TDR) soil water sensors per site and one automatic weather station per site installed and operational.
c	Infrastructure required for vine chamber experiments ready for use.	30/06/2013	Six aluminium framed transparent chambers (1.6 m^3 volume) constructed and located at the NWGIC in Wagga Wagga, NSW. Design to suit 75 mature, potted, spur pruned Shiraz grapevines on a fixed single cordon trellis system. Three chambers with reduced CO_2 concentration air (CO_2 scrubbing) used to regulate total canopy photosynthesis and carbohydrate supply to fruit. Three chambers used as control (with ambient CO_2). CO_2 scrubbing system tested and functional for continuous operation over 5-10 days with capacity to reduce photosynthesis (relative to control vines) without altering other environmental variables.

Outputs and Activities 2013–14

Year 2	Output	Target Date	Activities
a	An established field trial site with canopy and crop	30/01/2014	Factorial experimental design planned with four canopy and crop load

	manipulation treatments imposed.		manipulation treatments (including control).
b	A preliminary dataset demonstrating the impact of canopy and crop load manipulation treatments on vine physiology, yield and fruit composition from the first season of field trials.	30/06/2014	Canopy and crop load manipulation treatments applied between flowering and véraison. Monthly measurements of canopy growth taken until full canopy achieved. Fruit samples taken at least monthly from fruit-set to harvest for molecular and compositional analysis (sugar, colour, pH, TA, total tannins). Yield components and pruning weights data collected and analysed. Compare traditional and UoA 'toolkit' technique to measure vine size. Provide fruit to UA1207 for sensory analysis.
c	A preliminary dataset demonstrating the impact of reduced photosynthetic carbon gain on vine physiology, yield and fruit composition from the first season of the vine chamber experiment. A preliminary understanding of carbon supply effects on berry metabolite, anthocyanin and tannin production during the period between fruit-set and véraison.	30/06/2014	Undertake first experiment with CO_2 scrubbing system to determine the influence of carbohydrate supply on berry tannin and anthocyanin production. Low (100 ppm) and control (380 ppm) CO_2 treatments applied to potted Shiraz grapevines at three different periods (5-10 consecutive days each) between fruit-set and véraison. Experiment to use a total of 18 vines (3 replicates x 2 treatments x 3 treatment periods). Berry samples to be collected from three low CO_2 and three control vines during each treatment period, and from all treated vines at harvest. Samples stored at -80°C for subsequent analysis. Additional berries collected at harvest for immediate analysis of sugar, anthocyanin, pH, total acid, total tannins.
d	Gene targets identified and key samples prepared for gene expression and metabolite studies.	30/06/2014	Database searches will be conducted to establish candidate key genes from other plant species with known roles in carbohydrate metabolism and storage. This information will be used to isolate and characterise similar grapevine genes by designing specific primers and conducting PCR, sequencing reactions and sequence analysis. Flavonoid gene expression will also be examined, but as this is already done routinely in the

			CSIRO laboratory further development is not required. Selected samples from the first season identified and processed for expression analysis by extraction of RNA followed by cDNA synthesis.

Outputs and Activities 2014–15

Year 3	Output	Target Date	Activities
a	A set of bottled experimental wines from fruit from the first season of field trials and vine chamber experiment. A data set of the composition at bottling of experimental wines from the first season of field trials and vine chamber experiment. Initial understanding of direct role of vine balance and carbon supply effects on wine quality attributes.	30/10/2014	Three experimental winemaking replicates produced for each of four treatments for all three field trial sites (36 in total). Micro-scale experimental winemaking replicates (approx. 3 kg grapes/vine) to be produced for individual vines for each vine chamber treatment (3 vines x 2 treatments x 3 sampling times = 18 samples) Standard analysis (free and bound SO2, pH, TA, VA, alcohol, total tannins, anthocyanin and phenolics) to be undertaken on wines from field trials and vine chamber experiment prior to bottling. Analyse data sets to elucidate effects of vine balance in field trial and effects of carbon supply on berry metabolite and tannin production in vine chamber trial.
b	Information available on the impact of canopy, crop load and carbon availability manipulation treatments on wine sensory properties. Preliminary knowledge on carbon vine balance disseminated to industry through at least one extension mechanism.	31/12/2014	Organise and hold first sensory evaluation workshop with industry partners from Langhorne Creek, Young and Mildura to evaluate wines from first year of in field and vine chamber experiments. Prepare and submit an article on preliminary research outcomes to at least one industry journal.
c	A preliminary dataset demonstrating the impact of canopy and crop load manipulation treatments on vine physiology, yield and fruit composition from the	30/06/2015	Previously applied canopy and crop load manipulation treatments re-imposed in field trials. Monthly measurements taken of canopy growth until full canopy achieved.

	first two seasons of field trials. Preliminary understanding of the influence of treatments imposed on vine balance and impact of fruit quality and maturation rate achieved.		Fruit samples taken at least monthly from fruit-set to harvest for molecular and compositional analysis (sugar, colour, pH, TA, total tannins). Yield components and pruning weights data collected and analysed to determine measures of vine balance. Compare traditional and UoA 'toolkit' technique to measure vine size. Provide fruit to UA1207 for sensory analysis.
d	A preliminary dataset demonstrating the impact of CO_2 controlled photosynthesis reductions on vine physiology, yield and fruit composition from the first two seasons of the vine chamber experiment. Preliminary understanding of carbon supply effects on berry metabolite, anthocyanin and tannin production during the period between véraison and harvest.	30/06/2015	Undertake second experiment with CO_2 scrubbing system to determine the influence of carbohydrate supply on berry tannin and anthocyanin production. Low (100 ppm) and control (380 ppm) CO_2 treatments applied to potted Shiraz grapevines at three different periods (5-10 consecutive days each) between véraison to harvest. Experiment to use a total of 18 vines (3 replicates x 2 treatments x 3 treatment periods).. Berry samples to be collected from three low CO_2 and three control vines during each treatment period, and from all treated vines at harvest. Samples stored at -80^oC for subsequent analysis. Additional berries collected at harvest for immediate analysis of sugar, anthocyanin, pH, total acid, total tannins.
e	Preliminary understanding of gene expression and berry metabolite production in flavonoid pathway during key stages of berry development established.	30/06/2015	Expression of flavonoid genes and critical carbohydrate genes from season two measured by qPCR and analysed in selected field and chamber samples and correlated with changes in berry metabolites and measures of vine balance.
f	Progress report, including a review of progress and recommendations for change accepted by GWRDC and changes executed.	30/06/2015	Hold an industry reference group meeting to review and discuss project findings and outcomes. Undertake a project review meeting with GWRDC to discuss future direction of project.

Outputs and Activities 2015–16

Year 4	Output	Target Date	Activities
a	A set of bottled experimental wines from fruit from the second season of field trials and vine chamber experiment. A data set of the composition at bottling of experimental wines from the first two seasons of field trials and vine chamber experiment. A preliminary understanding of direct role of vine balance and carbon supply effects on wine quality attributes.	30/10/2015	Three experimental winemaking replicates produced for each of four treatments for all three field trial sites (36 in total) produced. Micro-scale experimental winemaking replicates (approx. 3 kg grapes/vine) to be produced for individual vines for each vine chamber treatment (3 vines x 2 treatments x 3 sampling times = 18 samples) Standard analysis (free and bound SO2, pH, TA, VA, alcohol, total tannins, anthocyanin and phenolics) to be undertaken on wines from field trials and vine chamber experiment prior to bottling. Berry and wine analysis data to be compared with equivalent analysis data from first season. Analyse data sets to elucidate effects of vine balance in field trial and effects of carbon supply on gene expression, berry metabolite and tannin production in vine chamber trial.
b	Information available on the impact of canopy, crop load and carbon availability manipulation treatments on wine sensory properties. Knowledge on carbon vine balance disseminated to industry through at least one extension mechanism.	31/12/2015	Organise and hold second sensory evaluation workshop with industry partners at Langhorne Creek, Griffith and Mildura to evaluate wines from second year of in field and vine chamber experiments. Prepare and submit an article on preliminary research outcomes to at least one industry journal.
c	A dataset demonstrating the impact of canopy and crop load manipulation treatments on vine physiology, yield and fruit composition from the first three seasons of field trials.	30/06/2016	Previously applied canopy and crop load manipulation treatments altered in consideration of previous results and re-imposed in field trials.

	Understanding of influence of treatments imposed on vine balance and impact of fruit quality and maturation rate achieved.		Monthly measurements of canopy growth until full canopy achieved. Fruit samples taken at least monthly from pre-véraison to harvest for molecular and compositional analysis (sugar, colour, pH, TA, total tannins). Yield components and pruning weights data collected and analysed. Compare traditional and UoA 'toolkit' technique to measure vine size. Provide some fruit to UA1207 for sensory analysis.
d	A dataset demonstrating the interactive effects of photosynthetic carbon supply and light exposure on fruit composition from the third season of the vine chamber experiments. A preliminary understanding of the relative influence of carbon supply and light exposure on berry metabolite, anthocyanin and tannin production during the period between fruit-set and véraison.	30/06/2016	Undertake third experiment with CO_2 scrubbing system to determine interaction between reduced photosynthetic carbon supply and bunch exposure. Low (100 ppm) and control (380 ppm) CO_2 treatments applied to potted Shiraz grapevines between fruit-set and veriason with three levels of bunch light exposure applied within each chamber. Timing and duration of treatments to simulate field leaf removal and shoot thinning treatments, with refinements according the results of the first two seasons and discussions with industry reference group. Berry samples to be collected from light exposure treatments from both CO_2 levels control during each treatment period, and again at harvest. Samples stored at -80°C for subsequent analysis of sugar, anthocyanins, pH, total acid and total tannins.
e	~~Potential molecular markers for vine balance identified.~~ Preliminary understanding of gene expression and berry metabolite production in flavonoid pathway	30/06/2016	Preliminary analysis of year 3 berry metabolite and gene expression data from field and whole vine chamber trials analysed and compared to year 1 and 2 analysis. ~~Analyse data to~~

	during key stages of berry development improved.		~~elucidate potential markers of vine balance.~~
~~f~~	~~Extension of project outcomes through at least four extension mechanisms.~~	~~30/06/2016~~	~~Organise and hold third sensory evaluation workshop with industry partners at Langhorne Creek, Young and Mildura to evaluate wines from third year of in field and vine chamber experiments.~~ ~~Research outcomes to be compiled and prepared for at least one industry journal article and at least two scientific papers published in refereed journals. At least one extension event held in collaboration with UA1207.~~

Outputs and Activities 2016–17

Year 4	Output	Target Date	Activities
a	A set of bottled experimental wines from fruit from the third season of field trials and vine chamber experiment. A data set of the composition at bottling of experimental wines from all three seasons of field trials and vine chamber experiment.	30/10/2016	Four experimental winemaking replicates produced for each of five treatments for all three field trial sites (60 in total) produced. Micro-scale experimental winemaking replicates (approx. 3 kg grapes/vine) to be produced for individual vines for each vine chamber treatment (3 vines x 2 treatments x 2 experiments = 12 samples) Standard analysis (free and bound SO2, pH, TA, alcohol, total tannins, anthocyanin and phenolics) to be undertaken on wines from field trials and vine chamber experiment at bottling.
b	Information available on the impact of canopy, crop load and carbon availability manipulation treatments on wine sensory properties.	31/12/2016	Organise and hold third sensory evaluation workshop with industry partners at Langhorne Creek, Griffith and Mildura to evaluate wines from third year of field experiments.
c	Potential molecular markers for vine balance identified.	30/06/2017	Identify and analyse additional fruit samples for expression of key genes: (a) based on wine sensory data from

			seasons 1-3 and (b) matched pre- and immediately post-treatment (24 hrs) from season 3 samples. All metabolite and gene expression data from field and whole vine chamber trials in seasons 1-3 compared to elucidate potential markers of vine balance/imbalance.
d	Detailed dataset of fruit and wine composition one month post-bottling from seasons 1-3 available.	30/06/2017	Analyse anthocyanin composition of berries at harvest and wines after bottling using UPLC. Analyse these data sets to elucidate effects of vine balance in field trial and effects of carbon supply in vine chamber trial on wine composition and contrast with effects on fruit composition.
e	Detailed dataset of wine composition ten months post-bottling from seasons 1-3 available.	30/06/2017	Analyse wines from all field trials ten months post bottling using industry standards, UPLC and difference testing. Analyse these data sets to determine wine stability with an emphasis on colour.
f	Extension of project outcomes through at least four extension mechanisms.	30/06/2017	Research outcomes to be compiled and prepared for Wine Australia final report, at least one industry journal article and at least two scientific papers for refereed journals. Extension workshop held in collaboration with UA1207.

Appendix 6

Budget reconciliation

The budget reconciliation has been provided separately, to Wine Australia.