An investigation into the effect of grapevine age on vine performance, grape and wine composition, sensory evaluation and epigenetic characterisation.

by

Dylan Grigg

Thesis submitted to the School of Agriculture, Food and Wine of the University of Adelaide in fulfilment of the requirements for the degree of

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Table of Contents

Abstract

The grapevine *Vitis vinifera* L. is a perennial cropping plant capable of surviving for decades or centuries. Examples exist of cultivated grapevines still fruitful after more than 400 years. However, in commercial viticulture multiple biotic and abiotic factors challenge longevity. Vineyards of greater vine age are often highly regarded due to the perceived quality of fruit and wine they produce. This is an entrenched phenomenon in the wine industry and media which has barely been exposed to critical examination.

The aim of this research was to assess the influence of vine age on grape and wine production. This research is ambitious and the goal is to serve as an investigation into the potential influence of vine age and a guide to future studies. Several key areas were selected for investigation: grapevine performance, fruit and wine composition and sensory analysis, wine metabolomic analysis and molecular (genome and epigenome) analysis.

Five Shiraz vineyards with genetically related 'young' and 'old' plantings in close proximity were selected with an average age difference of over 97 years. To date, this represents the greatest spread and extreme of vine ages to be subjected to scientific scrutiny.

Vine age was found to influence reproductive performance; older vines produced greater yields however, all vines were influenced by seasonal variation irrespective of vine age. Greater trunk circumference may be a key determining factor in increased reproductive capacity with age. Other measures of vine performance such as Y/P, shoot number, count shoots or shoot mass did not differ with age.

Wine quality is largely determined by the characteristics of the fruit from which it is made. This research used common chemical and modern 'omics techniques to elucidate quality traits that may be unique with increased vine age. Large differences in vine age did not produce differences in basic grape composition. Interestingly, older vines had a lower pH at similar Brix level. Compositional measures did not differentiate vine age categories and they were more indicative of the region where the fruit was grown. Analysis of secondary metabolites such as tannins and phenolics showed greater differentiation of growing region. Phenolic profiling revealed regional based influences of key compounds of known sensory outcomes, these results were supported by sensory analysis.

Sensory Descriptive analysis was undertaken on both grapes and wine over three seasons. Despite similar maturity profiles, differences were detected associated with grapevine age in both grape and wine samples. Grapes and wine from older vines showed consistent sensory characteristics across seasons, generally described as a lighter fruit profile. Targeted metabolomic analysis of fermentation derived volatile compounds also differentiated between vine-age groups at most sites.

Using modern next generation sequencing and reduced representation libraries, analysis of the genome and epigenome was undertaken. Genetic similarity between sites and ages was not detected, however, global DNA methylation level differed with vine age. DNA methylation was also associated with geographic distance and method of propagation. Differential methylation markers (DMMs) were found via pairwise comparisons between the sites one to four and the oldest two sites only. Site five presented no DMMs and a unique global methylation profile attributed to propagation technique, despite an age difference of 87 years.

Despite large differences in grapevine age, both site and season are highly influential in a broad range of qualitative assessments. The greater perceived quality attributed to grapevine age is subject to environmental influence and is more complex than previously thought

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references has been made in text.

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Dylan Grigg Date

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Chapter 1. Introduction

1.1 Introduction

It is a common belief that grapes from older grapevines produce more superior wine when compared to wines produced from younger vines (Koblet and Perret 1980, Smart 1993, Gladstones 2011, Dry 2013). Allegedly, wine produced from younger vines lacks particular characteristics and complexity compared to wines made from older vines (Reynolds et al. 2008). World renowned wine critic Jancis Robinson (2006) states that '*Vine age is widely considered a factor affecting wine quality, with widespread consensus that; in general, older vines make better wines'.* While acknowledged as a phenomenon, it is also widely noted that the causes of this perception are neither clear, nor scientifically validated (Smart 1993, Dry 2008, Reynolds et al. 2008, Dry 2013). This is likely due to the challenge presented by the many variables that can confound such a study, as well as the availability of a suitable resource of old vines. To make a direct comparison of significant vine age as a treatment factor with minimal variables requires a unique combination of circumstances. To link final wine quality to planted vine age requires a study with a wide initial scope that covers multiple disciplines.

There is an abundance of peer reviewed literature that investigates many vine performance measures and how these impact grape composition and wine quality. Wine composition is determined by many factors, ranging from viticultural practices to environmental and genetic factors (Roullier-Gall et al. 2014). Following this, the judgment of quality is resultant upon the complex composition of the wine matrix and its final perception via its interaction with the palate.

Current scientific literature has rarely investigated vine age as a stand-alone variable. Where studies have included vine age, significant age, i.e. >100 years; is yet to be reported. With careful experimental design around pre-existing sites with unique viticultural resources, the influence of vine age on wine quality can be investigated. The overall aim of this study is to report the findings of a multi-seasonal replicated trial, from vine to wine. This will inform greater knowledge into grapevine ageing and potentially pinpoint characteristics that are associated with high value wines. Further knowledge of factors associated with vine age may have implications for viticultural production, wine making and marketing.

1.2 Objectives of the research

The primary objectives of this research were to: i) investigate vine age in relation to grapevine vegetative and reproductive performance; ii) examine the chemical composition of grapes and wines produced directly from them; iii) ascertain if sensory analysis of grapes and wine could differentiate between vine age categories; iv) characterise molecular diversity at the genome and epigenome level amongst Shiraz vines in this study.

1.3 Linking statement

The research presented in this thesis is ordered into chapters. The first chapter is an introduction to the topic followed by chapter 2 which presents a literature review of aspects which may be pertinent to this study. Following this are four research chapters. These chapters are presented as manuscripts for publication in the respective journals indicated, and formatted appropriately. The individual manuscripts presented in this thesis reflect the timeline and evolution of this project.

Chapter 1 is a general introduction to the topic, its importance and indicates the novel addition this research makes to the field of viticultural science.

Chapter 2 contains a thorough literature review which broadly addresses the past and present status of research in the various fields presented in this thesis. Since few studies have approached this topic directly this literature review analyses the various paths, interactions and consequences that are presented in subsequent chapters but also in future direction.

Chapter 3 presents an investigation of measures relating to grapevine performance. This chapter specifically analyses the vegetative and reproductive characteristics associated with vine age. The aim of this study was to determine if vine performance characteristics were influenced by grapevine age.

Publication title: Vine age effects on vine performance of Shiraz in the Barossa Valley. Journal format: The Australian Journal of Grape and Wine Research.

Chapter 4 contains the manuscript of the results of study two. Study two investigated the chemical composition of fruit and wine. This study aimed to explore the chemical composition of fruit and wine in relation to grapevine age. This was to determine if from a compositional aspect vine age could be discriminated.

Publication title: Vine age and site effects on grape and wine chemistry in Shiraz from the Barossa wine zone in South Australia.
Journal format: Molecules

Chapter 5 study three investigates the sensory profiles of both grapes and wine. In addition to descriptive sensory analysis, in the final season wine was also subjected to targeted metabolomic analysis to investigate its volatile composition and its relationship with the sensory results.

Publication title: Sensory properties of grapes and wine from grapevines of contrasting ages.
Journal format: Molecules

Chapter 6 study four presents a molecular investigation into the genetic and epigenetic characteristics associated with vine age.

Publication title: The multiple influences of DNA methylation in *Vitis vinifera* L. cv. Shiraz; chronological age, environment and propagation technique.

Journal format: Nature: Scientific Reports

Chapter 7 presents a general discussion of the results reported in this thesis. This discussion combines the entire body of research through all previous chapters. Highlighted in the discussion are key findings, their implications and limitations encountered throughout this study. In addition, the general discussion outlines possible avenues for future research that would both complement the present study and make further advancements in relation to grape vine aging.

These areas of investigation are considered independently and have been presented as such in the form suitable for publication in respective journals.

Chapter 2. Background and Literature review

This literature review will aim to focus several main themes that emerged in the current literature. These themes relate to factors that may be important when attempting to elucidate the differences between vines (and wines produced) with contrasting ages. The discussion begins with viticulture and grape production, followed by the wine composition and components that may influence sensory assessment of finished wine quality. Finally, molecular characterisation of vine age is discussed focussing on genetic and epigenetic differences. The themes presented in this review cover a range of research contexts due to a lack of direct investigation in the field of vine ageing. Where possible this review will focus on potential sources of influence that might be associated with grapevine age and wine quality.

2.1 Old vines in the new world

The Barossa Valley in South Australia, is home to some of the world's oldest producing vineyards reportedly dating back to 1847 (Ioannou 1997, BGWA 2014). Many European countries have a much longer history of wine production than Australia; however, the introduction of phylloxera from America in the 1860s decimated the majority of these vineyards (Gale 2011). Widespread replanting of European vineyards onto American rootstocks from the 1880s has meant that production and heritage values continue. However, original pre-phylloxera plantings are now isolated and rare, surviving only in conditions where the phylloxera cannot. As in Europe, North America's plantings of European noble varieties (mainly in California) were largely lost also due to *Vitis vinifera's* lack of tolerance to phylloxera from 1873 (Winkler 1970, Gale 2011). While this pest has been present in Australia since 1877 (Gale 2011), through strict quarantine, it has remained isolated and, to date, has not been reported in South Australia. South Australia, and the Barossa Valley in particular are in a unique position with large areas of surviving phylloxera-free vines, some with continual production that dates back almost 200 years.

The Barossa Valley as a region has been proactive to market their old vines, as have other producing regions. To do this, the 'old vine charter' was developed in 2007 as a guide for producers when communicating the exclusivity that vine age presents in relation to wine production (Yalumba 2014). The identification of vine age as a point of difference in marketing or quality is not limited to Australia or even the Barossa Valley. In California there are small isolated pockets of vines dating back to the 1870s, these are monitored by a 'historical vineyard society' which was established to raise awareness of, protect, and market the oldest sites (Historic Vineyard Society 2014). In France the label designation *vieilles vignes* is used to

designate old vines; however, much like the term 'Reserve' on a label it is unregulated. Between regions and countries it is therefore not surprising that the use and reference to 'old' is far from consistent. Despite this, it is internationally common and widespread for trade journals, magazines and book authors to reference vine age, or 'old vine character' as a precursor or implication of a wine's potential quality (Smart 1993, Goode 2005, Robinson 2006, Parker 2007, Dry 2013).

2.2 Wine quality and marketing

Comparing the physiological aspects of vine growth between vines of different ages may be relatively straight forward, however comparison of finished wine is intrinsically linked to the perception of quality which is much harder to measure. The definition of wine quality can also be highly variable amongst critics, winemakers and consumers. Quality is ultimately assessed by the sensory attributes of a finished wine including taste, sight and smell (Jackson and Lombard 1993). These sensory attributes are derived mainly from intrinsic factors, it is important to note that packaging and marketing which form extrinsic factors can also influence the value and quality perception of wine (Sáenz-Navajas et al. 2013). The aforementioned implication of vine age and wine quality forms an extrinsic driver for perceived quality independent from the measurable intrinsic qualities. Implied wine quality is a significant factor in wine marketing, as observed by several studies which demonstrate that wine critic's grades influence wine pricing and demand (Ali et al. 2008, Gibbs et al. 2009, Friberg and Grönqvist 2012). It is common for winemakers to place extra value or prestige on wine from old vineyards (Ross 1999) which further cultivates the perception that older vines produce better wines. It has been suggested that implied quality in relation to wine quality began in France in 1855 with the first official Bordeaux classification of cru's (McIntyre 2011). Since quality is difficult to precisely define, this has led to the focus being on perceived rather than actual quality (Charters and Pettigrew 2007).

2.3 Climate

The climate in which grapes are grown plays a crucial role in determinations of both the quality and quantity of fruit production (Matese et al. 2012). Three levels of climate are identified in relation to viticulture; macroclimate, mesoclimate and microclimate. These refer to the region, site and canopy respectively (Smart and Robinson 1991). Growing season or climate is widely understood to influence vine growth and development (Freeman et al. 1979, Gladstones 1992, Jones and Davis 2000, van Leeuwen et al. 2004, Keller 2010, Roullier-Gall et al. 2014). Specifically van Leeuwen et al. (2004) reports that while studying the effects of climate, soil

and cultivar, it was climate that had the greatest effect. The limitation this places on the study of old and young vines will need to be accounted for and acknowledged. Statistical methods will be required to determine relationships within single seasons and across multiple seasons.

2.4 Terroir

Terroir is a widely used French term when discussing wine which has no exact equivalent in English (Gladstones 2011). Terroir commonly refers to the interactive ecosystem of a given place (van Leeuwen et al. 2004) including soil (White 2003), growing conditions or climate (Gladstones 1992) and the vine, including cultivar and rootstock (Seguin 1986). Recent studies suggest that the microbiome of the grape and soil also play a significant part in terroir (Gilbert et al. 2014, Bokulich et al. 2016). Also an often overlooked aspect of terroir is the interaction of root distribution within the soil-plant-atmosphere environment (Tomasi et al. 2015). Terroir is not limited to environmental factors, it has also recently been acknowledged to be shaped by human factors (Lenglet 2014). In one study it was observed that technological choices in both viticulture and production methods have a greater impact on quality than natural (terroir related) endowments (Gergaud and Ginsburgh 2010).

The importance of terroir is often debated due to its commercial and marketing relevance. White (2003) suggested that descriptions of terroir are so broad as to be too complex to measure as a direct relationship with wine. Regardless, studies have investigated the influence of terroir on a range of factors (Reynolds et al. 2013). Advances in technology and bioinformatics processing are now seeing multi-omics approaches to elucidate the influence of terroir at a very fine resolution (Bramley et al. 2011). Metabolomic profiles have been used to identify differences in fruit and wine on a regional level (Son et al. 2009, Gambetta et al. 2016) and at an even finer scale of a few kilometres (Roullier-Gall et al. 2014). This latter study highlights the resolution that can be achieved with Omics-related terroir investigations. This has been further exploited by combining metabolomics and transcriptomics to discover terroir-dependent traits in a single clone of the Italian variety Corvina over 3 years (Dal Santo et al. 2013, Anesi et al. 2015). The study of Anesi et al. (2015) found a clear persistent terroir-specific effect on the transcriptome and metabolome which allowed characterisation of the individual vineyards. Just as the grape or plant microbiome influences the expression of terroir so does the soil microbiome via root endophytes (Bokulich et al. 2016). As demonstrated by these studies increasing the resolution of measurement of terroir requires careful sampling to ensure reliable results. It has been demonstrated that terroir is spatially variable within in individual vineyard using precision viticulture Bramley et al. (2011). The influence of terroir is broad and

yet to be fully understood, particularly how quality traits are influenced via the complex interaction of terroir or the consistency of terroir over long time periods.

Modern targeted vineyard management may take the place of letting strong or weak sites succeed or fail on their own merit. This could lead to vineyards remaining in production, not because of excellence but for being in the right hands at the right time. Generally, commercial vineyards can remain productive for 40-50 or even 60 years; after this, they may become uneconomical (Gutierrez et al. 1985, White 2003, Robinson 2006). Due to these complexities a site considered to have good terroir is more than likely well established, well understood and well managed and as such more likely to contain older vines than a site without these qualities. This further adds to the complexity of the description. It is clear that there are a broad range of influences that can make a wine from a particular vineyard unique and marketable, many of which may be claimed as resulting from terroir.

2.4.2 The role of soil in vine growth and fruit composition

2.4.3 Soil structure

Soil is essential for providing structure and supplying water and mineral nutrition to grapevines. Various viticultural practices may influence soil structure over time, in both positive and negative ways depending on the actions undertaken. Rooting depth is anecdotally cited as a major factor in older vines being superior to younger. Evidence of this is mixed; in soils of widely different structure the majority of roots have been found in the top 60 cm (Saayman and Huyssteen 1980). Soil structure depth and texture have been shown to influence root density (Saayman and Huyssteen 1980, Nagarajah 1987, Tomasi et al. 2015). Potential root area can be limited through mechanical resistance by the presence of root impenetrable layers or depth to bedrock (Richards 1983, Wheeler and Pickering 2003). Despite this, exactly how rooting depth alone influences vine performance is yet to be determined (Smart et al. 2006). Individual roots have been noted beyond 6 m (Richards 1983). A review of rooting patterns surmised that soil structure, stoniness, and depth to water table were more important drivers for vertical root distribution than texture (Smart et al. 2006). Root exploration in relation to vine age is yet to be accounted for or fully understood. Perhaps through scientific studies with technological advances, less destructive methods of investigation can be undertaken in old high value vineyards.

2.4.4 Soil quality

Soil quality may refer to many factors that are inherently linked. Most simply, soil quality is defined as the soil's ability to support crop production or being 'fit for purpose' (Riches et al.

2013). Soil quality is known to be impacted by agricultural management practices, increasing its vulnerability to structural decline (Oliver et al. 2013, Riches et al. 2013). Through common viticultural management this may occur through physical contact of implements with the soil or via applied irrigation, soil amendments or chemicals for pest and disease control. One common example is the foliar application of copper in vineyards to control downy mildew (*Plasmopara viticola*), which has been in use since the 1850s (Ruyters et al. 2013). Many studies have reported the accumulation of copper in soil from long term use as a fungicide (Eijsackers et al. 2005, Toselli et al. 2009, Komárek et al. 2010). Therefore, soils with long agricultural or viticultural history are likely to have high levels of spray residues.

There are many ways that viticultural practices can influence soil quality over time in both positive and negative ways, and it is widely accepted that maintenance of soil condition physically, chemically and biologically is important for sustained yield and vine longevity (White 2003). The potentially long cropping life of a vineyard is likely to be exposed to unavoidable influences due to routine management over time. For example, physical compaction of the soil through contact with vehicle wheels (van Huyssteen 1983) and tillage is known to affect root distribution (Richards 1983, Williams 1990). This can greatly modify the natural soil structure and biological colonisation, particularly in regards to earthworms (White 2003, Eijsackers et al. 2005, Riches et al. 2013). In addition to mechanisation influencing macrobiota, some management practices have been shown to have long term effects on microorganisms such as fungal communities. For example tillage is known to decrease abundance and have long term effects on fungal communities (Steenwerth et al. 2008). Maintenance or otherwise of soil quality is therefore of importance in recognising the impacts that continued farming may potentially have on long lived perennial crops.

2.4.5 Nutrient supply

Soil mineral composition has an influence on vine development, fruit ripening (van Leeuwen et al. 2004) and vine balance (Keller 2005) with subsequent impacts on wine quality. It is not just the presence of nutrients that is important but also the ability of the vine roots to uptake them (Keller 2005). An abundance of both available nutrients and water supply can result in large and dense canopies, which are associated with both reduced total soluble solids and colour and high acidity (Jackson and Lombard 1993, Dry and Loveys 1998). A common practice is to use minor deficits of nutrient and water which is suggested to be beneficial to fruit and therefore wine quality (Keller 2005). In one study comparing terroir components of soil, cultivar and climate, vine mineral uptake measured in the petioles found no apparent relationship between

petiole N, K or Mg and berry composition (van Leeuwen et al. 2004). While a more recent study found significant differences in both petiole and grape berry nutrient status from vines of contrasting performance and soil properties (Bramley et al. 2011). White (2009) for one considers vine nutrition as secondary to climate and soil water relations in regards to viticultural potential since it is able to be manipulated. However, the presence or absence of conditions or attributes either positive or negative will no doubt have some underlying effect on the influence of terroir.

2.4.6 Soil water balance

The presence and management of plant available soil water can be a contributing and determining factor in the success of a particular vineyard site. The oldest vineyards were planted prior to the invention and adoption of modern irrigation methods. Therefore old vineyards if farmed successfully without irrigation would likely have had soil conditions and water availability suitable to sustain vine growth. Soil water relations are well known to influence vine vigour and fruit quality (Jackson and Lombard 1993, Dry and Loveys 1998, Wheeler and Pickering 2003, Keller 2005, Koundouras et al. 2006, White 2009). In light of old vineyards being planted well before irrigation was common, the modern view is that where evaporation exceeds rainfall, irrigation is required (McCarthy 1997, White 2003). By this definition, old vineyards must have either unique soil conditions to survive, or potentially have been manually irrigated in the past. The application of irrigation, not just for survival, can be used as a powerful tool to improve vine performance and manage vigour (Dry and Loveys 1998, Keller 2005). However, water application must be managed carefully; excessive irrigation can be detrimental to fruit quality, specifically via increased vigour (Dry and Loveys 1998, Wheeler and Pickering 2003). Alternatively water deficit during berry ripening is known to affect growth and composition of the fruit but not the rate and timing of ripening (Williams 1990). Where irrigation is required for a profitable crop, if well managed, it can be beneficial to both yield and quality (McCarthy 1983, 1997).

2.5 Carbohydrates

2.5.2 The importance of stored reserves

Carbohydrates stored in permanent woody tissues of grapevines are essential to provide a carbon source to support growth following budburst (Mullins 1992, Wheeler and Pickering 2003, Holzapfel et al. 2010). Furthermore, these reserves are used during times of reduced or absent photosynthesis (Mullins 1992). Nitrogen too is an important stored reserve; leaf chlorophyll concentration in early season growth has been shown to correlate closely with the

amount of nitrogen stored in the perennial parts of the vine (Keller and Koblet 1995, Treeby and Wheatley 2006). Based on this, stored reserves are essential for maintenance of early season growth and function. Woody vine structures, including the trunk and roots, have water content of approximately 60%, serving as a solvent for ions and organic molecules (Keller 2015). Not only are the reserves important for vegetative growth but also reproductive growth. Carbohydrate reserves have been reported to influence floral differentiation and inferred to affect inflorescence branching and retention (Holzapfel et al. 2010), thereby influencing fruit yield and variation in yield components from season to season (Holzapfel et al. 2010). Removal of basal leaves throughout the season reduced carbohydrate storage amounts and reduced inflorescence number per shoot and flower number per inflorescence by up to 50% (Bennett et al. 2005). Flower abortion has been inversely correlated with sugar (reserve) availability during flower formation, specifically female meiosis (Lebon et al. 2008). These results lead towards the thinking that there may be an effect of vine age resulting from greater potential carbohydrate storage due to physical size accumulation over time.

2.5.3 Carbohydrate location and role

It is widely accepted that non-structural carbohydrate reserves fluctuate annually in perennial wood, from being most depleted between budburst and flowering and accumulating until dormancy (Mullins 1992, Holzapfel et al. 2010, Zufferey et al. 2012). Non-structural carbohydrate reserves are shared among perennial parts of the plant. The above ground perennial tissues make up approximately 18-27% of total reserves (Holzapfel et al. 2010). The relative contribution that roots make to total carbohydrate reserves varies widely from 18-75% across varieties (Treeby and Wheatley 2006, Holzapfel et al. 2010). Total non-structural carbohydrates play a key role in vine longevity and potential harvest quality (Zufferey et al. 2012). The leaf to fruit ratio has been shown to be of importance in maintaining total non-structural carbohydrates reserves (Zufferey et al. 2012). Adjustment of this ratio both up and down can substantially modify reserve status, which has implications over multiple subsequent seasons (Holzapfel and Smith 2012). Reducing carbohydrate reserve status can make vines more prone to biotic stress (Holzapfel et al. 2010). Smart (1993) suggested that stable carbohydrate reserves are important for vine growth; he anecdotally dispels the notion that older vines have more stored carbohydrate reserves. Trunk cross-sectional analysis has shown a linear correlation between trunk diameter and vine age (Tyminski 2013). This study only assessed vines from 3 to 24 years of age; however, following this trend, increasing vine age is likely to result in greater trunk diameter and therefore as a consequent increase carbohydrate storage potential.

2.5.4 Carbohydrate accumulation with age; above ground

In general, older vines are visibly larger having a greater girth and volume of perennial wood than younger vines. This is supported by a study of narrow vine age that found a significant linear relationship of 0.673 between age and diameter (Tyminski 2013). This perennial wood, as noted previously, is the major storage organ for reserve carbohydrate. It has been calculated through destructive sampling that 18 year old Cabernet Sauvignon vines accumulated on average 240g $vine^{-1}$ $year^{-1}$ in trunk mass (Williams and Biscay 1991). In comparison, a study in South Africa of 10 year old Chenin Blanc vines found average total trunk and cordon accumulation to be approximately 360g $vine^{-1}$ $year^{-1}$ since planting (Saayman and Huyssteen 1980). The oldest sampled vines were 50-60 years old and had a trunk mass accumulation of approximately 50g $vine^{-1}$ $year^{-1}$. These studies show varied accumulation rates and further work would need to be conducted to determine at what point the rate of accumulation slowed beyond the initial high values. This also suggests that, if the work of Tyminski (2013) encompassed a greater range of vine age, the authors might re-consider the findings of a linear relationship. Biologically it is not likely that even a long lived plant will continue biomass accumulation in a linear cumulative fashion. In a study of several tree species relative growth rate (kg/year) is reported to reduce rapidly early on in the trees life and slow over chronological time spaning 1-36 years to a maximum of 32-269 years (Mencuccini et al. 2005). The study of Mencuccini et al. (2005) fitted a power correlation which better represents growth rate and time, this would have been more appropriate for the study undertaken by Tyminski (2013). It is important to consider that many studies on aging and size in perennial species do not consider effects associated with routine annual pruning.

Accumulation of carbohydrate reserves provides the plant body with a buffer against seasonal conditions, be they biotic or abiotic (Gladstones 1992, Mullins 1992). In a study comparing vines aged from 6 to 50 years, increased yields and enhanced fruit maturity was credited to the presence of additional wood due to the above ground vine training system (Reynolds et al. 2008). Water storage of older vines with more carbohydrate storage had a greater capacity to buffer against stress, making them less vulnerable to xylem cavitation and other stress factors (Keller 2005). These findings support the suggestion that older vines might have a greater ability to handle seasonal stresses than younger vines.

2.5.5 Carbohydrate accumulation with age; below ground, roots.

Root maturity is suggested to occur at a time when the roots have fully occupied their available soil volume; following this, further maturation occurs via radial thickening (Gladstones 2011).

Woody framework roots of *Vitis vinifera* have been found to be up to 100mm in diameter at a depth of 300-350mm from the surface. This depth is suggested to not increase after the third year from planting (Richards 1983, Jackson 2008). This would be dependent on the soil type with vine roots taking 7-8 years to fully colonise the soil (Saayman 1982). A destructive study of 18 year old Cabernet Sauvignon vines found there was no relationship between vine root and trunk dry weights (Williams and Biscay 1991). The specific soil characteristics, such as soil texture and water holding capacity will ultimately determine the vines potential availability to water. This in turn will have implications for the structure and growth over time. In sandy soils vines show a greater root to shoot ratio to compensate for the increased possibility of cavitation (Keller 2015). Therefore carbohydrate reserves in the below ground permanent framework may provide greater differences between old and young vines than those stored in the above ground structures. Without destructive sampling or excavation this is difficult to measure.

2.6 Vigour

2.6.2 Vine vigour and vine capacity

Vigour is commonly used to describe vegetative vine growth, in terms of both rate and extent of growth. Vine vigour is often associated with changes to fruit and wine quality (Clingeleffer 2000). Vine capacity more accurately describes the total production of the vine (Dry and Loveys 1998). Winkler (1970) uses the rate of growth of an individual shoot as an example of vigour, referring to vine capacity as the individual shoots potential for production and not just the rate of growth. Dry and Loveys (1998) provide a very succinct overview of vigour and capacity, outlining that (vine) vigour in itself is not always detrimental to fruit quality. It is important to note from this discussion the suggestion that if a vine of high vigour can produce a high volume of quality fruit then it is said to be 'balanced' (Dry and Loveys 1998). Many factors can influence vigour irrespective of vine age, both viticultural and environmental. An excess or deficit of water and/or nutrients can result in vigour issues and unbalanced vine growth (Keller 2005). It is not simply the presence of high or low shoot vigour that is undesirable. When too much vigour competes with fruit production or there is not enough vigour to maintain the crop, this is when undesirable consequences for fruit quality may arise (Dry and Loveys 1998).

2.6.3 High or excessive vigour

High vine vigour can be associated with a large number of variables from site to management. For example, deep fertile soils with high water holding capacity (Smart and Robinson 1991)

and high mineral nutrition specifically due to nitrogen (Jackson and Lombard 1993) are related to high vigour. Excessive pruning by removing too many buds also can result in high vigour of remaining shoots (Keller 2010). High vigour can be characterised principally by: long internodes, excessive shoot growth late into the season, strong lateral growth and lateral leaf area greater than primary leaf area (Smart and Robinson 1991, Wheeler and Pickering 2003). If the vine trellis or support system is inadequate, this can create a dense shaded canopy exhibiting a high leaf layer number (Terry and Kurtural 2011). Dense canopies can be detrimental to grape quality, noted by: reduced total soluble solids, reduced colour, higher acidity, increased disease pressure and in the majority of situations—lower wine quality (Jackson and Lombard 1993, Dry and Loveys 1998). In addition to these fruit quality effects, the reduction of light infiltration into the canopy near the renewal zone reduces node fruitfulness perpetuating a vegetative growth cycle (Dry 2000, Sommer et al. 2000, Terry and Kurtural 2011). Initial site selection should attempt to avoid factors that will lead to excessive vigour and the creation of a vegetative growth cycle. If this is unavoidable there are many options to ameliorate high vigour situations; however, these can be relatively costly requiring technically complicated and labour intensive divided canopy trellis systems.

2.6.4 Low or reduced vigour

Low vigour vine growth is characterised directly by short internodes, short shoots and low pruning weights and indirectly by high leaf and fruit exposure and high light exposure to the interior canopy (Smart 1985, Smart 1993, Dokoozlian and Kliewer 1995, Cortell et al. 2007). As with high vigour situations there can be many causes for low vine vigour. Vine shoots are very sensitive to deficit in water and nutrient status (Keller 2005). Extreme low vigour can be detrimental to potential quality, due to a high degree of bunch exposure, which especially in hot climates, has been shown to inhibit anthocyanin production in berries (Haselgrove et al. 2000, Spayd et al. 2002). Both sunlight and temperature were shown to influence grape berry composition (Spayd et al. 2002) where temperature was later shown to have the greatest influence on anthocyanin profile (Tarara et al. 2008). Furthermore, excessive fruit exposure can result in physical evaporation and degradation of pigments and aromatics as berry temperature can exceed 12-15^{o}C above ambient (Gladstones 2011).

2.6.5 Vigour moderation and vine balance

Vine vigour has a strong influence over both vine structure and fruit composition. Due to the number of variables involved and sheer complexity of managing them, site selection is often

the first opportunity to control vigour (Dry and Loveys 1998). Fruit with good exposure to sunlight will generally have higher concentrations of sugars, anthocyanins and total phenolics along with lower concentrations of malic acid, potassium and a lower pH (Smart and Robinson 1991, Bergqvist et al. 2001). Kliewer and Dokoozlian (2005) observed that fruit tasted from vines with well exposed leaves and fruit obtained the highest quality score in terms of flavour. Very low yields have been found to improve berry skin anthocyanin levels with higher acetate esters and lower secondary alcohols giving a more intense aroma (Jackson and Lombard 1993) and generally increased positive aspects of fruit composition and wine quality (Smart 1985). However, it is important to note that insufficient leaf area and fruit exposure can be as detrimental to fruit quality as excessive leaf cover (Jackson 2008). In addition very low yields are not typical of well balanced vines.

It has been noted that a reduction in vigour may be evident in older vineyards over time as observed in Bordeaux (Smart 1993). Smart (1993) suggests that vigour reduction in old vines is due to impoverished nutrient-depleted vineyard soils combined with an accumulation of pruning wounds allowing the vine to become infected with fungi. This suggestion is also supported by Goode (2005) who mirrors the statement that old vines produce better fruit, due to less vigour from disease and depleted soils. It has been shown that vine age was negatively correlated with vigour (*pruning weight)* and berry number per bunch; however, vine age was not strongly correlated with overall yield or yield components (Considine 2004). Reducing vigour either by management or other effects is essentially re-balancing the relationship between reproductive growth and vegetative growth. When a suitable relationship is achieved between these elements discussed this is termed ‘vine balance’ (Reynolds and Vanden Heuvel 2009).

2.7 Vine balance

2.7.2 What is vine balance

Vine balance is a term used to identify when measurable parameters of vine growth are ideal for a situation, resulting in growth conditions that produce fruit with the highest possible quality attributes for a targeted style (Iland et al. 2011). Most definitions from literature conform to the above statement, and generally include reference to maximising yield without negative impacts (Howell 2001), or achieving an equilibrium of growth (Wessner and Kurtural 2013). When vegetative growth and fruit load are in balance, management of variables aims to result in grapes of ideal ripeness (Gladstones 1992, White 2003). This makes vine balance a critical

factor in viticultural production, directly relating to wine quality and potential longevity of production.

2.7.3 Vine balance and age

Vine balance provides a method to empirically assess vine differences, this is not without its challenges. These arise due to the many factors involved with the regulation and interdependence of vigour, capacity and crop load (Keller 2015). For example, vine balance can tie in with the previous discussion of vigour where several examples of reduced vigour, resulting in increased balance, are attributed to improved wine or fruit quality (Smart 1985, Jackson and Lombard 1993, Smart 1993, Goode 2005). However there is a distinct lack of literature directly assessing vine age and balance, especially to establish if increased age results in greater balance.

2.7.4 Measuring vine balance

The first method proposing a measure of vine balance was developed over 100 years ago in the south of France by M.L Ravaz (Dry et al. 2004). This method suggests that a particular ratio of fruit to wood (now known as the Ravaz index) is the key to consistently producing good quality fruit. This Ravaz index is still somewhat relevant today; however, due to vine balance having many interacting variables this same concept has been re-interpreted in many ways; see Iland et al. (2011) for many examples.

Vine balance is most often expressed as one of two ratios: the ratio between fruit weight and pruning weight per vine (Y/P) or leaf area to yield (LA/Y). It should be noted that there are several other indices, and indicators used for discussing vine balance, their suitability and limitations are well summarised in Dry et al. (2004) and Howell (2001). The most common measures of vine balance that may be applied to a study comparing young and old vines are described below.

2.7.5 Fruit yield to pruning weight (Y/P)

The Ravaz index as noted above utilises pruning weight and fruit weight as an assessment of balance. In the 1920s Partridge built on this suggesting pruning weight could be also used to determine the upper level of fruit production in the following year, referring to this as ‘the growth yield relationship’ (Howell 2001). The ideal Y/P ratio is generally between 5 and 10 and towards the upper end in hotter climates (Dry et al. 2004). The spread in this value is due to differences in variety, canopy management or trellis system that may exist (Kliewer and Dokoozlian 2005).

2.7.6 Leaf area

Early work into Y/P noted the importance of leaf area (Howell 2001) when ripening a given crop load; however, it was Smart (1985) whose work on canopy microclimate highlighted the need to consider leaf area. The photosynthetic capacity of the plant is directly linked to leaf area, this can be considered the most critical factor in determining final berry total soluble solids (Jackson and Lombard 1993). Smart and Robinson (1991) called this the leaf area to yield ratio or LA/Y and assigned optimal values. LA/Y takes into account canopy management and light interception as shown by Kliewer and Dokoozlian (2005) who demonstrated that a single canopy trellis LA/Y of 0.8 to 1.2m^2/kg was required compared to 0.5 to 0.8m^2/kg for a divided canopy. LA/Y is inversely related to Y/P with high values indicating high or excessive vigour (Dry et al. 2004).

It is suggested by Dry et al. (2004) that these measurements and more are useful but should not be used in isolation but in a more holistic manner when assessing vine performance considering both vine and wine attributes. Likewise of the current accepted measures Lakso (2009) suggests that they are not static concepts having many interacting components and therefore cannot be defined by simple indices.

2.8 Quality

The definition of quality can be varied however, the Oxford dictionary states it as: "the standard of something as measured against other things of a similar kind; the degree of excellence of something". Wine is routinely assessed in relation to its quality, commonly measured against similar products from the same region and variety or even against global benchmarks. Quality is such a powerful term in association with wine that entire industries are supported by its discussion and publication. Due to the price premium and prestige that quality determinations can provide, prediction and objective measurements of quality are often sought (Gishen et al. 2001, Francis et al. 2005, Gambetta et al. 2016). These objective measures are commonly aligned with sensory studies to assist in the identification of attributes and components associated with quality.

The final assessment of wine quality is via its sensory attributes, many of which originate in the grape berry. Therefore grape quality is inextricably linked to potential wine quality (Dry and Coombe 2004, Iland et al. 2011). The final composition of wine, while derived from fresh grapes, is a highly complex mixture of compounds that originate from not only grapes but the yeast, bacteria and vessel used during fermentation (Swiegers et al. 2005). During the fermentation process there are a large number of potential sources of influence, providing a

near infinite unique combination of compounds that when tasted give rise to the ultimate signal of quality, pleasure.

Sensory science is a recognised field for examining the complex nature of flavours and aroma where the judgments of a panel are considered more reliable than those of an individual (Robinson et al. 2014). Sensory descriptive analysis uses a trained panel to assess attributes within presented samples (Mantilla et al. 2012) via multiple modalities including taste, touch and smell (Lawless and Heymann 2010). This method of sensory assessment is commonly applied to both grapes and wine.

2.8.2 Grape quality

Grape quality assessment is a precursor to potential wine quality. The physical and chemical composition of the grape is the foundation for wine production, this clearly affects wine composition (Mercurio et al. 2010). All the components of the grape are utilised in red wine production: the pulp, skin and seeds are all combined during fermentation prior to being separated on completion of fermentation. During this time even the grape seeds contribute to red wine composition (Ristic and Iland 2005). The main chemical constituents of the grape are sugars, acids and pigments (Gladstones 1992, Iland 1997). The composition of these elements depends on many factors, such as: variety, climate, degree of maturity, soil characteristics, water availability and management (Cozzolino et al. 2010). Specifically it has recently been reported that water availability has a significant effect on the perceived astringency of Syrah berries (Kyraleou et al. 2016). Despite the chemical composition being so important, fruit characteristics are governed by the personal perception of taste and flavour (Keller 2015) with organoleptic assessments of grapes often made before harvest (Dry and Coombe 2004, Mantilla et al. 2012).

Measures of grape chemical composition can be used to make objective measures of quality. The most common of which are: total soluble solids (TSS), titratable acidity (TA), pH and phenolic and anthocyanin concentrations—including colour (Jackson and Lombard 1993, Kennedy 2001, Francis et al. 2005). Grape colour for example has been associated with wine quality and has even been used for commercial quality assessment (Gishen et al. 2001, Kennedy 2001, Francis et al. 2005, Herderich and Smith 2005). Despite this and even utilising a broad range of measures, linking grape quality to wine quality is challenging. This is further complicated by the fact that in isolation these measures are not always an adequate predictor of potential quality or style (Iland et al. 2011). Nor should some measures be used interchangeably as predictors such as tannins and phenolics (Mercurio et al. 2010). The use of

TSS, TA and pH to determine optimum grape maturity has been implemented for some time. Over time it has also been noted that these measures should be considered together with other indices to determine optimal maturity (Du Plessis 1984).

In addition to the chemical composition, the elemental composition of wine grapes has also been used to make connections with quality parameters. Unsupervised multivariate statistical analysis is often employed to uncover relationships. Above the aroma recognition threshold these fermentation-derived compounds have descriptors of *sweet* and *perfume* for hexyl acetate and *roses* for 2-phenylethanol (Siebert et al. 2005). It has been shown that both Zn and Cu were positively correlated with the presence of hexyl acetate and negatively correlated with 2-phenylethanol (Gambetta et al. 2016). The correlation of minerals in grapes and aroma active volatiles in wine is, however, not a direct link, as it does not account for yeast metabolic function. The metals Cu and Zn are suggested to be among the most important to influence fermentative processes (Walker 2004). In addition to Zn, Mn is also known to dramatically influence fermentative performance, and modulate environmental stress, while Cu is required as a component for redox pigments (Walker 2004).

In this way grapes that satisfy optimal values for many of these compositional measures are likely to produce a higher quality wine than grapes that do not. There have been many studies undertaken to attempt to link grape or wine composition to wine quality. (Mercurio et al. 2010) reports on a comprehensive list of 21 studies which vary in terms of parameters assessed and quality attributes measured. Some studies have shown that basic fruit measures do not always align with wine quality scores and are therefore not a good measure of quality. For example, fruit destined for wines of contrasting quality levels (low and high) was found to have no significant difference between pH, TSS, anthocyanin and phenolic concentrations (Bramley 2005). Holt et al. (2008) found that high berry anthocyanins, total phenolics and tannin concentration levels were not good indicators of wine quality scores. Grape phenolic composition and concentration can be influenced by a broad range of variables such as; cultivar, microclimate and management (Downey et al. 2004, Koundouras et al. 2006, Cohen and Kennedy 2010). Therefore these measures can lead to different interpretations of quality and different quality ratings depending on the drivers of difference. As Iland et al. (2011) states, grape quality should be linked to the wine produced, with final wine quality an indicator of grape quality.

Berry sensory analysis (BSA) is the application of sensory descriptive analysis to fresh grape berries. BSA is commonly undertaken in a less formal fashion by winemakers to determine harvest decisions based of fruit characteristics in the field. Practical implementation of BSA has been thoroughly described by Winter et al. (2004). BSA has been successfully applied in a range of research applications including canopy manipulations and response to environmental stress among others (Lohitnavy et al. 2010 Bonada et al. 2015). Le Moigne et al. (2008) used BSA in conjunction with berry deformity to identify stages of ripeness in Cabernet Franc. This methodology has been applied to compositional measures of Shiraz (Olarte Mantilla et al. 2015), and in the same way it can potentially be used to identify differences in grapes based on vine age. Furthermore, identifying if characters of difference are within common descriptions of quality can improve our understanding of sensory attributes of grape quality.

2.8.3 Wine quality

Quality in relation to wine can mean different things to different people and is based on personal judgment (Charters and Pettigrew 2007). Wine has been shown to comprise of a number of dimensions, both intrinsic and extrinsic (Charters and Pettigrew 2007). The results of different drivers have led to a range of descriptors used to signify quality, from being 'fit for purpose' or having an 'absence of faults' or being 'representative of a style category' (Charters and Pettigrew 2007, Iland et al. 2011). Quality perception has even been linked to associations with landscape images of greater visual impact (Tempesta et al. 2010). While these descriptions of quality are difficult to assess, there are several descriptors that are used to signify wine quality. The most common elements of quality identified are: complexity, balance, personality, length, intensity of flavour and varietal purity (Charters and Pettigrew 2007, Iland et al. 2011).

Quality in relation to vine age has been the subject of peer reviewed studies. In a multi-season multi-varietal study in Switzerland wines produced from older vines were preferred to those produced from vines 29 years younger (Zufferey and Maigre 2008). The Shiraz wines in particular were noted as having a lighter, more intense red fruit profile compared to the younger vine wines. In California commercial Cabernet Sauvignon wines from older vines were judged to have higher wine quality ratings (Heymann and Noble 1987). Vine age was positively correlated with berry aroma and fruit flavour where young vine wines were correlated with green bean and vegetative flavours. These studies suggest a link between vine age and measures of wine quality, however, the study of Reynolds et al. (2008) did not a find consistent effect of age; possibly due to the 10 year age difference studied.

Wine is made up of numerous small chemical molecules and the makeup of these molecules is mainly derived from three sources: the grape, the yeast strain used to ferment the berry and the fermentation vessel (Cuadros-Inostroza et al. 2010). This leaves a very large scope to assess and attempt to align quality scores with wine compositional parameters.

Of the many qualitative parameters used to assess wine quality, colour has shown the largest correlations (Mercurio et al. 2010). In young red wines colour was positively correlated with expert wine scores and deeper colour was more highly rated than lighter colour (Somers and Evans 1974). Colour in general has been shown in many cases to correlate to quality assessments or bottle price (Somers and Evans 1974, Jackson et al. 1978, Francis et al. 2005, Kassara and Kennedy 2011). These studies generally show that higher wine colour measures result in higher wine quality score or higher bottle price. Other studies have shown positive correlations with anthocyanin concentration and wine quality scores (Jackson et al. 1978, Francis and Newton 2005).

Total phenolics too have also been positively associated with wine quality scores (Ristic et al. 2007, Ristic et al. 2010). Methods of tannin and wine phenolic concentration measurements have allowed these parameters to be successfully used in wine assessment (Sarneckis et al. 2006, Mercurio et al. 2007). For a large number of wines (n=1643) over three vintages, higher scores were given to wines that had greater phenolic and tannin concentrations (Mercurio et al. 2010). These secondary metabolites such as phenolics and tannins are known to have specific sensory attributes such as mouthfeel and astringency (Mercurio et al. 2010) which can drive quality rating or assessments. Many studies have drawn correlations between secondary metabolites and wine quality (Johnstone et al. 1996, Ristic and Iland 2005, Ristic et al. 2007, Mercurio et al. 2010). Further detail into the chemistry of grape and wine composition identifies several classes of secondary metabolites of grape derived flavanoids. These are; flavonols (quercetin, kaempferol, myricetin, isorhammenetin, laricitrin, syringetin), anthocyanins (malvidin, petunidin, peonidin, delphinidin, cyaniding, pelargonidin) and flavan-3-ols (catechin, epicatechin, gallocatechin, procyanidins, condensed tannins) (Casassa and Harbertson 2014). While the origin of these metabolites is in the grape berry prior to fermentation, these metabolites are the principal sources of wine colour, aroma, and flavour.

Grape phenolic composition and concentration is known to affect the appearance, taste and mouthfeel of wine (Casassa and Harbertson 2014). Specifically, the flavonols quercetin and kaempferol which are commonly found in red grape skins. Quercetin and kaempferol have

been identified as being discriminating phenolic compounds in characterising geographic origin (Makris et al. 2006). Thus the potential quality relating to these compounds is driven by environmental conditions perhaps as a result of terroir. The sensory effects of quercetin derivatives have been linked to bitterness in red wine (Rudnitskaya et al. 2010) adding further weight to the discrimination of wines via terroir linking composition and sensory outcomes. Links between the terroir or site effect in relation to quality have been made using a range of compositional measures, including; flavonoid composition (Brossaud et al. 1999, Tarr et al. 2013) and total phenol content (Hooper et al. 1985).

Recent advances in the identification and quantification of compounds that contribute to wine flavour have provided a greater understanding to the complex nature of sensory perception. One of the aims of these advances is to deconstruct the chemical components of wine flavour in order to identify and associate these to sensory properties. There are many techniques that may be employed to purify and quantify the numerous compounds that can contribute to sensory perception. Gas chromatography (GC) has been widely used in assessing volatile compounds that contribute to both grape and wine aroma for over 60 years (Ebeler 2012). GC is a favoured method as it can provide quantitative information and can also allow tentative identification of molecules from the data collected (Robinson et al. 2014). The combination of GC and mass spectroscopy (MS) is even more powerful allowing molecule detection based on the ratio of their mass to charge. The combination of GC-MS is one of the most widely used techniques for targeted quantification of trace analytes in wine (Ebeler 2012, Vilanova et al. 2012). This has resulted in a very broad range of research questions having been addressed. For example GC-MS has been used to discriminate between cultivars (Ferreira et al. 2000), determine the effect of growing season (Schueuermann et al. 2016) and the indication of terroir (Anesi et al. 2015).

Combining sensory and chemical compositional data can lead to greater understanding of the drivers of wine quality (Vilanova et al. 2012, Gamero et al. 2014). The data obtained from these studies is often combined with multivariate statistical techniques such as PCA (Reynolds et al. 1996, Forde et al. 2011, Hopfer et al. 2015, Olarte Mantilla et al. 2015). This technique allows determination of relationships that may be otherwise hidden (Heymann and Noble 1989). Use of PCA to make statistical evaluations of sensory data is well established and widespread in a range of wine related studies; including comparisons such as soil texture and vine size (Reynolds et al. 2013), sodium chloride concentration (de Loryn et al. 2013) or

interrogating the volatile composition of grapes and wine (Arias et al. 2004, Gambetta et al. 2016).

2.9 Grapevine genetics

2.9.2 Conservation of genotype

The cultivated grape vine *Vitis vinifera* does not breed true from seed (Mullins 1992). Vegetative propagation, however, provides relative genetic stability, as well as phenotypic consistency of desirable traits (Jackson 2008). For this reason and to ensure perpetuation of elite selected cultivars (phenotypes), grapevine cultivars have been selected, conserved and maintained via (asexual) vegetative propagation since domestication thousands of years ago (Mullins 1992, Thomas et al. 1994, This et al. 2006, Pelsy 2010). Vegetative propagules from a single common ancestor are referred to as clones (Forneck 2005). One drawback associated with clonal propagation is the loss of genetic diversity generated by genome recombination during sexual reproduction (Emanuelli et al. 2013).

Despite the relative genetic stability that vegetative propagation provides, somatic mutations are still possible (Alizadeh and Singh 2009, Anhalt et al. 2011, Meneghetti et al. 2012). Although such mutations have the potential to disrupt quality traits, they can also result in the appearance of superior qualities or distinct characters producing an individual sport or new cultivar (Anhalt et al. 2011). Somatic mutations may be fixed via asexual propagation leading to intra-varietal diversity within cultivars resulting in several clonal lines with differing phenotypes (Franks et al. 2002).

A range of techniques have been utilised to study genetic diversity within cultivars including sequence specific amplification polymorphism (S-SAP) (Stajner et al. 2009, Wegscheider et al. 2009), microsatellites or simple sequence repeats (SSRs) (Imazio et al. 2002) inter simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) (Forneck 2005). The advent of next generation sequencing (NGS) and the use of single nucleotide polymorphism (SNP) markers allows the economic and reliable screening of tens or hundreds of thousands of markers per assay (de Lorenzis et al. 2017). SNPs are able to be identified from short reads created by NGS platforms, through aligning to a reference genome or *de novo* genome assembly (Nielsen et al. 2011). The use of reduced representation libraries (RRLs), as a form of complexity reduction combined with barcoded sampling allows for multiplexing of multiple samples in a single sequencing lane, has significantly reduced the cost and complexity

of SNP genotyping (Elshire et al. 2011). Compared to some other genotyping techniques, SNPs are highly stable and reproducible between laboratories (Cabezas et al. 2011).

However, many clonal lines of *V. vinifera* can often be indistinguishable using genetic information alone (Blaich et al. 2007, de Lorenzis et al. 2017). A recent example of this was presented in two cultivars; Aglianico and Muscat, whereby an 18k SNP array could not discriminate clonal lines (de Lorenzis et al. 2017). However, this study did discriminate clones based on chemical diversity of secondary metabolites; specifically anthocyanins and phenolic substances (de Lorenzis et al. 2017). This is a method of discrimination via phenotype not unlike the physical practice of ampelography (Galet 1979) albeit at a finer resolution. The authors speculated that epigenetic regulatory mechanisms, although not included in their study, this could contribute to clonal diversity in grapevine. This was hypothesised much earlier some 15 years ago by Imazio et al. (2002) who suggested that morphological differences amongst clones (in this case Traminer) could be the result of differential expression owing to clone specific epigenetic differences. Since then, the use of epigenetic approaches has shown to be better suited for the identification of clonal lines (Ocana et al. 2013).

The process of vegetative propagation in plants shortcuts the developmental transitions from seedling to adult plant. This vegetative phase change has already occurred in woody perennials sometimes at the expense of juvenile potential (such as rooting). This phase change can be reversed and is known as rejuvenation. Various techniques of propagation from mature tissues have resulted in observations of change due to rejuvenation. Phase change following rejuvenation has been shown to present altered DNA methylation states compared with juvenile and mature tissues in *Sequoia sempervrens* (Huang et al. 2012). Propagation based methylation differences have been observed several species including; *Sequoiadendron giganteum* (giant sequioa) (Monteuuis et al. 2008), *Manihot esculenta* (cassava) (Kitimu et al. 2015) *Pinus radiata* (radiata pine) (Fraga et al. 2002) and *Vitis vinifera* (grapevine) (Baranek et al. 2015). These differences across species suggest a consistent alteration in methylation may be due to the conditions of propagation or the process of plant rejuvenation.

2.9.3 Epigenetic mechanisms

Epigenetic mechanisms are molecular modifications that result in changes in gene-expression and phenotype without affecting the underlying DNA sequence (Verhoeven et al. 2010, Becker and Weigel 2012, De Paoli 2013). Epigenetic variation is essential (Vanyushin and Ashapkin 2011), inheritable (Verhoeven et al. 2010) and reversible (Ocana et al. 2013). Several forms of epigenetic modification can influence transcription and gene expression. A specific feature of

the plant genome is a high degree of DNA cytosine methylation (5-methylcytosine: m^5C) (Vanyushin 2006). Cytosine methylation is a covalent modification to DNA with dual roles of regulating gene expression and silencing transposable elements, or foreign DNA (Torregrosa et al. 2011). In plants this is the most studied modification in the context of heritable epigenetic variation (Verhoeven and Preite 2014). In experiments where DNA methylation is suppressed, a large number of inheritable phenotypic effects can be observed and are caused by unusual reactivation of suppressed genes (Vanyushin and Ashapkin 2011). DNA methylation is considered a mechanism that enables species to adapt to changes in environmental variables at a finer timescale than is possible via the relative stability of the underlying genome without the need for recombination or random beneficial mutations (Tricker 2015). This means that even with homogeneity of DNA amongst clones of the same genotype, clones have been proven to present phenotypic plasticity (Dal Santo et al. 2013). Phenotypic plasticity refers to the extent that the environment modifies the phenotype (Via and Lande 1985). Alteration of phenotype due to gene expression can be regulated by a range of biological mechanisms. Epigenetic modification is one mechanism which can modify gene expression under specific environmental conditions (Thomas 2013) resulting in phenotypic modification to adapt to a changing environment.

The genotype x environment (GxE) interaction is defined as the differential response of genotypes under changes in the environment (Via and Lande 1985). Since first being described some 30 years ago many studies now attribute this interaction of genotype and environment to epigenetic regulation of transcription such as via DNA methylation. In plants DNA methylation has been shown to be accumulatively triggered by environmental stimuli such as temperature. For example, *Arabidopsis thaliana* presents an epigenetic regulation of flowering preferentially during optimal conditions only after prolonged exposure to cold (Song et al. 2012, Turck and Coupland 2014). Many other environmental cues have also been recorded to affect DNA methylation in plants (Schellenbaum et al. 2008, Becker and Weigel 2012). Specific examples are atmospheric relative humidity (Tricker et al. 2012), drought (Rico et al. 2014), salinity (Lira-Medeiros et al. 2010) and herbivory (Herrera and Bazaga 2013) across various species. As such, DNA methylation has been described as “hidden variation” that can be released under particular environmental conditions (Turck and Coupland 2014). This epigenetically driven phenotypic plasticity is believed to buffer against environmental extremes thereby maintaining homeostasis of primary metabolism (Dal Santo et al. 2013).

The presence of cytosine methylation can be measured via several molecular techniques. In non-model organisms where the entire genome is unknown methylation levels can be observed via the methylation-sensitive amplified polymorphism (MSAP) technique (Fulnecek and Kovarik 2014). This MSAP technique is a modified AFLP technique which uses restriction enzymes (isoschizomers; e.g. *Hpa*II and *Msp*I) with differential sensitivity to cytosine methylation at restriction sites (CCGG for *Hpa*II and *Msp*I) (Ocana et al. 2013). The methylation state of the CCGG site will determine if the restriction enzyme can cut the DNA producing a fragment. The presence/absence and relative abundance of such fragments can then be measured resulting in determination of methylation patterns across the studied genomes. The drawback to this method is the anonymous nature of the resulting markers (no sequence information is generated). The highest resolution DNA methylation analysis is via bi-sulphite treatment and whole genome mapping (Cokus et al. 2008). The negative aspect to this is the computational time and cost associated with whole genome sequencing. The advancement of next generation sequencing (NGS) provides many opportunities to study not only methylation, but genome sequences at a reduced cost. The use of RRLs whereby a fraction of the genome is often highly multiplexed and then sequenced presents new opportunities to explore both genotyping and epi-genotyping.

2.9.4 Epigenetics in Vitis vinifera

A great deal of research has been undertaken using genetic fingerprinting techniques to differentiate and identify grapevine varieties and clones. For instance, using the grapevine clones of Traminer both AFLP and MSAP techniques highlighted that methylation differences exist within the single cultivar (Imazio et al. 2002). Using AFLP (*viz.* genetic markers) alone does have limitations, for instance a study of 86 Riesling clones were unable to be grouped according to either age, sub-clonal lineage or origin (Anhalt et al. 2011). Imazio et al. (2002) suggests that morphological differences observed among clones could be due to differential expression of genes owing to epigenetic regulation, hence it is important to investigate epigenetic markers for comparative diversity and potential phenotypic segregation.

In *V. vinifera* MSAP has been used to highlight DNA methylation differences of grapevine mother clones and somaclones produced via somatic embryogenesis (Schellenbaum et al. 2008). MSAP has also been used to show intra-varietal diversity in Pinot Noir clones on an epigenetic level (Ocana et al. 2013). Again Blaich et al. (2007) found limitations with using AFLP markers alone when assessing Pinot Noir clones showing that 17 out of 70 common clones had ‘identical’ genotypes, and a further 48 clones were within 99% genetic similarity.

The author then went on to suggest that AFLP markers may serve as a basic tool but additional markers are necessary for detecting clonal variation (Blaich et al. 2007) such as epigenetic markers.

For a low cost mass screening technique AFLP combined with MSAP analysis can be used to provide a genetic map of relatedness between historic plantings, quasi-clones[1] and known clones of a single variety. However both techniques present some characteristics that work against their ability to differentiate between closely related genotypes, such as vegetatively propagated clones (Benjak et al. 2006). The generated markers are dominant (i.e. heterozygotes are scored as homozygotes) and size homoplasy (co-migration of bands) cannot be avoided. In addition large numbers of samples can be tedious to score introducing scorer's errors and subjectivity of band intensity (Bonin et al. 2004, Blaich et al. 2007). Also reproducibility between laboratories can be difficult due to extraction method, scoring system and by fragment separation technologies and platforms.

In order to avoid the negative characteristics associated with the AFLP/MSAP platform a new technology named methylation sensitive Genotyping By Sequencing (ms-GBS) that uses the same restriction enzyme principle of MSAPs has been recently developed (Kitimu et al. 2015). This technology combines a Genotyping By Sequencing (GBS) approach (Elshire et al. 2011) to generate sequence based SNPs with the use of methylation sensitive restriction enzymes to generate markers capable of detect changes to the DNA methylation profiles of the samples analysed. The use of next generation sequencing and reduced representation libraries provide a rapid cost effective method of determination of both genetic and epigenetic diversity between grapevine genomes in one pass.

2.10 Summary

As highlighted in this literature review there are many factors which can influence vine growth and grape composition. The review presents evidence of many studies that investigate individual factors that contribute to vine growth and grape and wine quality. Viticultural research has covered much ground since early pioneering studies (Antcliff et al. 1957, Winkler 1958, Winkler 1970) which identified relationships of vine growth, fruitfulness and production to modern 'omics' based studies investigating gene transcription and metabolite levels at very high resolution (Dal Santo et al. 2013, Roullier-Gall et al. 2014, Anesi et al. 2015, Roullier-

[1] Quasi-clone refers to vines that are propagated from a known population of vines NOT one specific vine as a true clone would be.

Gall et al. 2015). As identified in this review, the challenge lies in linking these fragmented but intertwined disciplines of viticulture and oenology to one single variable.

Despite the extensive resource of viticultural and oenological science across many fields and years, few examples exist that link vine age with wine quality. While this is scarcely investigated it is increasingly important as all grapevines are subject to rising chronological age. Therefore the influence that age may present over multiple cropping cycles is an important consideration to understand.

The ability to study plant age in any system is limited by the availability of suitable subjects to study. This research will need to take into consideration the many avenues of research and knowledge already published in order to apply this to grapevines with significant age differences. This novel and challenging subject will require the integration of various scientific disciplines such as; viticulture, oenology, chemistry, sensory science and molecular biology. In order to elucidate the effect that vine aging may have will require the linking and integration of a range of data-sets. Furthermore, this research will utilise both traditional and new technologies in order to achieve these project aims.

The complex nature of viticultural production highlighted in this review demands that to undertake a study, investigating vine age as a factor would certainly address gaps in the present literature. In addition, results would be of great interest and value to the wine industry adding to the knowledge of the discipline. The results of such a study would not be limited to the wine industry but informative to a range of horticultural and forestry industries that cultivate long lived perennial crops. A study where confounding factors are minimised employing age as the treatment would be truly unique and enhance the field of viticulture. Furthermore, to undertake a study using vines of globally significant age differences would hopefully assist in elucidating differences that may exist.

Chapter 3. Published Article 1: Vine age effects on vine performance in Shiraz in the Barossa Valley.

Statement of Authorship

Title of Paper	Vine age effects on vine performance of Shiraz in the Barossa Valley, Australia
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Australian Journal of Grape and Wine Research

Principal Author

Name of Principal Author (Candidate)	Dylan Grigg
Contribution to the Paper	Grigg conducted the research experiments, analysed the data, drafted and constructed the manuscript.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Cassandra Collins
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript editing and evaluation.
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Contribution to the Paper	Dry contributed to research ideas, interpretation and editing of the manuscript.
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Vine age effects on vine performance of Shiraz in the Barossa Valley, Australia

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Abstract

Background and Aims: Old vineyards are often suggested to be superior to younger ones a claim that has scarcely been scientifically investigated. The aim of this study was to investigate if vineyard age influences vine performance.

Methods and Results: Five commercial Shiraz vineyards in the Barossa Valley, South Australia, were selected, each site contained younger vines which had been vegetatively propagated from older vines on the same site. Measurements of vegetative growth and reproductive development were obtained over three seasons. Results show that the effect of vine age is difficult to measure and separate from seasonal and site interactions. Vine age was strongly correlated with trunk circumference. Vines aged 49 years showed more similarities with older vines (93-168 years old) than with younger vines (6-28 years old).

Conclusions: Vine age had an impact on grapevine reproductive performance. Older vines produced greater yields than younger vines. Greater vine size, measured by increases in trunk circumference over time, may be a key determining factor in increased reproductive capacity.

Significance of study: Vine age is commonly attributed to superior vine performance, as a precursor to fruit and wine quality. Our findings suggest that increasing vine age had a positive effect on reproductive performance in terms of yield, contrary to popular belief. This establishes a starting point for future studies that are underway to quantify if these findings have an effect on fruit and wine quality.

Keywords: *Old vines, Shiraz, Vitis vinifera, vine balance, fruit weight*

Introduction

The grapevine *Vitis vinifera* L. is a long-lived perennial plant which has the ability to produce fruit over many cropping cycles, potentially exceeding 400 years (Vršič et al. 2011). Furthermore, 'old' vineyards are often highly regarded; producing wines that are sought-after and highly priced and prized due to perceived wine quality. The concept that greater vine age positively influences wine quality is long standing (Koblet and Perret 1980, Heymann and Noble 1987, Howell 2001, Considine 2004, Reynolds et al. 2008, Gladstones 2011).

There is a broad range of factors that may prevent a vineyard from reaching significant vine age. Generally, commercial vineyards can remain productive for 40-50 or even 60 years, after this they may become uneconomical (Gutierrez et al. 1985, White 2003, Robinson 2006) due to a reduction in yield relative to the crop value. The causes for yield reduction are diverse and varied, factors such as pathogens in the soil, wood or growing tissue or management issues such as soil compaction and degradation may all limit yield and viability if poorly managed over time (White 2003).

Many European countries have a much longer history of wine production than Australia; however, the introduction of phylloxera from America in the 1860s decimated the majority of these vineyards (Gale 2011). Widespread reconstitution of European vineyards onto American rootstocks from the 1880s has resulted in original pre-phylloxera plantings being isolated and rare, surviving only in conditions where the phylloxera cannot. North America's plantings of European noble varieties (mainly in California) were also largely lost also due to phylloxera (Winkler 1970, Gale 2011). While this pest has been present in Australia since 1877 (Gale 2011) through strict quarantine it has remained isolated and to date it has not been reported in South Australia. The Barossa Valley in South Australia is unique with large areas of phylloxera free vines in continual production that dates back almost 200 years.

Several studies have investigated the influence of vine age in the past and the main findings from these are summarised in Table 1. While the effect of vine age on vine growth and quality measures has been explored in scientific publications, as a phenomenon, the drivers of age-based differences are currently neither clear nor scientifically validated (Smart 1993, Dry 2008, Reynolds et al. 2008, Dry 2013). Even so, suggestion of causation is not uncommon, one such example being vine size, noted as a potential mechanism for the greater quality reported for fruit from older vines (Howell 2001). To date, there has been a distinct lack of published literature which attempts to investigate vine age in relation to vine performance.

Despite this, it is not uncommon to find acknowledgment of the phenomenon in a wide range of media from trade journals (Smart 1993, Dry 2008) to books (Goode 2005) and peer reviewed journals already cited.

Effects of vine age on fruit and wine quality are very difficult to evaluate due to the contribution and interaction of many factors. A starting point for the investigation into the effect of vine age in this study is via the analysis of vegetative and reproductive measures and the assessment of vine balance. The term vine balance is commonly used to identify the state when parameters of vine growth (fruit mass and pruning mass) are ideal, resulting in growth that produces fruit with the highest possible quality attributes for a targeted style (Iland et al. 2011). In this study, all sites are within the same region, allowing balance measures to be utilised for comparison. While vine balance itself is not a direct measure of final wine quality, high quality wine is less likely to be achievable from vines that have high vegetative vigour or are over-cropped (Lakso 2009).

In Australia and California, many vineyards greater than 100 years in age are still found, some reportedly dating back to the 1850s and 1880s respectively (BGWA 2014, Historic Vineyard Society 2014). To the best of our knowledge, studies that include vines of these ages have not been undertaken. Grapevine vegetative and reproductive measures were taken from five sites over three seasons using vines of substantially different ages. Details of compositional and sensory aspects of grapes and wine from this study will be reported in future publications. This manuscript aims to scientifically investigate if vine age measurably influences vine vegetative and reproductive performance.

Materials and Methods

Sites, experimental design and plant material

Five commercial Shiraz (*Vitis vinifera* L.) vineyards containing vines of contrasting planted age were selected in 2012 within the Barossa Valley zone, South Australia (Table 2). Local climatic data for the region can be found in Table 3 sourced from the Australian Bureau of Meteorology weather station at Nuriootpa (numbered 23373). Long term average temperature and rainfall were obtained from weather data archived on the Australian Bureau of Meteorology website (Bureau Of Meteorology 2015).

Effective degree days (E°days) were used as a general climatic statistic for seasonal comparison (Table 3). E°days were calculated after Gladstones (2011), briefly; mean

temperature values were truncated at 10°C and 19°C and adjusted for latitude to account for day length in months where mean temperature was below <19°C (average multiplier value 0.983). Further adjustment to account for the diurnal temperature range was applied via the updated mean daily range method. Mean temperatures for months October – January only were adjusted by -0.25°C for every 1°C wider than 12°C daily range and +0.25 for every 1°C narrower. Meteorological conditions for each of the growing seasons 2012/13, 2013/14 and 2014/15 are presented in Figure S1. The period from October to April corresponds to the average growing season in the Southern Hemisphere. References to growing season are within this period only. The Barossa zone is divided into two regions: the Barossa Valley and the Eden Valley. Sites 3, 4 and 5 are found in the Barossa Valley, which is generally drier and, warmer and earlier maturing than the Eden Valley region, which contains sites 1 and 2.

Sites were chosen with the assistance of local industry members and vineyard owners to ensure an accurate record of vine age, and to minimise differences between vines within each site. Consideration was given to relative homogeneity of site and vine characteristics between vine age groups within each site such as: soil type, row orientation, pruning method, trellis system and vineyard floor management. End vines and outside rows were excluded, each replicate row of vines was chosen to avoid missing vines internally preventing reduced competition effects. Each selected site contains vines established at two unique times, with old vines ranging from 93 to 168 years and, for comparison, young vines aged from 6 to 49 years at project inception. All young vines at each site were vegetatively propagated via hardwood cuttings from the older vines located at the same site, with the only exception being site 5, which was propagated via layering. All vines are growing on own roots. Virus status is unknown, vine health was assessed visually based on uniformity and representative growth, and appearance with the assistance of each vineyard manager / owner. *Eutypa lata* is known to affect vines in this region and vines with obvious symptoms or in decline were avoided. Clonal status of vine material is unknown but, as all vines were propagated via anonymous cuttings, they are considered to be a mass selection or quasi-clones.

While there is no universally agreed definition of when a vine is old, for the purpose of this discussion at each site older vines will be referred to as ‘old’ while younger vines will be referred to as ‘young’.

At each vineyard, three replicate blocks containing 4 or 5 vines in adjacent rows were selected resulting in 12-15 vines per age treatment per site. At sites 1-4 each vineyard block

was a discrete unit surrounded by vines of the same age. The younger plants at each site were planted in land specifically prepared for establishment with new trellis infrastructure. Due to the propagation technique at site 5, old and young vines are interspersed resulting in two adjacent rows of vines being selected and grouped to form treatment replicates. While use of this technique allows the mature vine to support the layered vine, potential effects of competition prior to, and post separation are duly noted. Vine management decisions at each site were made by respective property managers and were consistent between age groups. Irrigation, where present, followed standard regional practices. Details of each vineyard site and age group can be found in Table 4.

Vegetative measures

Mass of all one year old wood was measured at the time of pruning during dormancy in each season. At this time, average cane length was determined by measuring 10 randomly selected canes greater than 5 nodes in length from each vine. Node numbers retained the year prior were counted before pruning along with count and non-count shoot emergence in seasons 2013/14 and 2014/15 only. Average cane mass was calculated by dividing total cane mass by total main shoot number. Count shoots were classed as any shoots which emerged from nodes that were intentionally retained at pruning, either spur or cane. All other shoots were classed as non-count shoots. Budburst percentage is defined as total number of shoots divided by nodes retained. Cordon length for spur-pruned vines was measured as the total length of established cordon wood per vine. When vines were cane pruned the term ‘cordon length’ describes the total length of one year old cane wrapped onto the wire. Trunk diameter and circumference were measured at the conclusion of field data sampling in 2015. Measurement was taken approximately 100mm from ground level around the circumference of the main supporting trunk.

Reproductive and vine balance measures

Harvest date was determined based on technical maturity as assessed by the respective winemaker responsible for commercial wine production from each site. Details of harvest date and TSS for each site and season are provided in Table 5. Total bunch number and fruit mass (yield) (kg/vine) were measured in the vineyard at harvest for each vine in each treatment and replicate. Average bunch mass (g) was determined by dividing the fruit mass per vine by bunch number per vine. Berry number per bunch was determined by dividing bunch mass by berry mass. Berry mass (g) was estimated from a 50 berry sub-sample taken from each replicate at harvest. The contribution of the rachis mass was considered constant and ignored. Yield to

pruning weight ratio (Y/P) was determined on a per vine basis from harvested fruit mass (kg/vine) and pruning mass (kg/vine).

Statistical analysis

Statistical analysis and interpretation was guided by The University of Adelaide Biometry team from the School of Mathematical Sciences. ANOVA and repeated measures ANOVA were performed using Genstat version 15 (VSN international, Hemel Hempstead, UK). Further ANOVA, Principal Component Analysis (PCA), t-tests and Correlation matrices were generated using XLSTAT Version 2015.4.01.20116 (Addinsoft SARL, Paris, France). Details of individual analysis or method of mean separation is found in the text or captions.

Results

Climatic summary of seasons

Growing season 2012/13 had the greatest accumulation of E°days for the period October to April (Table 3) combined with 26 days over 30°C in the two months preceding harvest (Figure S1). Rainfall was below average with no significant rain during the ripening months. Growing season 2013/14 had the second highest E°days and the highest growing season rainfall (Table 3). During October 2013, a low temperature 0.1°C event was recorded (Figure S1). January had the highest mean January temperature (MJT) for the study, while together with February had 28 days over 30°C (Table 3). Rainfall for season 2013/14 was 255 mm, 107 mm of this occurred in 3 days close to harvest (13^{th} – 15^{th} February 2014, Figure S1). Therefore, rainfall was below average for the majority of the growing season. Season 2014/15 recorded the lowest MJT and E°days, below that of the long term average (Table 3). There were 25 days above 30°C in January and February, 17 of these occurred in February resulting in the highest mean February temperature (MFT) of the study (Table 3). Rainfall close to veraison was the only significant event for the growing season (Figure S1).

Season, site and age

The influence of season, site and age was analysed via repeated measures ANOVA using key mean vegetative and reproductive variables, these results are summarised in Table 6. Growing season showed significant differences among all variables with the exception of trunk diameter (not measured seasonally). After the effect of season and site, age-based differences were observed for several variables including: berry number per bunch, bunch mass, fruit mass and trunk circumference.

Site by site overview

Mean summary tables of differences for variables measured in each season and site are presented in Table 6, Table 7 and Table 8. This study aims to assess the effect of vine age, therefore our interpretation of the data is primarily focussed on age-based findings, discussion of site and seasonal differences are presented where necessary.

Site 1

Pruning mass per metre of canopy was greater in old vines in both 2013 and 2015. The number of non-count shoots on old vines was significantly higher in 2015. Old vines had significantly greater mean trunk circumference (397 mm) compared to young vines (289 mm). Fruit mass per metre of canopy, bunch number and fruit mass per node were all significantly higher in old vines in 2014 only. At harvest, in 2013, old vines had significantly fewer berries per bunch, which did not affect overall fruit mass.

Site 2

Pruning mass per metre was significantly lower in old vines in 2015. Old vines had significantly more nodes retained at pruning in both 2014 and 2015. Nodes retained as a proportion of fruit mass was significantly higher in old vines in 2014 and 2015. Mean cane mass was significantly lower in old vines in 2015 while total shoot number per metre was significantly higher. In 2014 percentage budburst was also significantly lower for old vines. Old vines had significantly higher count shoots in 2014. Old vines had significantly greater mean trunk circumference of 507 mm compared to 295 mm. Old vines had greater yield per metre at harvest in 2013 and 2014 as a result of higher bunch number per metre of canopy. Bunch mass was significantly greater in old vines at harvest in 2013 as a result of greater berry number per bunch. Old vines had significantly more nodes retained at pruning per kilogram of fruit in both 2014 and 2015. Bunch number per shoot was greater in old vines in 2014. Y/P was significantly greater for old vines in all three seasons showing a broad range, from 1.7 to 4.1.

Site 3

Cordon length was greater in old vines and unchanged each season due to the permanent arm training system. In 2015 node number retained per vine was significantly lower for old vines. Shoot number per metre of canopy was not significantly different in 2014 or 2015; however, in 2014 count shoot number was higher while non-count shoots were lower in old vines. Percentage budburst was greater in old vines in 2015 only. Old vines had a significantly greater mean trunk circumference of 628 mm compared to 230 mm. This site recorded the largest

average trunk circumference. Fruit mass per metre of canopy was significantly lower at harvest in old vines in 2014. Old vines had fewer bunches per metre and per shoot as well as lower berry number per bunch. Fruit mass per node retained was significantly less in old vines in 2014. In 2014 average bunch mass was greater for old vines. Y/P was not significantly different between age groups in any season and was consistently low compared to all other sites. Node number retained per kg of pruning mass was significantly greater for young vines in 2015.

Site 4

Pruning mass per metre of canopy was significantly greater for old vines in 2013 while cordon length was lower. Count shoot number and budburst percentage were significantly higher for old vines in 2014. Mean cane mass at pruning was significantly higher for old vines in both 2014 and 2015. Mean cane length was lower for old vines in 2014 while cane mass was lower in 2014 and 2015. Old vines had significantly greater mean trunk circumference of 439 mm compared to 229 mm. Average berry mass at harvest was greater for old vines in 2014. Old vines had fewer bunches per shoot at harvest in 2014. Fruit mass per node was greater in old vines in 2014.

Site 5

Old vines had greater pruning mass per metre in 2014 and 2015. Cordon length was significantly greater for old vines in seasons 2013 and 2014. A greater number of nodes were retained per metre on young vines in 2014. Nodes retained per kg of pruning wood was lower for old vines in both 2014 and 2015. Count shoots were significantly for lower for old vines in 2014. Mean cane length and mass were greater for old vines at pruning in 2014 and 2015. Budburst was significantly greater for old vines in 2014. Old vines had significantly greater mean trunk circumference of 402 mm compared to 132 mm. Fruit mass was significantly greater for old vines in 2014 and 2015; this was accompanied by lower berry number and bunch mass. Fruit mass per node was greater for old vines in 2014 and 2015. Y/P was significantly lower for young vines in 2013.

Age differences in vine performance

PCA was undertaken on the data set to visualise key vegetative and reproductive variables and their relationships over the three seasons. The first two principal components explain 73.7% of the variation in the dataset (Figure 1). PC2 explains 26% of the variation in the data and separates old and young vine samples mainly by fruit yield per metre and berry number per bunch. Both vine ages at site 1 are relatively close together. This site presents the closest age

gap between young and old of 56 years with young vines that are 49 years of age. Eigen vectors are presented in Table S1 for further detail.

Correlation analysis across all sites and seasons revealed that bunch number was the main determinant of fruit yield per vine, followed by bunch weight and berries per bunch with Pearson correlation coefficients of 0.729, 0.612 and 0.502 respectively (all significant at $P<0.0001$). Bunch mass is the result of berry number per bunch and individual berry mass, of which berry number had a greater correlation (0.794) with bunch mass. Berry mass was more closely correlated with the vegetative measure pruning mass per metre than with any yield components.

Further PCA was performed to explore how the overall trends in these reproductive measures relate to vine age (Figure 2). Young vines at site one (black circle, 49 years of age) again are in closer proximity to the old vines; these 'young' vines are more than 21 years older than their nearest other 'young' vine. This PCA shows that vine yield (fruit mass kg/m) was closely related to berry number followed by bunch number and bunch mass. Eigen vectors are presented in Table S2 for further detail.

Following the significant interactions presented the same vegetative and reproductive performance variables were analysed not by discrete classes of age, but using age as a continuous variable. This analysis across seasons allowed greater clarity into the comparisons between and within sites. Significant differences were calculated for all measures (Table 7). Values associated with the youngest vines were generally lower than the oldest and more so for reproductive variables than vegetative. Across seasons significant interactions were observed; these include:

Site 5: Greater cane length in old vines, greater fruit mass per bud left at pruning and more nodes retained per kg/yield. Greater berry number, bunch mass and fruit mass/m recorded in old vines.

Site 2: More buds retained per m in old vines, lower budburst percentage in old vines and lower mean cane mass. Old vines had lower berry mass and bunch number but greater fruit mass per m and Y/P. Pruning mass per m was lower in old vines compared to young.

Site 1: Older vines had greater individual berry mass, fruit mass/m and pruning mass than younger vines.

Vine trunk circumference is statistically greater amongst the old vine age group. This is a long term measure of a vine's accumulation of physical size. Its relationship with age is demonstrated via a power trend R^2 of 0.88 via regression analysis (Figure 3). Seasonal accumulation of fresh matter showed that the older vines with larger trunk size were more stable in their range of pruning mass: young vines ranged from 0.27-2.1 kg/m while older vines ranged from 0.35-1.2 kg/m (Table 8).

Discussion

General

To the best of our knowledge this study is the first to utilise long established vineyards to principally investigate whether vine age has any measurable effect on vine performance. Previous studies in this field have included vine age as a variable (Ezzili 1992, Considine 2004, Zufferey and Maigre 2007, Reynolds et al. 2008) but with less substantial age differences than presented here. This data set and its analysis provide a currently-lacking documented resource of growth characteristics between vines of greatly contrasting age.

There are many variables which can influence a study of this type. Prior to commencing this study rigorous selection criteria reduced the pool of prospective vineyards by more than 50%, however, some relatively minor concessions were required to ensure this topic could be approached. It is commonly accepted that as vineyards age their inherent uniformity will often decrease. Considering the unique vine ages represented in this study a selection was made that represented the best possible example of vines of substantial age. Therefore, areas of reduced health or obvious decline were intentionally avoided, as they would be routinely excluded from a commercial harvest of these sites given the high price of fruit. The measures of vine performance presented also include unavoidable responses to management and site influences. Vine management can potentially impact on vine longevity, a mismanaged or poorly managed site may receive fewer inputs thereby suffering early decline. The sites presented here are noteworthy for their age and therefore due to the price premium of the fruit produced are managed to the highest standard in regards to industry best practice and sustainability. In addition, with the exception of site 4 the current owners and managers are direct descendants of the persons responsible for establishment. In these cases management is consistent and traditional, specifically in regards to pruning.

It is well known that many aspects of vine growth are influenced by seasonal differences relating to climate (Freeman et al. 1979, Gladstones 1992, Jones and Davis 2000,

Keller 2010, Roullier-Gall et al. 2014). This study was no different: the contribution that season makes to the data is well illustrated in Table 5. Sites 1 and 2 which are located in the cooler Eden Valley region were at least seven days later to reach maturity each season (Table 5). Specific climatic indices of the seasons can be found in Table 3 and Figure S1. Due to the proximity of these sites within the Barossa region, climatic influences within each season at each site are considered to be consistent.

Due to the challenges associated with the differences and interactions presented in Table 6 and discussed above, the data have been presented across seasons presenting age as a continuous variable. These results highlight the differences between individual sites and ages; this is presented in Table 7 for discussion.

Vegetative performance

An indication of vegetative growth can be deduced through investigation of pruning mass and its components: cane mass and cane number (Smart 2001). Pruning mass of 0.3–0.6 kg/m of row or canopy is generally considered an optimal indication of vine balance (Smart and Robinson 1991, Kliewer and Dokoozlian 2005, Keller 2015). Values greater than 1.0 kg/m are often indicative of overly vigorous vines and dense canopies (Keller 2015). Pruning mass per metre for old vines was equal to, or greater, than that of young vines in 11 out of 15 instances; however, only significantly so in 5 of these cases (Table 8). Across the seasons studied age-based differences were contradictory at two sites. At Site 1 older vines had greater pruning mass than young vines while at site 2 the younger vines had a greater pruning mass (Table 7). While the drivers of this are not clear the presence of catch wires may have encouraged greater vigour by altering the light environment of the canopy. An alternative theory may be that significantly lower bunch number and yield on younger vines at site 2 could have altered the balance for available resources. Site 2 was the only site to present a difference in the bud number retained; younger vines had significantly fewer buds retained which coincided with significantly higher budburst and mean cane mass. This cane mass, however, is largely driven by season 2015 where the individual cane mass in young vines was more than double that of the old vines (54 g versus 116.5 g per shoot). At site 5 it cannot be discounted that the differences observed in cane mass and cane length were in part due to competition from neighbouring vines.

Considering pruning mass as a surrogate for vigour, this contrasts with two other studies that reported vigour decline associated with vine age (Ezzili 1992, Considine 2004).

Furthermore, the study of Considine (2004) suggests that vigour decline occurs around 25-30 years of age in Zante Currant vines, with a maximum studied vine age of 50 years. It is noteworthy that the study of Considine (2004) used irrigated vineyards in a hot climate targeting high yield for dried fruit. This is a different cropping strategy to premium wine grapes in the Barossa Valley where limited water and moderate yields are more typical. This may result in differences observed between studies. Here we report vines of significantly greater age, some beyond 160 years, showing no discernible decline in vigour according to this measure.

Where significant differences in pruning mass were observed, old vines had the higher values for all sites excluding site 2. This suggests that, at the site level, in this study old vines had a tendency to produce greater vegetative mass even with similar pruning levels. This may be due to the greater capacity of old vines in relation to carbohydrate storage, and early shoot growth (Winkler 1958). The total carbohydrate pool of an individual vine is largely dependent on physical vine size resulting from old wood (Pellegrino et al. 2014). Trunk circumference as a measure of old wood was significantly greater in old vines in all cases (Table 7). Not surprisingly, site one had the narrowest gap in mean trunk circumference between old and young and the narrowest age gap in the study (56 years). Regression of trunk circumference in relation to vine age resulted in a positive relationship with a power trend line $R^2 = 0.88$ (Figure 3). This data set also suggests that differences in trunk circumference appear to be established early in the life of these vines. A similar positive relationship has been reported comparing age and diameter in destructive sampling of trunk cross sections (Tyminski 2013). In this case, a linear regression was reported ($R^2 = 0.673$); however, with lesser vine age differences of 3-24 years (Tyminski 2013). Further resolution via larger sample size would be beneficial to identify when trunk circumference or vine size begins to plateau.

Increased physical vine size, due to the accumulation of wood, has been suggested to be beneficial for early season shoot growth and canopy development (Koblet and Perret 1980, Sommer et al. 1995). Since no measure of actual shoot vigour in terms of rate of growth was made in those studies, any reference to vine or shoot vigour in this case is implied from a greater mass of dormant canes, most likely accrued through increased growth rate. Individual cane mass ranging from 35 g to 45 g is considered to be ideal for balanced vines (Smart and Robinson 1991). In our study cane mass varied greatly between sites, with young vines at site 2 showing the highest mean cane mass for season 2013/14 and 2014/15 (60.2 g and 116.5 g respectively). These values are well above the suggested ideal range noted above. Values

ranged from 0.27-2.1 kg/m for younger vines to 0.35-1.2 kg/m for older vines; values within, and well outside the optimal reported range, highlighting the variability often present within vineyards and between sites. The old vines at site 2 also had the highest mean cane mass for the same period (59.9g and 54.2g). This suggests that site 2 has high vigour for both young and old vines based on individual cane mass and pruning mass per metre. In contrast, according to the same measures, site 4 can be considered a low vigour site for both young and old vines despite the presence of irrigation in the young vines only.

Pruning level has long been known to influence cane mass (Winkler 1958, May et al. 1973, Freeman et al. 1979) and shoot vigour (Smart and Smith 1988, Dry and Loveys 1998). An optimum pruning level to maintain vine balance is suggested to be 30 nodes per kg of pruning mass (Smart and Robinson 1991, Smart 2001). In this study in 2014 and 2015, pruning that retained fewer than 30 nodes/kg pruning weight resulted in well above optimal cane mass, such as at site 2. It should be noted that it is possible that greater cane mass at this site may be a result of trellis type. The presence of catch wires may have altered leaf area and encouraged greater growth although this was not directly measured. Site 4 was consistently pruned to greater than 30 nodes/kg resulting in individual cane mass well below the optimum range. This indicates that, even with anecdotal suggestions of older vines having a greater 'natural' balance, management decisions are still highly influential.

Since vine root volume is not often measured, Smart (2001) suggests that the canopy measure of pruning mass per metre can be used to indicate vine root volume, with less than 0.5 kg/m indicating a weak root system, and values above 1.0 kg/m indicating a large, strong root system. Based on this suggestion, a high proportion (73%) of old vines may be likely to possess a larger root system than young vines at the same site. It is not clear if this refers to overall area exploited, or girth and storage capacity of the root area. In general, older vines may have a larger root volume by this guide although it was not always statistically significant in this study. This implied measure is subject to seasonal variation and as such longer term data would make an interesting study for future exploration.

Accumulated wood is thought to confer greater resilience under adverse conditions (Howell 2001) and buffer against biotic and abiotic stress (Mullins 1992). Given the increase in trunk circumference with age, further investigation of stress adaption in response to age is warranted to determine if it exists to a greater extent in old vines as reported by Zufferey and Maigre (2007).

Reproductive performance

Fruit mass per metre of cordon in old vines was equal to or greater than young vines in 12 out of 15 situations across all sites and seasons; however, this was not significant in all cases (Table 8). Old vines were found to have a statistically higher yield at three out of five sites across seasons: sites 1, 2 and 5 (Table 7). Previously reported age-based differences in yield have been diverse, ranging from not strongly related (Considine 2004, Zufferey and Maigre 2008) to significant but seasonally inconsistent (Reynolds et al. 2008). Fruit yield per metre in this study is a product of bunch mass per metre and berry number per bunch (Figure 3).

Bunch mass was generally greater for old vines according to principal component analysis. Further investigation found that the only significant effect of age on bunch mass across seasons was found at site 5 (Table 7). This effect may potentially be confounded by effects of competition amongst neighbouring vines. Greater bunch mass was a result of increased berry number per bunch. Berry number is determined by the number of flowers that turn into berries, while flower number is influenced by branching of inflorescence primordia in the bud (May 2004). Reduced fruitset has been observed with greater vine age in Alicante Grenache (Ezzili 1992). It is unclear in our study, since no measures were taken, if differences in berry numbers were a result of differences in flower number or fruitset, or both. Total flower number and fruitset can be influenced by temperature (May 2004, Rogiers and Clarke 2013). Since all vines were subjected to similar environmental conditions, the assumption of comparable flowering and fruitset can be made between age groups (Dry et al. 2010). Young vines at site 5 were found to have consistently fewer berries per bunch which may have also have been a result of competition due to the propagation technique. It is likely that competition would also impact on berry size which was not observed in this study. Further work is required to determine if the effect that vine age has on yield components relates to flowering and fruitset, via calculation of the Coulure and Millerandage Indices (Collins and Dry 2009).

The greater physical size of old vines, and the inherently larger reserves that size represents, may be a factor influencing fruitfulness via increased capacity. Reynolds et al. (1994) found higher crop load in Riesling vines with a greater volume of old wood—this was due to bunch number not berry number per bunch. Nevertheless, Lebon et al. (2008) showed that, in the grapevine, flower abortion is inversely correlated with sugar (or carbohydrate) availability during flower formation. As the overall reserve status of the vine increases, the number of flowers formed per inflorescence and per vine increases (Holzapfel et al. 2010). In addition, stored carbohydrate and nitrogen are both important for flower development, early

season leaf growth, vigour and overall canopy size (Smith and Holzapfel 2003, 2009, Holzapfel et al. 2010, Keller 2015). This total carbohydrate pool is known to be largely dependent on vine size; comprising both root system and trunk/cordon (Pellegrino et al. 2014). In addition to carbohydrate storage, the most significant source of nitrogen in 50-60 year old Sultana vines was the roots, followed by trunk and 1-2 year old wood (Treeby and Wheatley 2006). The interaction of nutrient storage and fruitfulness in this situation is not clear; however, the large differences in vine size provide some strength to the assumption that this may be a driver of difference in observed vine performance. It is therefore tempting to speculate that the higher crop load observed in old vines could be linked to the supply of sugar reserves in woody parts.

Individual berry size is influenced by many environmental factors, particularly early in the season (Keller 2010). In this study, the environment (mesoclimate and soil type) were assumed to be uniform within each site as noted. Individual berry size at harvest was also relatively constant between age groups (Table 8). The effect of age on berry size was found only at two sites; site 1 had greater berry size in old vines while site 2 had smaller berries in old vines. These contradictory results are possibly driven by site specific factors complicated by vigour, as vines with greater pruning mass also had greater berry size in both cases. Water relations, through differences in soil type as well as a range of cultural practices, have been found to influence berry mass (Roby et al. 2004, Keller 2005, Matthews and Nuzzo 2007). Individual berry mass was found in our study to have a closer correlation with pruning mass/m than with other yield components. This implies that individual berry mass in these sites has an important relationship with vigour rather than physical trunk size. Individual berry mass was also closely associated with season (Table 6). This is not surprising since soil conditions and management at each site are near identical between vine age groups.

Bunch number is the main determinant of yield in this study. Yield is influenced by its components: bunch number, berry number and berry mass. Standardised bunch number per metre was greater in older vines at site 1 (in 2014) and 2 (in 2013 and 2014) only while it was greater in young vines in one site and season only: site 3, 2014 (Table 8). PCA of these yield components displayed in Figure 2 suggests that yield is influenced to a similar extent by bunch mass and berry number and less so by individual berry mass. This PCA reveals that berry mass was less correlated with yield than berry number per bunch. Management differences at site 2 should be noted as a potential driver for age-based yield differences. Young vines at site 2 had significantly fewer nodes retained in both 2014 and 2015 thereby potentially reducing the yield

potential. Old vines had a greater bunch number per shoot which if applied to greater shoot (and count shoot) numbers would systematically result in greater yield in these vines.

Vegetative and Reproductive

Yield to pruning mass ratio (Y/P) is commonly used as a measure of vine balance (Smart and Robinson 1991, Howell 2001, Dry et al. 2004, Lakso 2009). In this study Y/P values ranged from 1.1 to 7.3 (Table 8) across all seasons, sites and age groups. Low Y/P values (<4) are said to indicate an excess of vegetative growth in relation to reproductive yield, while high numbers (>10) indicate high reproductive yield and low vegetative growth. The range of these values is influenced by differences in climate (Smart 2001), variety, canopy management and trellis system (Kliewer and Dokoozlian 2005). Hot sunny climates may be closer to 10, while for cooler climates values closer to 5 are more appropriate (Smart 2001). Therefore in this study we would expect Y/P values ranging from 5-10 considering the climate in the Barossa Valley. Recent research with Shiraz in the Barossa Valley, over a three year period, found a similar seasonal variation and range of Y/P values from 1.6-7.0 (Kidman et al. 2014) as reported in this study. Over three seasons, mean values of Y/P ranged from 2.0-6.6 in old vines and 1.2-6.1 in younger vines representing a range of 4.6 and 4.9 for old and young respectively.

There were no consistent significant differences in Y/P related to age in this study. Across seasons Y/P was only different between ages at site 2, where older vines had a significantly higher reproductive yield and lower vegetative growth. Y/P measures demonstrated site specific trends only and were not a useful index to differentiate vines of varying ages.

Limitations and suggested improvements

This study was unconventional in its approach in that no treatment in a traditional sense was applied to measure a response. In contrast, the time in years since planting was the treatment. Due to the challenging and ambitious subject matter of this study, limitations were difficult or near impossible to avoid. The greatest challenge was finding suitable vines for comparison; in this regard some minor management differences were unavoidable between study sites. Within-site differences in management were carefully selected to be minimal with reference to the pool of vineyards available for such a study. With the exception of fruit harvesting and pruning no destructive sampling or manipulation was undertaken, the hypothesis being that greater vine age will have a measureable influence on vegetative performance under uniform management as implemented by each property owner.

Future studies that may approach this subject would benefit from greater individual vine numbers to reduce variability. Further improvement via manipulation of bud number and bunch number may simplify analysis and interpretation of results. Suggested avenues for future research should target specific physiological processes. These may include: carbohydrate storage and composition, sap flow and photosynthesis measurements and destructive or semi-destructive root volume analysis.

Conclusion

Vine age, in this study, was difficult to separate in terms of vine performance. According to PCA, older vines had higher fruit mass /m due to higher berry number per bunch. The proposed explanation for this is based on vine capacity, which would appear to be due to an increased amount of perennial wood as measured above-ground. Below-ground root structure and size while not measured is likely to follow the same trend as above-ground. This may provide further evidence that physical size is a determining factor in vine performance based on age. Even with this larger store of reserves, significant seasonal and managerial variation is still observed. However, the extent to which greater reserves provide an ability to 'buffer' vegetative and reproductive performance in extreme stress was not revealed by the seasons or time period of this study. This work represents part of a multi-disciplinary investigation into vine age, vine performance and reproduction. Further work is underway to examine if vine age presents differences in relation to fruit and wine composition and sensory perception.

Importantly, even after more than 160 years of existence, a vine's ability to produce balanced growth, both vegetatively and reproductively, can still be influenced by management and season. Therefore, these findings suggest that vine age alone is not an overriding factor in balanced production and subsequent quality potential. Within the scope of this study the effects associated with site were greater than the effect of age.

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Tables

Table 1. Summary of findings from previous vine age related studies in literature.

Measures	Vine age		Location	Varieties	Findings	Reference
Vegetative, Fruit, Wine	4	14	Canada (Ontario)	Cabernet Sauvignon, Cabernet Franc, Pinot Noir and Riesling	Old higher yield, bunch number, bunch mass and berry mass-one and lower brix in one season only. Age had little impact in second season. Wine pH and TA was contrasting each season, young vines were more vegetal in 2002 and not 2003.	(Reynolds et al. 2008)
Vegetative, Fruit, Wine	5	34	Switzerland (Wadenswil)	Chasselas, Pinot Blanc, Arvine, Gamay, Syrah	Older vines had higher TA, YAN and pruning weight. Age had no impact on sugar levels. Old vine wines were more preferred early and after 4 years of aging.	(Zufferey and Maigre 2007, 2008)
Vegetative, Fruit	6	50	Australia (Western Australia)	Zante Currant	Older vines had lower vigour and berry number per bunch. Vine age was not related to total yield, bunch number or berry volume.	(Considine 2004)
Fruit set kinetics	13	50	El Khanguet (Tunisia)	Alicante Grenache Noir	Older vines had lower vigour and reduced fruit set.	(Ezzili 1992)
Wine only	5	20	USA (California)	Cabernet Sauvignon	Vine age was correlated with berry aroma and fruit flavour in finished wines. Old vines had higher ratings. Negative correlation between wine age and green bean and vegetative flavour in wines. Younger vines from cooler areas produced more vegetative wines.	(Heymann and Noble 1987)

Table 2. Closest township, vine establishment dates and relative age gap (in brackets) between old and young vines for all Barossa Valley sites.

		Year of establishment		Relative	
Site	**Location**	**Old**	**Young**	**age gap***	**Approximate coordinates**
Site 1	Angaston	1908 (105)	1964 (49)	56	-34.47;139.07
Site 2	Eden Valley	1906 (107)	1985 (28)	79	-34.49;139.12
Site 3	Nuriootpa	1885 (128)	2001 (12)	116	-34.41;139.00
Site 4	Tanunda	1845 (168)	1996 (17)	151	-34.50;138.96
Site 5	Rowland	1920 (93)	2007 (6)	87	-34.56;138.94

*Vine age and relative age difference in years since establishment, as at commencement of study in 2013

Table 3. Climatic data for Nuriootpa, Barossa Valley, South Australia. For growing seasons 2013 – 2015, October - April.

Growing season	Mean daily	Mean daily	E°days**	Rainfall	MJT	MFT
Season 1 2012-2013	27.0	12.2	1686	110	21.8	22.5
Season 2 2013-2014	26.0	11.5	1536	255	23.3	21.8
Season 3 2014-2015	26.0	11.3	1520	145	20.8	23.6
*Long term average**	*25.2*	*11.3*	*1545*	*203*	*21.5*	*21.4*

*Long term average from 1952- 2015, Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m (Bureau Of Meteorology 2015). **Calculated as per Gladstones (2011).
MJT = mean January temperature. MFT = mean February temperature.

Table 4. Site specific details for each Barossa Valley Shiraz vineyard included in the study.

Site	Age	Pruning	Cane or	Cordon	Trellis configuration	Management		Soil group-	Average
						Floor	Undervine	Sub group*	Irrigation
Site 1	Old	Cane	2-3	1.0	Single cordon wire	Cultivated	Mulch	B-B4	0.4ML/ha
	Young	Cane	2-3	1.1,1.3	Two wires 200mm apart	Cultivated	Mulch	B-B4	" "
Site 2	Old	Cane	2-3	1.0	Single wire	Sward	Mulch	D-D2	0.3ML/ha
	Young	Cane	2	1.0	Single wire pus four catch wires	Sward	Mulch	D-D2	" "
Site 3	Old	Spur bilateral cordon	2	1.2	Single wire + roll wire 250mm above	Cultivated	Cultivated	G-G3	0.48ML/ha
	Young	Spur bilateral cordon	2	1.2	Single wire + roll wire 250mm above	Cultivated	Cultivated	G-G3	" "
Site 4	Old	Cane	2-4	1.0,1.3	Two wires	Sward	Cultivated	D-D2	**
	Young	Cane	2-4	1.1,1.4	Two wires	Sward	Cultivated	D-D2	0.2ML/ha
Site 5	Old	Cane	2	1.0	Single wire	Sward	Herbicide	D-D2	0.2ML/ha
	Young	Cane	2	1.0	Single wire	Sward	Herbicide	D-D2	" "

*Australian soil classification subgroup codes (Isbell 2002, Hall 2009, ARIS 2011) represent:

B-Shallow soil on calcrete or limestone

B4-Shallow red loam on limestone

D-Hard red-brown texture-contrast soils with alkaline subsoil

D2-Loam over red clay

G-Sand over clay soils

G3-Thick sand over clay

**Only young vines at site 4 have irrigation.

Table 5. Details of harvest date for Shiraz fruit from the Barossa zone from three seasons, five sites and two age classes.

		TSS (Brix°)		Harvest date	
Site	**Season**	**Old**	**Young**	**Old**	**Young**
Site 1	2013	27.7	28.1	22-Feb	22-Feb
	2014	24.0	24.6	18-Mar	18-Mar
	2015	26.6	27.3	26-Feb	26-Feb
Site 2	2013	24.4	26.9	22-Feb	22-Feb
	2014	23.5	23.4	18-Mar	18-Mar
	2015	24.1	24.7	6-Mar	10-Apr
Site 3	2013	27.2	27.7	15-Feb	15-Feb
	2014	24.7	24.6	5-Mar	5-Mar
	2015	26.1	25.9	18-Feb	18-Feb
Site 4	2013	28.6	27.6	15-Feb	15-Feb
	2014	24b	25.3a	5-Mar	5-Mar
	2015	25.6b	27.6a	18-Feb	18-Feb
Site 5	2013	27.3	27.5	5-Mar	5-Mar
	2014	25.7	25.1	19-Feb	19-Feb
	2015	24.0	25.0	18-Feb	18-Feb

Table 6. Results of repeated measures ANOVA performed on data obtained from Shiraz grown in the Barossa Valley during seasons 2012-13, 2013-14, and 2014-15, two age groups, three replicates of over five sites and three seasons were included in the model.

	P values						
Variable	**Site**	**Age**	**Season**	**Site.Age**	**Season.Site**	**Season.Age**	**Season.Site.Age**
Berry number (#/bunch)	0.026	0.008	<0.001	0.002	<0.001	0.029	0.5
Berry mass (g)	<0.001	ns	<0.001	<0.001	<0.001	0.009	<0.001
Bunch mass (g)	ns	<0.001	<0.001	<0.001	<0.001	0.007	0.29
Bunch number (#/m)	<0.001	ns	<0.001	ns	<0.001	ns	0.012
Fruit mass (kg/m)	ns	<0.001	<0.001	ns	<0.001	ns	ns
Pruning mass (kg/m)	<0.001	ns	<0.001	<0.001	<0.001	ns	<0.001
Yield / Pruning mass (Y/P)†	<0.001	ns	<0.001	ns	<0.001	ns	ns
Trunk Circumference (mm)	<0.001	<0.001	*	<0.001	*	*	*

†Yield = Fruit mass

*no seasonal change as only one measurement taken in 2015.

Table 7. Vine performance measures of Shiraz combining both reproductive and vegetative variables for five sites and ten age groups over two[†] or three growing seasons 2012-2015 in the Barossa Valley. Within site standard errors are presented in Table S3.

	Vine age since planting									
	6	**12**	**17**	**28**	**49**	**93**	**105**	**107**	**128**	**168**
Variables	Site 5	Site 3	Site 4	Site 2	Site 1	Site 5	Site 1	Site 2	Site 3	Site 4
Berry number (#/bunch)	58e	74.1cde	83.7abcd	66.8de	91.6ab	91.9a	79.8abcd	83abc	74.6bcde	96.8a
Berry mass (g)	1.2bc	1.1c	0.9e	1.3a	1.0d	1.2ab	1.2c	1.1c	1.2abc	0.9e
Bunch mass (g)	66.7d	81.7bcd	72.2cd	87.3bc	87.1bc	113.9a	90.7b	91.2b	86.4bc	77.3bcd
Bunch number (#/m)	23.4bc	20.2cd	27.9ab	14.9e	20.2cd	23.9bc	23.5bc	23.5bc	17.5de	29.9a
Fruit mass (kg/m)	1.6d	1.7cd	1.9bcd	1.5d	1.7cd	2.6a	2.2ab	2.4ab	1.5d	2.2abc
Pruning mass (kg/m)	0.48def	0.74bc	0.34f	1.2a	0.47ef	0.57de	0.62cd	0.86b	0.76bc	0.39f
Yield / Pruning mass (Y/P)	4.3cd	2.5ef	6.1ab	1.2g	4.3cd	5.1bc	4.3cd	3.2de	2.0fg	6.4a
Trunk circumference (mm)	132.5g	230.1f	229.2f	295.4e	288.8e	395.6d	396.8d	507.2b	628.4a	439.1c
Buds retained (/m) [†]	15.8bcd	16.9abcd	18.1abc	12.5d	17.3abc	15cd	20ab	17.6abc	14.9cd	20.9a
Count shoots (/m) [†]	12.1c	15.5abc	15.7abc	13.8bc	15.0abc	13.4bc	17.2ab	15.1abc	17.3ab	18.4a
Non Count shoots (/m) [†]	2.6cde	6.9a	2.6cde	4.7abc	2.4de	2.1e	3.1cde	6.4ab	6ab	4.4bcd
Shoot number (/m) [†]	14.7d	22.3ab	18.3abcd	18.5abcd	17.5bcd	15.4cd	20.3abc	21.5ab	23.3a	22.8a
Budburst (%)[†]	95e	136bc	104de	161a	103de	104de	103de	120cd	156ab	111de
Cane length (cm) [†]	80.1d	105.3bc	84.2cd	124.1a	100.4b	99.3bc	104.4b	112.3ab	112.1ab	82.2de
Fruit mass / Bud (g/bud retained) [†]	97.6bcd	112.6abc	111.7abc	148.1abc	102.4bc	159.9a	121abc	152.1ab	86.1c	108.3abc
Nodes retained (/kg yield) [†]	47.4a	22.7cde	50.1a	8.4f	36.2b	31.4bcd	31.7bc	18.1ef	20.7de	58.2a
Bunch number (/shoot) [†]	1.6ab	1cd	1.7a	1cd	1.1cd	1.6ab	1.2bcd	1.2abcd	0.8d	1.4abc
Mean cane mass (g) [†]	25.9cde	35.1cd	20.3de	88.4a	30.7cde	38.3c	34.7cd	57.1b	33.6cd	17e

Rows with different superscript are significantly different following ANOVA using Tukeys HSD at 5% level. All variables were significantly different at P<0.0001.

[†]Data collected over growing seasons 2013-14 and 2014-15 only.

Table 8. Reproductive and vegetative measures of Shiraz vines from five vineyards in the Barossa Valley. Means are presented for each three growing seasons by vine age.

	2013			2014			2015		
SITE 1†	Old	Young	P-value	Old	Young	P-value	Old	Young	P-value
Pruning mass (kg/m)	0.45	0.33	0.047	0.49	0.42	ns	0.91	0.64	0.041
Fruit mass (kg/m)	2.0	1.6	ns	2.4	1.3	0.009	2.2	2.2	ns
Bunch number (#/m)	24	21	ns	26	18	0.036	20	22	ns
Bunch mass (g)	83	86	ns	86	80	ns	103	96	ns
Berry number (#/bunch)	90	121	0.040	71	78	ns	78	75	ns
Berry mass (g)	0.9	0.7	ns	1.2	1.0	ns	1.3	1.3	ns
Yield / Pruning mass (Y/P)	4.9	5.6	ns	5.1	3.4	ns	2.9	3.8	ns
SITE 2‡									
Pruning mass (kg/m)	0.46	0.42	ns	0.92	1.1	ns	1.2	2.1	0.000
Fruit mass (kg/m)	1.7	0.4	0.000	1.4	0.7	0.019	4.0	3.5	ns
Bunch number (#/m)	22	9	0.000	20	10	0.044	28	26	ns
Bunch mass (g)	77	49	0.010	70	70	ns	126	142	ns
Berry number (#/bunch)	102	58	0.006	59	54	ns	88	88	ns
Berry mass (g)	0.8	0.9	ns	1.2	1.3	ns	1.4	1.6	ns
Yield / Pruning mass (Y/P)	4.1	1.1	0.000	1.7	0.7	0.023	3.8	1.8	0.018
SITE 3‡									
Pruning mass (kg/m)	0.75	0.66	ns	0.69	0.73	ns	0.84	0.80	ns
Fruit mass (kg/m)	1.8	1.5	ns	1.3	1.9	0.014	1.3	1.7	ns
Bunch number (#/m)	16	18	ns	18	23	0.013	19	19	ns
Bunch mass (g)	116	85	0.008	70	81	ns	72	79	ns
Berry number (#/bunch)	110	89	ns	62	78	0.013	51	55	ns
Berry mass (g)	1.1	0.9	0.033	1.1	1.0	ns	1.4	1.4	ns
Yield / Pruning mass (Y/P)	2.5	2.4	ns	2.0	2.7	ns	1.7	2.3	ns
SITE 4‡									
Pruning mass (kg/m)	0.41	0.27	0.024	0.35	0.35	ns	0.41	0.38	ns
Fruit mass (kg/m)	2.3	1.9	ns	1.7	1.8	ns	2.6	2.2	ns
Bunch number (#/m)	28	22	ns	27	32	ns	35	30	ns
Bunch mass (g)	100	88	ns	59	54	ns	72	75	ns
Berry number (#/bunch)	151	107	ns	68	74	ns	71	70	ns
Berry mass (g)	0.7	0.8	ns	0.9	0.7	0.016	1.0	1.1	ns
Yield / Pruning mass (Y/P)	7.1	7.3	ns	5.0	5.0	ns	6.9	5.8	ns
SITE 5‡									
Pruning mass (kg/m)	0.58	0.72	ns	0.59	0.41	0.004	0.52	0.30	0.001
Fruit mass (kg/m)	3.0	1.9	0.008	1.7	1.3	ns	3.1	1.7	0.000
Bunch number (#/m)	22	22	ns	19	23	ns	32	25	ns
Bunch mass (g)	143	79	0.000	90	51	0.000	107	70	0.000
Berry number (#/bunch)	120	75	0.000	75	44	0.000	79	55	0.000
Berry mass (g)	1.2	1.1	ns	1.2	1.2	ns	1.4	1.3	ns
Yield / Pruning mass (Y/P)	5.2	3.6	0.027	3.4	3.3	ns	6.6	5.9	ns

Sample sizes indicated as follows: † n=15, ‡ n=12. Means were separated by ANOVA using Fishers least significant difference (LSD) test (P=0.05). ns, not statistically significant. Pruning mass was recorded in dormancy all other variables measured at harvest.

Table 9. Reproductive and vegetative measures of Shiraz vines from five vineyards in the Barossa Valley. Means are presented for two growing seasons by age

SITE 1†	2014			2015		
	Old	Young	P-value	Old	Young	P-value
Nodes retained (#/m)	18	16	ns	22	18	ns
Count Shoot (#/m)	16	14	ns	18	16	ns
Non count shoot (#/m)	2	2	ns	4	2	0.047
Shoot (#/m)	18	16	ns	22	19	ns
Budburst (%/vine)	102	101	ns	103	106	ns
Cane length (cm)	85.4	85.7	ns	123.4	114.9	ns
Mean cane mass (g)	27.6	26.3	ns	41.7	35.2	ns
Nodes retained (#/kg)	38	41	ns	26	31	ns
Bunch number (/shoot)	1.5	1.1	ns	0.9	1.2	ns
Fruit mass / node (g/node)§	135.9	82.8	0.016	106	122	ns
SITE 2‡						
Nodes retained (#/m)	14	9	0.000	21	16	0.011
Count Shoot (#/m)	12	13	ns	19	14	0.030
Non count shoot (#/m)	5	5	ns	8	4	0.008
Shoot (#/m)	16	19	ns	27	18	0.017
Budburst (%/vine)	118	205	< 0.001	123	118	ns
Cane length (cm)	109.4	121.4	ns	115.1	126.6	ns
Mean cane mass (g)	59.9	60.2	ns	54.2	116.5	<0.001
Nodes retained (#/kg)	16	9	<0.001	20	8	<0.001
Bunch number (/shoot)	1.1	0.6	0.031	1.2	1.5	ns
Fruit mass / node (g/node)§	103.1	70.6	ns	201	225.5	ns
SITE 3‡						
Nodes retained (#/m)	15	14	ns	15	20	0.001
Count Shoot (#/m)	17	14	0.045	17	17	ns
Non count shoot (#/m)	4	7	0.000	8	7	ns
Shoot (#/m)	21	20	ns	25	24	ns
Budburst (%/vine)	143	147	ns	168	125	<0.001
Cane length (cm)	121.1	112.7	ns	103	97.8	ns
Mean cane mass (g)	33.3	36.4	ns	33.9	33.8	ns
Nodes retained (#/kg)	23	20	ns	19	25	0.011
Bunch number (/shoot)	0.8	1.2	0.005	0.7	0.8	ns
Fruit mass / node (g/node)§	83.4	138.6	0.002	88.8	86.6	ns
SITE 4‡						
Nodes retained (#/m)	21	18	ns	21	18	ns
Count Shoot (#/m)	18	16	ns	19	16	ns
Non count shoot (#/m)	3	2	0.004	5	3	ns
Shoot (#/m)	22	17	0.039	21	17	ns
Budburst (%/vine)	106	97	ns	116	111	ns
Cane length (cm)	76.1	86.8	0.014	88.2	81.4	ns
Mean cane mass (g)	16.8	20.5	0.029	17.2	20.1	0.142
Nodes retained (#/kg)	60	53	ns	56	47	ns
Bunch number (/shoot)	1.2	1.9	0.005	1.6	1.5	ns
Fruit mass / node (g/node)§	83.6	96.9	ns	132.8	126.3	ns
SITE 5‡						
Nodes retained (#/m)	13	15	0.007	17	16	ns
Count Shoot (#/m)	12	11	ns	15	13	ns
Non count shoot (#/m)	2	3	ns	2	2	ns
Shoot (#/m)	15	14	ns	17	15	ns
Budburst (%/vine)	109	97	0.002	98	92	ns
Cane length (cm)	102.7	83	0.005	95.9	77.1	0.000
Mean cane mass (g)	46.2	30.6	0.017	30.5	21.1	0.002
Nodes retained (#/kg)	24	39	0.003	39	56	0.018
Bunch number (/shoot)	1.4	1.6	ns	1.8	1.7	ns
Fruit mass / node (g/node)§	136.2	88.1	0.038	183.5	107	0.000

Sample sizes indicated as follows: † n=15, ‡ n=12. Means were separated by ANOVA using Fishers least significant difference (LSD) test (P=0.05). ns, not statistically significant. § node refers to count nodes retained at pruning.

Figures

Figure 1. PCA biplot of mean vegetative and reproductive data for measures taken from five sites and 10 vine ages over three seasons 2012-13, 2013-14 and 2014-15 from Shiraz vines in the Barossa Valley. Shared colours indicate common sites, triangle markers (▲) indicate the older vines while circle (●) markers indicate younger vines.

Figure 2. PCA biplot of selected variables taken from five sites and 10 vine ages over three seasons 2012-13, 2013-14 and 2014-15 from Shiraz vines in the Barossa Valley. Shared colours indicate common sites, triangle markers (▲) indicate the older vines while circle (●) markers indicate younger vines.

Figure 3. Regression of mean trunk circumference and vine age ±SE for Barossa Valley Shiraz from five sites and ten ages taken in 2015, power trend line is shown ($R^2 = 0.88$). Triangle markers (▲) indicate the older vines while circle (●) markers indicate younger vines.

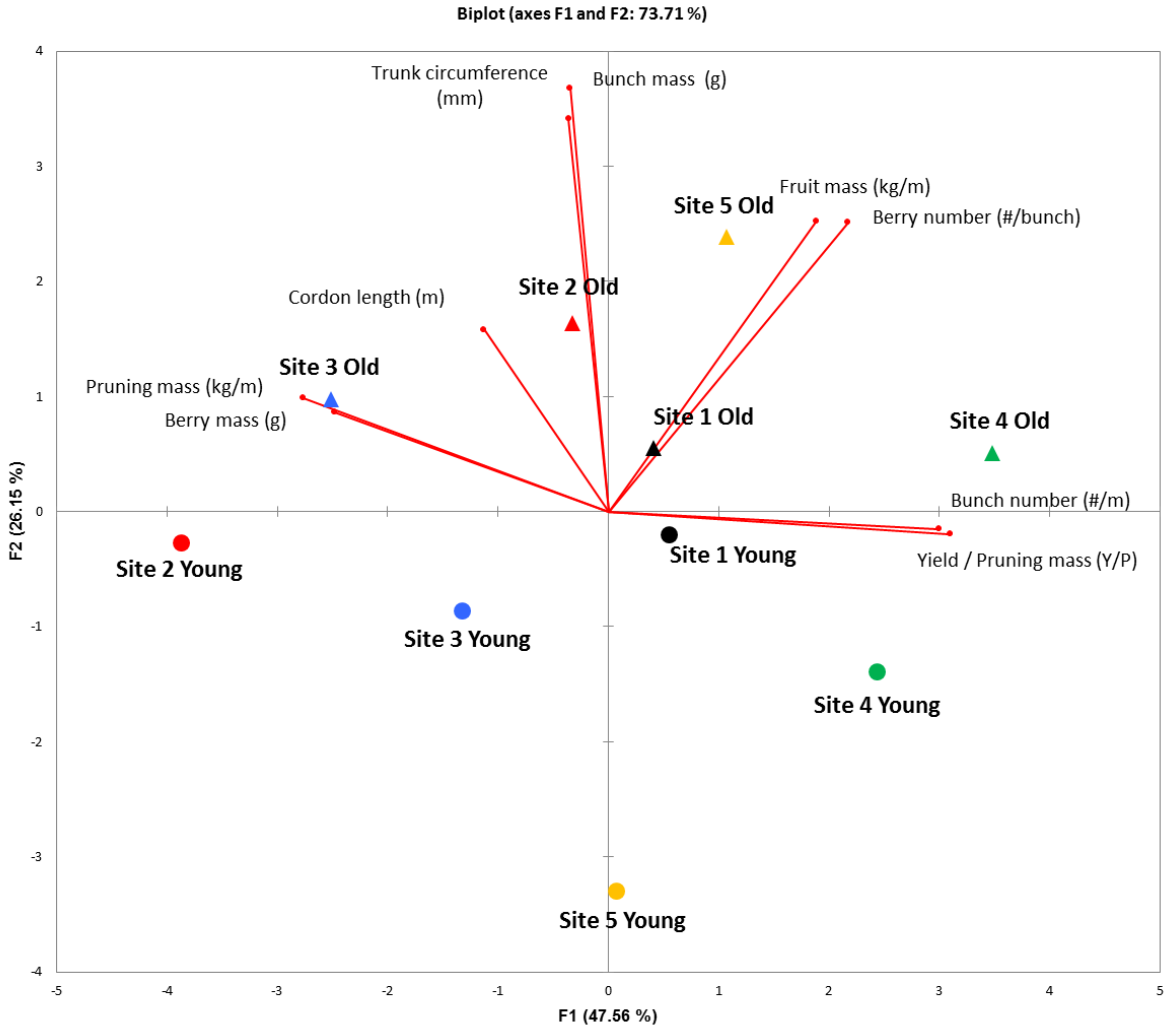

Figure 1. PCA biplot of mean vegetative and reproductive data for measures taken from five sites and 10 vine ages over three seasons 2012-13, 2013-14 and 2014-15 from Shiraz vines in the Barossa Valley. Shared colours indicate common sites, triangle markers (▲) indicate the older vines while circle (●) markers indicate younger vines.

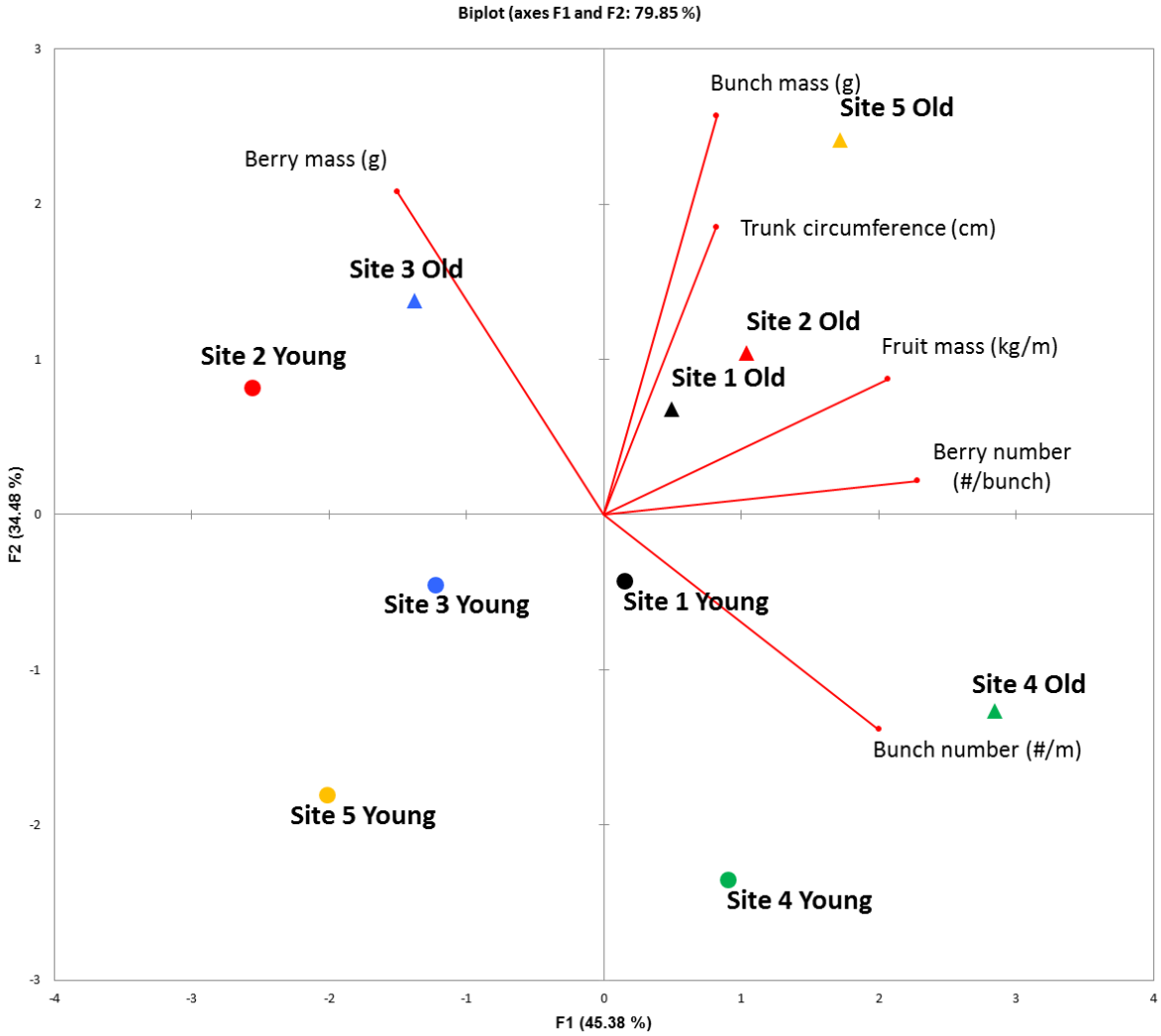

Figure 2. PCA biplot of selected variables taken from five sites and 10 vine ages over three seasons 2012-13, 2013-14 and 2014-15 from Shiraz vines in the Barossa Valley. Shared colours indicate common sites, triangle markers (▲) indicate the older vines while circle (●) markers indicate younger vines.

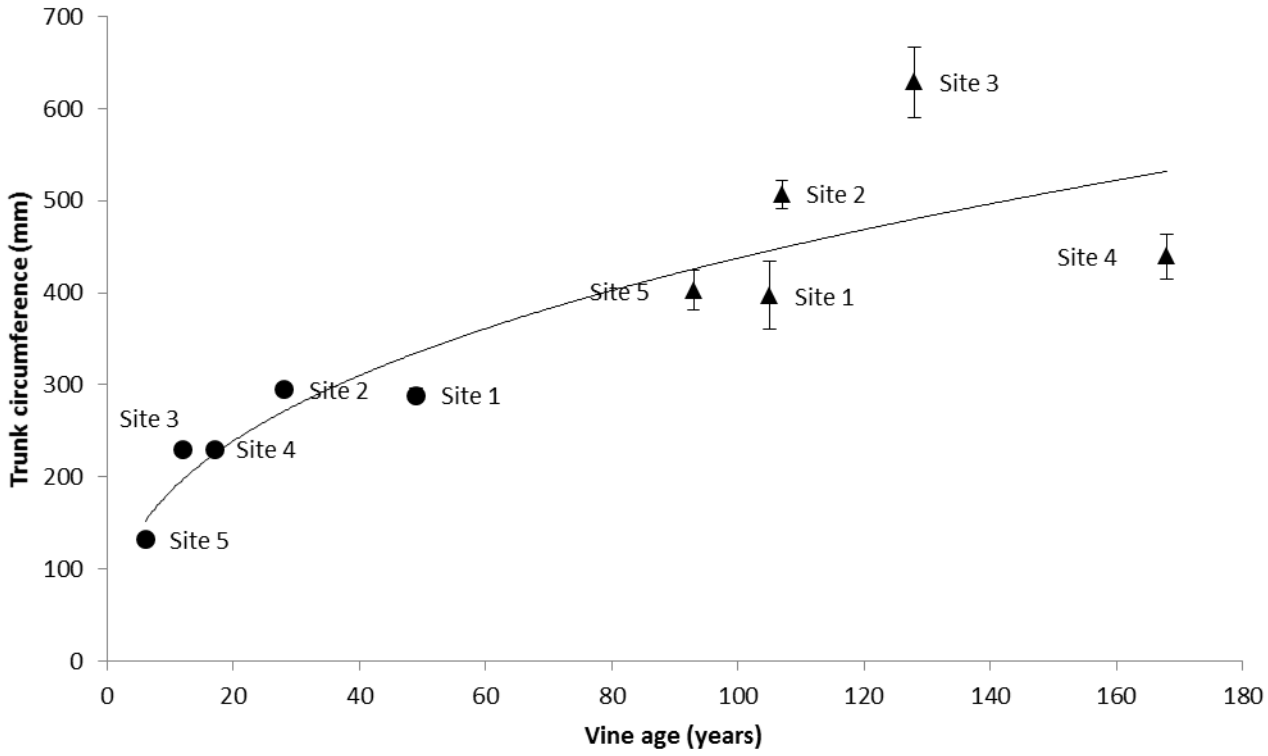

Figure 3. Regression of mean trunk circumference and vine age ±SE for Barossa Valley Shiraz vine from five sites and ten ages taken in 2015, power trend line is shown ($R^2 = 0.88$). Triangle markers (▲) indicate old vines while circle (●) markers indicate young vines at each site.

Supplementary Figures

Figure S1. Summaries of seasonal climatic data for each season, approximate growth stages and harvest periods are indicated by arrows.
Bureau of meteorology site 23373 in Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m.

Supplementary Tables

Table S1. Eigen values and Eigen vectors for Principal Component Analysis presented in Figure 1.

Table S2. Eigen values and Eigen vectors for Principal Component Analysis presented in Figure 2.

Table S3. Standard error of the multi season means for each variable presented in Table 6.

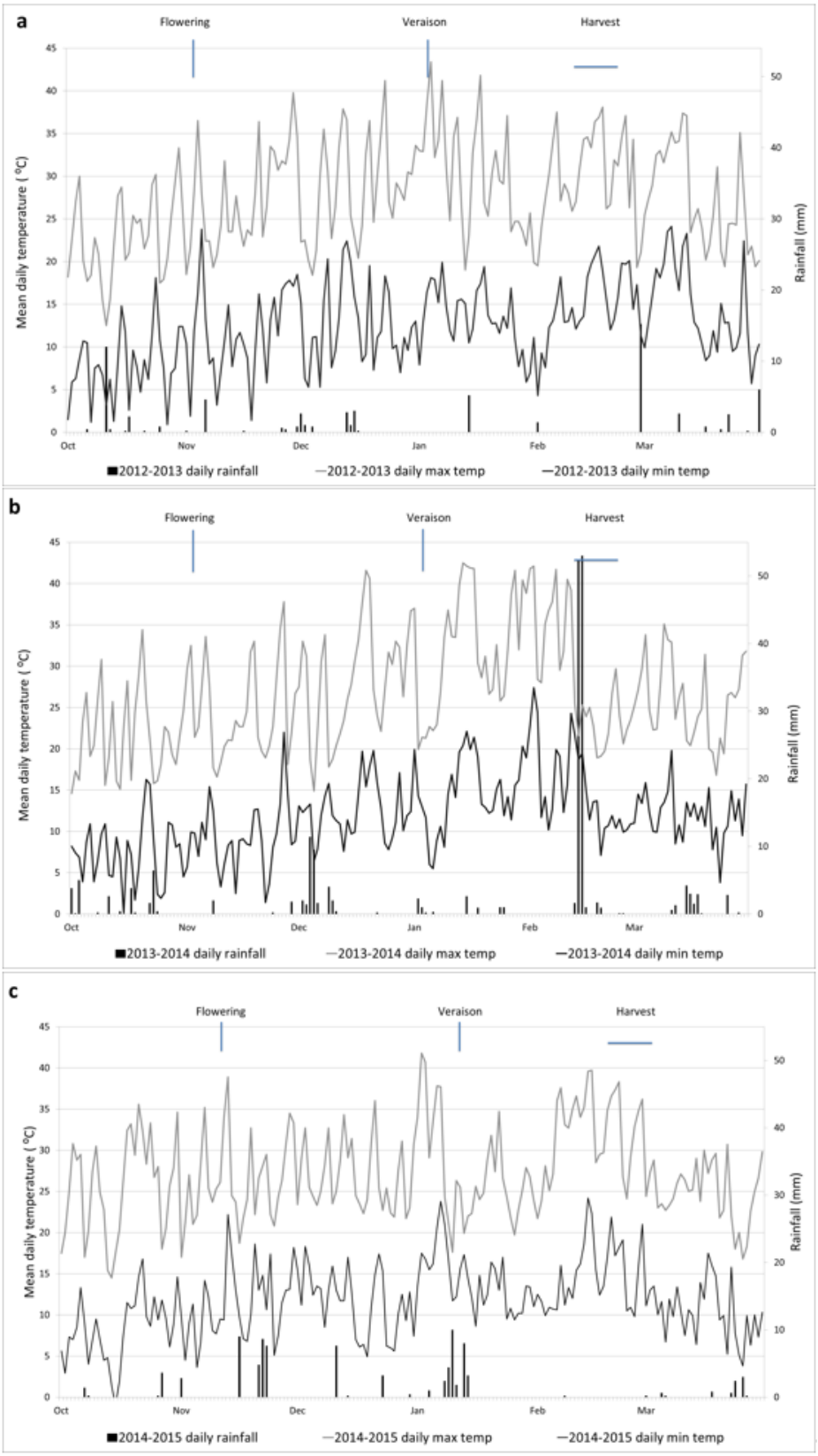

Figure S1. Summaries of seasonal climatic data for each season, approximate growth stages and harvest periods are indicated. Bureau of Meteorology site 23373 Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, altitude 275m.

Table S1. Eigen values and Eigen vectors from Principal Component Analysis presented in Figure 1.

Eigen values	F1	F2	F3	F4	F5	F6	F7	F8	F9
Eigenvalue	4.280	2.354	1.506	0.413	0.307	0.099	0.031	0.010	0.000
Variability (%)	47.557	26.152	16.730	4.585	3.415	1.103	0.346	0.110	0.002
Cumulative %	47.557	73.709	90.439	95.024	98.439	99.542	99.888	99.998	100.000

Eigen vectors:	F1	F2	F3	F4	F5	F6	F7	F8	F9
Berry mass (g)	-0.382	0.133	-0.447	0.127	-0.237	0.253	0.156	0.464	-0.510
Bunch mass (g)	-0.054	0.567	-0.310	-0.404	-0.219	0.175	0.186	-0.063	0.547
Berry number (/bunch)	0.335	0.387	0.220	-0.413	-0.026	-0.469	-0.129	0.427	-0.317
Cordon length (m)	-0.174	0.244	0.670	-0.152	0.172	0.610	0.105	0.014	-0.162
Yield / Pruning mass (Y/P)	0.477	-0.030	-0.047	-0.004	-0.110	0.026	0.763	-0.295	-0.297
Bunch number (#/m)	0.461	-0.023	-0.047	0.339	0.293	0.255	0.120	0.613	0.359
Pruning mass (kg/m)	-0.426	0.152	-0.048	0.020	0.691	-0.377	0.412	0.029	0.049
Yield (kg/m)	0.291	0.389	-0.368	0.109	0.453	0.225	-0.384	-0.351	-0.302
Trunk circumference (cm)	-0.056	0.526	0.256	0.707	-0.292	-0.235	0.020	-0.110	0.056

Table S2. Eigen values and Eigen vectors for Principal Component Analysis presented in Figure 2.

Eigen values:	F1	F2	F3	F4	F5	F6
Eigenvalue	2.723	2.069	0.778	0.392	0.031	0.007
Variability (%)	45.376	34.476	12.973	6.540	0.514	0.120
Cumulative %	45.376	79.852	92.825	99.365	99.880	100.000

Eigen vectors:	F1	F2	F3	F4	F5	F6
Harvest Mean berry weight (g)	-0.365	0.504	-0.337	0.217	0.323	0.590
Bunch Weight (g)	0.198	0.622	-0.208	-0.375	0.296	-0.550
Berry number (#/bunch)	0.552	0.052	0.265	-0.525	0.116	0.577
Bunch number (#/m)	0.484	-0.335	-0.243	0.428	0.639	-0.060
Fruit mass (kg/m)	0.500	0.211	-0.492	0.262	-0.622	0.092
Trunk circumference (cm)	0.197	0.448	0.687	0.534	-0.017	-0.056

Table S3. Standard error of the multi season means for each variable presented in Table 6.

	Vine age since planting									
	6	**12**	**17**	**28**	**49**	**93**	**105**	**107**	**128**	**168**
Variables	Site	Site 3	Site 4	Site 2	Site 1	Site 5	Site 1	Site 2	Site	Site 4
Berry number (#/bunch)	3.95	3.45	3.60	4.89	6.07	4.26	4.46	6.18	5.54	8.55
Berry mass (g)	0.01	0.04	0.03	0.05	0.04	0.02	0.03	0.06	0.03	0.04
Bunch mass (g)	4.03	3.13	3.03	8.33	5.29	4.99	4.99	7.16	5.08	9.85
Bunch number (#/m)	1.23	1.31	1.58	1.85	1.30	2.14	1.51	2.31	1.10	2.38
Yield (kg/m)	0.15	0.14	0.10	0.26	0.13	0.16	0.20	0.33	0.11	0.24
Pruning mass (kg/m)	0.06	0.03	0.02	0.13	0.03	0.03	0.05	0.07	0.03	0.03
Yield / Pruning mass (Y/P)	0.38	0.24	0.33	0.13	0.41	0.36	0.40	0.38	0.16	0.80
Trunk circumference (mm)	3.3	2.8	3.0	3.2	4.8	12.0	19.8	8.8	22.0	14.3
Bunch number (#/vine)†	2.16	4.75	3.86	4.62	4.96	5.79	4.00	6.69	3.60	6.79
Buds retained (#/m) †	0.77	0.78	1.19	0.72	0.68	0.77	1.69	1.26	0.58	1.16
Count shoots (#/m) †	0.75	0.55	0.92	0.82	0.52	0.68	1.30	1.23	0.92	0.96
Non count shoots (#/m) †	0.46	0.37	0.35	0.55	0.24	0.36	0.41	0.82	0.59	0.66
Shoot number (#/m) †	0.79	0.67	1.16	0.73	0.59	0.84	1.60	1.96	1.19	1.44
Budburst (%)†	4.67	3.76	3.32	11.74	3.42	2.75	2.55	5.11	4.94	4.32
Cane length (cm) †	2.04	2.62	1.91	3.60	4.51	3.58	4.51	3.16	2.92	2.72
Fruit mass / Bud†	10.16	11.69	6.53	19.68	10.32	13.00	13.19	21.69	6.38	14.57
Nodes retained (#/kg) †	3.62	1.39	2.15	0.43	2.39	3.79	1.93	1.58	1.21	3.50
Bunch number (#/shoot) †	0.07	0.07	0.07	0.15	0.09	0.14	0.10	0.16	0.06	0.15
Mean cane mass (g) †	2.22	1.52	0.77	7.34	2.06	3.29	2.29	5.55	1.71	0.91

†Data for 2014 and 2015 growing season only.

Chapter 4. Prepared Manuscript 2: Vine age and site effects on grape and wine chemistry in Shiraz from the Barossa zone in South Australia.

Statement of Authorship

Title of Paper	Vine age and site effects on grape and wine chemistry in Shiraz from the Barossa wine region in South Australia
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Molecules

Principal Author

Name of Principal Author (Candidate)	Dylan Grigg
Contribution to the Paper	Designed and conducted the research experiments, analysed the data, drafted and constructed the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 17/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roberta De Bei
Contribution to the Paper	Supervised research, contributed to research ideas and design and editing of the manuscript.
Signature	Date 15.05.17

Name of Co-Author	Keren Bindon
Contribution to the Paper	Assisted in analysis and interpretation of tannin composition data and editing of the manuscript
Signature	Date 19/5/17

Name of Co-Author	Natolya Lloyd
Contribution to the Paper	Assisted with metabolomic data collection and interpretation and editing of the manuscript
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Name of Co-Author	Carlos Rodriguez Lopez
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Signature	Date 14/05/17

Name of Co-Author	Paul Smith
Contribution to the Paper	Contributed to interpretation and editing of the manuscript
Signature	Date 18/5/17

Name of Co-Author	Cassandra Collins
Contribution to the Paper	Supervised research, contributed to research ideas and design and editing of the manuscript
Signature	Date 16/5/17

Vine Age and Site Effects on Grape and Wine Chemistry in Shiraz from the Barossa Wine zone in South Australia

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Abstract

Greater vine age is believed to lead to increased wine quality. However, there is little scientific evidence to support this proposition. This study was undertaken to test the hypothesis that vine age has an influence on non-volatile grape and wine quality. Five commercial vineyards of *Vitis vinifera* L. cv. Shiraz were selected within the Barossa Zone, South Australia. Each vineyard contained two age classes of grapevines based on chronological planted age: 'young' and 'old'. Over three consecutive seasons, grapes were harvested for analysis and experimental wines were made. Chemical measures of °Brix, alcohol, pH, TA, colour, phenolic and tannin were for both grape and wine samples. Tannin compositional analysis was undertaken in the final two seasons as well as non-targeted metabolomic analysis in the final season. Vine age was not a major contributor to differences in wine non-volatile composition comparison to the effect of season and site. Primary metabolites were less descriptive whereas components of the phenolics analysis were able to discriminate site and regional differences. Finally, sensory descriptive analysis results were found to correlate with secondary metabolite composition through principal component analysis. Despite large differences in vine age, commonly used non-volatile objective measures of grape and wine composition did not differ greatly. Wine composition was more closely influenced by season and site. This data set shows that regions could be differentiated by wine secondary metabolites, which were reflected in mouthfeel characteristics of the wine.

Keywords: berry composition, Shiraz, vine age, phenolics, terroir, metabolomics, grapevine

Introduction

In the wine industry there is a well-entrenched belief that increased fruit and wine quality is linked to greater grapevine age [1, 2]. As such, this often results in a price premium paid for both fruit and wines produced from 'old' vines. This suggestion frequently appears in a broad range of popular media, including industry journals, blogs, wine writers etc. and is also widely acknowledged within the scientific community [3-8]. However, this notion has scarcely been validated via scientific investigation, thereby leaving the measurable presence, potential mechanisms or source of quality difference unresolved.

As perennial plants grapevines can have a very long productive lifespan, in some cases in excess of 400 years [9]. Many 'old' vineyards planted prior to the 1850s and 1880s can still be found in Australia and California respectively [10, 11]. On the other hand in Europe, large areas of vines were removed and re-planted on rootstocks (due to the introduction of phylloxera in 1863) [12]; thus making 'old' vines less common. Several studies have been undertaken to assess the effect of vine age on grape and wine composition [5, 6, 13]. However, the age of vines used in these studies does not reflect vine ages that are often associated with claims of greater quality and price-premium. The greatest potential challenge in undertaking such a study is finding suitable sites with well-established vines that possess a minimum of variables, allowing vine age to be measured as a treatment.

Grape composition at harvest is a key driver of wine quality [14] which can be influenced by both environmental and cultural factors [15, 16]. Grape compositional measures are often used to provide an indication of potential quality or suitability for winemaking. Due to the inherent compositional diversity a measure of one component alone is unlikely to be consistently related to quality, therefore often a number are combined [17, 18].

Several measures of grape primary metabolites are well established as the first indication of quality, including sugar total soluble solids (TSS) or Brix, acidity measured as pH and titratable acidity (TA) [19, 20]. In addition, multiple secondary metabolites are known to influence the mouthfeel and astringency of wine, such as phenolic compounds and tannins [21]. A wide range of studies have drawn correlations between secondary metabolites and wine quality scores [21-24]. Secondary metabolites of grape-derived flavanoids are divided into three classes: flavonols (e.g. quercetin, kaempferol, myricetin), anthocyanins and flavan-3-ols (e.g. catechin, epicatechin, gallocatechin, procyanidins, condensed tannins) [25]. These grape-

derived secondary metabolites are the principal sources of wine colour, flavour and texture (astringency and bitterness) [26].

Grape colour has been positively associated with wine quality and used for commercial quality assessment [19, 27-29]. In addition, the contribution of coloured compounds and their presence in wine has been shown in many cases to correlate with quality assessments or bottle price [27, 30-32]. Total phenolic and tannin measures of wine have also been positively correlated with wine quality and higher red wine scores [21-24]. Generally, these studies show that higher wine colour, tannin or phenolic measures result in a higher wine quality score, or higher bottle price.

Grape phenolic composition and concentration can be influenced by a range of variables, such as: cultivar, microclimate and management [33-35]. They are also known to affect the appearance, taste and mouthfeel of wine [25]; specifically, there are the flavonols quercetin and kaempferol which are commonly found in grape skins. The sensory effects of quercetin derivatives have been linked to bitterness in red wine [36, 37]. Both quercetin and kaempferol have been identified as the most discriminating phenolic compounds for characterising geographic origin [38].

Whether primary or secondary metabolites are measured in research, they are also subject to the unique sum of their influences, encapsulated in the term: 'terroir'. Terroir has different interpretations ranging from meaning just "soil" or the "total natural environment" including soil, climate (temperature) and topography [39, 40]. Recent studies and new technologies are now uncovering influences of terroir through high resolution 'omics' analysis [41-44]. Irrespective of the definition or study undertaken, environment is well acknowledged as a major driver of difference in terroir [45-49]. Discrimination of the terroir or site effect has successfully been made using a range of compositional measures, including: flavonoid composition [50, 51], total phenol content [52] or 'omics'-type approaches [53, 54]. Terroir is of interest in relation to the investigation into vine age, as it is suggested that only as vines mature will terroir become fully expressed [55].

Quality determination of grapes and wine and their underlying causes are inherently complicated, in part due to the complex interplay between genotype, environment and management [15, 43, 56-59]. This study aimed to test the hypothesis that vine age would result in differences in grape and wine composition of Shiraz from the Barossa wine zone, Australia.

To achieve this, where possible, variables were minimised at five experimental sites to allow the most direct evaluation of age and site making use of a unique set of vineyards. To our knowledge, such a study has never been carried out on vines with this degree of age differences, making this both novel and informative.

Results

Grape and wine compositional data collected over three seasons were initially analysed via repeated measures ANOVA to determine the influence of season, site and age (Table 2). Not surprisingly the influence of both season and site were significant.

Grape composition

Overall the grape data showed few significant differences between compositional measures of fruit from young and old vines. All sites were harvested at commercial maturity. Out of 15 harvest comparisons only at site 4 in 2014 and 2015 was the maturity different between old and young vines, in each season young vines had a higher °Brix (Table S2). Harvest TA was significantly higher in juice from old vines in the following sites / seasons; site 1 in 2014, site 3 in 2013, site 5 in 2015 and site 4 in 2013 and 2015. At site 2, harvest TA was significantly higher in juice from young vines in 2014 and 2015. This observation was not consistent in every season nor at every site. A significantly lower pH was also observed in the juice from older vines compared to younger vines at site 1 in 2014 and site 4 in 2013 and 2015. Significant differences in berry mass were only observed at site 3 in 2013 and site 4 in 2014, where in each case older vines had larger berries. Total anthocyanin, phenolic and tannin concentrations in fresh grapes from old and young vines were not significantly different at each site following pairwise comparisons in any of the three seasons apart from phenolic concentrations at site 4 in 2015.

The basic grape compositional data was subjected to PCA to visualise any differences in the dataset. No clear relationships were present except for some grouping of sites 3 and 5 together (see supplementary Figure S3).

Wine composition

A greater number of significant differences were observed between wines from old and young vines than the corresponding grape composition measures from each site (Table S4). Wine from old vines had lower pH and higher TA levels compared to young vines. In most seasons final wine alcohol did not differ significantly between old and young vines. The exceptions to this were at site 2 in 2013 and site 4 in 2014 and 2015 where alcohol levels were lower in wines

made from old vine fruit. At site 3 in 2014 and site 4 in 2013 alcohol levels were higher in wines made from old vine fruit. Anthocyanin concentrations were greater in young vine wines in each instance where a statistical significance was observed; only at site 5 was no difference in anthocyanin concentration observed.

Generally wines produced from older vines had less colour, tannin and lower phenolic concentration levels (when significantly different). Visualisation of basic wine chemistry variables using PCA revealed that 64% of the variability is explained by the first two PCs (Figure 1). Variation on PC1 (40%); is explained predominantly by differences in pH and to a lesser extent TA. Sites 1, 3 and 5 show the greatest clustering with site 2 being the most clearly separated from the other sites along PC1. The separation of site 2 appears to be driven by wine pH, which was significantly different in two out of three seasons; at this site the highest pH of all sites and treatments was also recorded (Table S4).

Wine tannin composition was not found to be significantly different except in one instance between vine age pairs (Table S5). Interestingly, a consistent trend each season was observed whereby sites 1 and 2 (49, 105 years and 28, 107 years respectively) were consistently separated from the other sites when PCA was performed on tannin compositional measures (Figure 2). Both of these sites are located in the region of Eden Valley while the remainder of the sites were from the Barossa Valley region. This separation is generally the result of catechin and epicatechin subunit percentages being higher from the Eden Valley sites while Barossa Valley sites had generally higher tannin molecular mass (MM), mean degree of polymerisation (mDP) and epigallocatechin subunit percentage.

Non-targeted analysis of phenolics was undertaken using LC-MS/MS on the 2015 wines to determine features that were able to discriminate sites or vine age. A total number of 1598 molecular features were detected. PCA was used to visualise the data and 42 molecular features with the highest correlation loadings were selected. This sub-set was subjected to further PCA to determine those with the greatest influence on the dataset for potential identification. Based on the correlation loadings a shortlist of five molecular features were chosen for MS^2 experiments and their mass spectra matched against an in-house library containing reference mass spectra of pure reference compounds. Two of these were positively identified as flavonols: quercetin and kaempferol (Table 3).

A dataset resulting from sensory descriptive analysis (DA) of the same 2015 wines has been included for direct comparison with the chemical analysis reported here. Due to the known

influences of flavonoid composition on mouthfeel properties of wines attributes associated with mouthfeel and texture were specifically selected (Table S6). Both tannin composition and the phenolic compositional datasets were subjected to PCA with wine DA as supplementary variables. This is presented in Figure 3 which explained a total of 72% of the variation in the dataset. PC1 presents the greatest variation (52%) which is largely driven by variables associated with sites in the Eden Valley region (sites 1 and 2). Therefore PC1 describes this region well. Driving the regional separation on PC1 were the phenols quercetin and kaempferol in addition to tannin MM, mDP and epigallocatechin subunit percentage. A further 20% of the variation is described by PC2 which is driven by %galloyation of tannins and a number of undetermined phenolic features. No association of the variables with vine age was revealed by this analysis; however, some clustering of individual sites within regions is observed (Figure 3).

Discussion

As shown in previous studies, season and site have a large influence on fruit composition [49] which this study also supports. Repeated measures ANOVA found that both site and season had significant interactions in relation to differences observed within the dataset.

Seasonal effects

Temperature is known to be a significant factor of influence in grape quality and composition [15, 47, 49, 60] with measures of quality often linked to climatic differences between seasons [59, 61, 62].

Fruit quality potential is generally thought to be improved if temperatures during ripening are mild [55]. More specifically, higher fruit quality in the Barossa Valley has been reported when temperatures in the months leading to harvest are between 28-30°C combined with fewer days above 40°C [58]. Based on this it can be expected that the overall parameters relating to quality of each season in this study may differ, potentially due alone to the number of days over 40°C which were 3, 10 and 2 days for harvest seasons 2012/13, 2013/14 and 2014/15 respectively. Following preliminary ANOVA and PCA of basic grape and wine compositional measures, growing seasons 2013/14 and 2014/15 presented a closer relationship than 2012/13 (Figure S1). This may be due to the broad measure of growing degree days (GDDs) which were more similar in 2013/14 and 2014/15 (1536 and 1520 respectively) than in 2012/13 (1686) even though there were different numbers of days >40°C (Table S1). Seasonal grouping is at least partially explained by GDDs, however, other factors may be influencing these seasonal measures of quality.

Rainfall in conjunction with soil water availability is known to influence compositional parameters such as sugar, flavour and colour [59, 62-64]. Rainfall was shown to be inversely correlated with wine quality rankings across many years (1971-2002) in five Italian wine regions, suggesting rainfall was having a greater significance than temperature [63]. In a two year experiment on non-irrigated vineyards, water deficit due to climate and soil conditions was linked to higher quality potential in red grapes in Greece [35]. In the current study, growing season rainfall was variable between seasons, notably, 2013/14 had the highest recording by almost 100 mm. Despite the high total, 105 mm fell in one event very close to harvest therefore the majority of the growing season was similar to the other two seasons (Figure S2). The highest January rainfall for the three seasons was in 2014/15 when 31 mm was recorded just prior to veraison E-L 35 [65]. This is a likely driver for berry mass in growing season 2014/15 which had the highest mean, median and maximum mass for both vine age classes across all sites.

Berry size and quality

The impact of berry size on grape and wine quality has been the subject of much discussion [24, 57, 66]. Many compounds responsible for red wine character are found in the pericarp or skin [60]. Increases in berry size have been shown to alter the skin to pulp ratio and subsequent dilution of berry solutes [66-68]. Smaller berries have been found to produce higher quality wines than larger berries in some cases [24, 69]. In contrast, other studies have found larger berries to produce higher quality wines than smaller berries [70, 71]. While some studies found no difference in fruit composition despite large differences in berry size [72, 73]. In relation to vine age berry mass may explain differences between sites and seasons, however, few differences in berry mass were observed between vine ages in any of the seasons investigated. Repeated measures ANOVA found that site and season both had the strongest interactions in terms of berry mass (both $P<0.001$) while age had no influence ($P<0.61$). It has been suggested that the drivers determining berry size are more important than berry size alone in determining grape and wine quality [71, 74].

Grape and wine chemical composition

While ‘old’ grapevines have physically larger trunks and generally yielded more fruit (Chapter 3), this did not translate to changes in the magnitude of commonly used non-volatile chemical measures of quality in this study. In contrast, over three seasons Müller Thurgau yield and TA were consistently higher in vines which retained older cordon wood, following dormant pruning. This effect was not noted for Pinot Noir so it may be varietal dependent [75]. Grapes

from each age class were harvested at commercial maturity as judged by the respective winemaker at each site. No significant differences in Brix level at harvest were seen between the majority of comparisons; only at site 4 in 2014 and 2015, where Brix levels were higher in younger vines. This finding also translated into higher wine alcohol for the same seasons. Other sites which recorded differing alcohol percentages in wine but not Brix levels in fruit were site 2 in 2013 (old was higher) and site 3 in 2014 (young was higher). Overall there was no consistent difference related to vine age and no effect related to region.

Similar grouping trends are observed for wine compositional measures (Figure 1); however, separation of the sites is driven by the significant interaction of site effects in the acid measures (pH and TA) as presented in Table 2 (Eigenvectors presented in Table S6). These regional differences are to be expected as berry organic acid concentration is known to be influenced by the environment [15]. In terms of environmental influences, grapevines grown in cooler climates are known to have higher malic acid concentrations [76, 77] compared with warmer climates. Tartaric and malic acids account for 90% of acids in grape berries [77]. While malic acid was not directly measured in this study, it will be making a contribution to the measure of TA. Therefore, the influence of acidity measures would appear to be age independent, resulting from site-specific growing conditions.

Over three seasons few differences were observed in measures of anthocyanin and phenolic concentration in fresh grapes between vine age groups. Repeated measures analysis over three seasons found both site and season to be significantly different for all attributes. Minimal variation was present within sites due to age, and much greater variation of compositional measures was found between sites. This is similar to the results of Reynolds, *et al.* [49] where site-specific effects were dominated by site and vintage effects. PCA of basic grape composition at harvest (Figure S3) presented a tight grouping of sites 3 and 5 due to intra-site similarities; this is despite age differences of 116 and 87 years respectively at these sites. The remaining sites present regional associations without site specific grouping. This is especially evident at site 2 which occupies the positive quadrant of PC1 and PC2, which is principally driven by fruit TA and berry mass at this site.

Wine tannin composition

Final wine quality is determined by its sensory properties. In this study compositional measures are inferred to represent greater wine quality potential. Assessment of tannin composition has

been shown in past studies to positively correlate with quality rankings [21-24, 32]. Despite this, tannins are known to be tasteless or indistinctly flavoured [78]. However, tannin concentration has been strongly correlated with mouthfeel sensations of perceived astringency [79, 80] likely due to interactions with salivary proteins [81, 82]. It has also been shown that larger wine tannins by molecular mass tend to result in a more astringent mouthfeel sensation than smaller tannins [83]. In red wine varieties Merlot and Cabernet Sauvignon, mean degree of polymerisation (mDP) has also been positively correlated with intensity of astringency [84]. Therefore, if the quality of a wine is determined by its sensory properties relating to astringency, tannin size is likely to be associated with degrees of wine quality, for example; high astringency is not a positive trait of red wines. More specifically, Shiraz skin tannins were associated with quality grades in wine in addition to overall tannins [32]. The tannin subunit epigallocatechin has been found in higher concentrations in grape skin combined with higher mDP compared to seeds in Shiraz [32]. Our data suggest that sites 1 and 2 show reduced association with skin derived tannin indicators (Figure 2) which is supported by a lower perception of astringency and tannin intensity including; mouthfeel astringency and tannin intensity (mouthfeel and aftertaste) (Figure 3). These sites are both found in the cooler Eden Valley region, therefore the presence of these tannin descriptors might indicate that their presence and intensity is a site or 'terroir' effect and not age. Furthermore, catechin and epicatechin tannin subunits (as molar proportion) were consistently greater at sites 1 and 2, which may be indicators of seed extracted tannins [85]. Seed tannin extracts have been shown to be no less astringent than skin tannins in Merlot and Cabernet Sauvignon [84]. Conversely mDP and MM, which are indicators of skin extracted tannins were greater in Barossa Valley sites and have been shown to be positively associated with astringency intensity [84]. This suggests that there is a chemical and possibly sensory basis for discrimination of the site or terroir influence in these wines irrespective of age.

Wine phenolic profile

In the final season (2014/15) wines were subjected to non-targeted LC-MS/MS analysis to investigate the non-volatile metabolomic profile of the wines. Uniform winemaking practices and the exclusion of oak ensures the phenolic profiles of the wines are as representative as possible of the grapes from which they were produced. Following data processing steps, including deconvolution and filtering, further data analysis uncovered several discriminating molecular features in the finished wines (Figure 3). As shown in Figure 3 there was separation between regions and sites clustered together within these regions which indicates an

environmental influence independent of vine age. This environmental effect offers a potential explanation for the regional discrimination of these metabolites.

Increased sunlight is known to influence secondary metabolites, specifically quercetin is noted as an excellent indicator of sunlight available to bunches [77]. The exclusion of UV light results in large increases in flavonols including glycosides of quercetin, kaempferol and myricetin [86]. These reported correlations of temperature and sunlight on the production of flavonols could present a possible explanation for differences observed in our study based on regional differences. Phenolic composition has been shown to clearly differentiate wine samples based on region of origin [38]. It cannot be discounted that subtle differences in the phenolic signature of wines may be due to clonal variation, which has particularly been characterised by quercetin concentration [87, 88].

Sensory analysis

Compositional measures provided a chemical basis for regional discrimination, the translation of these findings into wine sensory profiles was then undertaken. Sensory analysis of bitterness, astringency and tannin intensity attributes support the regional discrimination using PCA (Figure 3). Tannin concentration has been shown to be strongly correlated with increased intensity of astringency [79, 80]. It has also been reported that the astringency characteristics of tannins from grape seeds and skins differ [28] due to their composition [89]. In our study compositional differences in relation to mDP and MM to mouthfeel characteristics relating to tannin intensity and physical length of drying sensation were highly correlated (Pearson correlation coefficients of 0.84 and 0.92 respectively). These attributes are found in the positive space of PC1 (Figure 3) which best explained the regional separation between sites. The regional differences in these sites is characterised by climatic differences in growing conditions (Table 1) particularly the temperature measure of degree days. Furthermore our study found that, astringency and bitterness were associated with higher levels of the phenolic compounds quercetin and kaempferol. These flavonols, particularly quercetin, are known to be both bitter [36, 90] and astringent [91]. The combination of tannin composition and phenolic composition collectively contribute to discrimination of growing region.

Terroir: region and site effects

It has long been documented that wine quality is subject to the characteristics of geographical location, encompassed in the term *terroir* [39]. This study demonstrated that growing region

(or the sum of the influences of site terroir) had a greater effect on non-volatile composition than grapevine age.

Differences in basic climatic data of MJT, rainfall and growing degree days are observed between regions within the Barossa zone (Table 1). The observations in this study are likely a result of such differences. Vintage effect on grape metabolite profiles has been shown to be greater than the soil effect [76], suggesting that a typical unirrigated vineyard could be defined by its climatic traits. The specific influences of grape and wine composition based on vineyard site have been widely reported using a range of approaches [38, 50, 53, 92]. The influence of the specific growing site was highly evident in this study in all seasons, and within season, regional effects were also evident. The influence of region is most notably highlighted via PCA whereby sites 1 and 2 were separated from the majority of other sites. This was most pronounced as the resolution of the measurement increased e.g. Figure S3 compared to Figure 2. Combined with the increased resolution of the measures there could also be an effect due to the susceptibility of the individual variables to environmental change [93]. For example under experimental conditions of environmental change, anthocyanin accumulation presented a far greater range of variation (148%) than sugars (39%) [56]. This supports our findings that anthocyanin (or secondary metabolites in general) have a greater ability to show regional differences in wine from differing regions.

Conclusion

Compositional (non-volatile) measures relating to fruit and wine quality were not readily separated based solely on vine age in this study. Some interesting differences are reported, specifically in relation to older vines having a more desirable (i.e. lower) pH at a similar Brix level compared to younger vines. This study concurs with previous findings that objective measures of quality in grapes are often challenging to align to crop price or wine quality (using price as a proxy) [70, 94]. As demonstrated by the identification of regional characteristics and not age based characteristics.

Wine compositional measures were often closely related at each site, irrespective of age. Non-volatile secondary metabolites showed sensitivity to regional influences allowing differentiation. Tannin compositional differences and key phenolic compounds were identified

as significant determinants of regional differences, and they did not show significant differences between vine age-based associations.

This regional differentiation was validated in the final season using sensory DA which demonstrated that, irrespective of vine age, wine mouthfeel properties were likely to be a result of regional differences. If greater quality or value is attributable to age it is likely to be associated with characters not measured here, such as volatile composition or via complex interactions in the wine chemical matrix. The drivers and interdependence of phenolic composition, flavour intensity and wine quality are again suggested to be more complex than previously thought [95, 96]. The inclusion of new technologies at ever increasing measurement resolutions may provide more insight into comparisons of composition in relation to vine age.

Materials and methods

Sites, experimental design and plant material

Five commercial Shiraz (*Vitis vinifera* L.) vineyards were selected in 2012 within the Barossa wine zone, South Australia (Table 1). General climatic statistics for the region are presented in Table 1 and specific climatic observations for the growing seasons 2012-2015 are presented in Figure S2. The Barossa zone is divided into two regions: the Barossa Valley with sites 3, 4 and 5 and Eden Valley which has sites 1 and 2. Elevation of vineyards in the Barossa Valley ranges from 200-300 m while the Eden Valley is between 400-500 m above sea level and is cooler and wetter than the Barossa Valley.

Sites were chosen with the assistance of local industry members to ensure an accurate record of age and to minimise differences between vines at each site. Consideration was given to relative homogeneity of site and vine characteristics between vine age groups within each site such as soil type, row orientation, pruning, trellis system and vineyard floor management. Each site had vines established at different times: older vines ranging from 93 to 168 years and comparatively younger vines aged 6 to 49 years. All young vines at sites 1-4 were vegetatively propagated via hardwood cuttings from the older vine material. Site 5 was propagated via layering with the connecting stem severed from the mother vine after several years. All vines were on own roots. Virus status was unknown and vine health was assessed visually based on uniformity, representative growth and appearance with the assistance of each vineyard manager or owner. *Eutypa lata* and *Botryosphaeria spp.* are known to affect vines in this region, therefore vines with obvious symptoms or in decline were avoided. Clonal status of vine

material is unknown, but as all vines were propagated via cuttings, they are considered to be a mass selection or quasi-clones. At each vineyard, three replicates containing 4 or 5 vines in adjacent rows resulted in 12-15 vines per age treatment being selected. Due to the propagation technique at site 5, old and young vines are interspersed resulting in two adjacent rows of vines being selected and grouped to form treatment replicates. No interference was made with respect to vine management at any time which was left to the respective vineyard management at each site. Specific details for each site and age group can be found in Table S3.

Berry chemical analysis

At each site and each season treatments were harvested at similar Brix levels where possible to yield comparable berry characteristics. The decision to harvest was determined by each site's winemaker to produce commercial wine of the highest quality. Measures were taken from a 100 berry sub-sample of each treatment replicate to measure pH and total acidity (TA) (autotitrator; Crison instruments Barcelona, Spain) [20]. Total soluble solids (TSS) of juice samples were measured with a digital refractometer (BRX-242 Erma inc. Tokyo, Japan).

A random sample of 50 berries was collected from bunches at harvest and frozen at -20°C for anthocyanin, phenolic and tannin analyses. Total grape tannins were measured by the methyl cellulose precipitable (MCP) tannin assay [97] using the protocol of Mercurio, *et al.* [98]. Total anthocyanin and phenolics were determined according to the method of Iland, *et al.* [20].

Winemaking

Fruit was harvested by hand from each treatment replicate into 20 kg vented crates and transported to the University of Adelaide, South Australia, Waite Campus for processing. Fruit was stored overnight in a 4°C cool room and processed the following day when the fruit reached 18-20°C in a temperature controlled room to ensure uniform and appropriate inoculation conditions. Fruit was crushed and de-stemmed using a small combination crusher de-stemmer (Grifo Machine Enologiche, Piadena. Italy) into 20 L plastic fermentation vessels and allowed to reach room temperature. At the crusher 50 ppm of potassium metabisulphite was added.

Post crushing, ferments were inoculated with 200ppm of commercial dried yeast (AWRI 796, Laffort, Bordeaux, France) and 200 ppm of nutrient (Dynastart, Laffort, Bordeaux, France). Following an initial drop of 2° Baume 200 ppm diammonium phosphate (DAP) was added

along with malo-lactic bacteria culture (Lalvin VP41, Lallemand, France) at 0.2 ppm to complete primary and secondary fermentation simultaneously.

Must was fermented on skins under controlled conditions at 22°C, plunged twice per day with a stainless steel plunger to break and wet the cap only. Baume (Hydrometer) and temperature were monitored and recorded twice daily. When Baume was <2 ° or after 5 days of skin contact wines were pressed off using a 20 L hydraulic water bag press (300 KPa max). Pressed wine was transferred into 10 L glass demijohns to complete malo-lactic fermentation (MLF). MLF was monitored approximately weekly using an L-Malic acid enzymatic test kit (Vintessential, Dromana, Australia). Following completion of MLF (≤0.05 g/L malic acid) wines were sulfured to a free level of 30 ppm. Wines were bottled directly into 750 mL glass claret bottles and sealed under screwcap. The wines were then stored at a constant 15°C for future wine sensory and chemical evaluations.

Wine chemical analysis

Wine pH and TA was determined as described by Iland, *et al.* [20]. Final alcohol levels were determined using an Alcolyzer Wine ME (Anton Paar, Graz, Austria). Colour, tannin concentration and tannin composition were analysed six months post-bottling in each season. Wine colour was determined using the modified Somers assay using a high throughput method in 96 well plates [98]. Wine tannin concentration was determined using the methyl cellulose precipitable (MCP) tannin assay of Mercurio, *et al.* [98] and is expressed as epicatechin equivalents (mg/L) using an 8-point epicatechin standard curve [99]. The modified Somers assay was used to determine; wine colour density (WCD), SO_2-corrected WCD, degree of anthocyanin ionisation, phenolic substances and anthocyanins (in mg/L). Wine tannin composition for 2014 and 2015 wines were determined using a two step process, briefly; solid phase extraction (SPE) was used to isolate total polymeric phenols which were subjected to acid catalysis in the presence of phloroglucinol (phloroglucinolysis), reaction products were analysed using HPLC as detailed by Kassara and Kennedy [32].

Metabolomic analysis

Non-targeted metabolomic analysis of the 2015 wine samples was performed using LC-MS/MS. The metabolites were isolated from bottled wine samples using solid-phase extraction (SPE) with Phenomenex Strata-X 33 um 85Å polymeric reverse-phase 60mg/3mL cartridges. A 2 mL aliquot of each sample was evaporated to dryness under nitrogen and 30°C. SPE conditions are presented in Table S8. A pooled mix of all samples was prepared and used to monitor instrument performance. The analysis was performed on an Agilent 1200SL HPLC

coupled to a Bruker microTOF-Q II in ESI negative mode. The operating conditions are described in Table S8.

Following data acquisition, mass calibration was performed on each file using Bruker Daltonic's DataAnalysisv4.1 "Enhanced Quadratic" calibration method (Bruker Singapore, The Helios, Singapore). Each file was exported from DataAnalysis in the mzXML generic file format for further processing. The files were processed using R (statistical programming environment) v3.1.0 and Bioconductor v2.14 under a Debian Linux 64-bot environment. Molecular features were extracted for each file using *xcmx* package and features that possessed a common mass and retention time across samples were grouped together.

Wine sensory descriptive analysis.

A panel of assessors was selected consisting of both male and female staff and students from the University of Adelaide. All assessors were highly experienced in sensory DA, and familiar with wine sensory analysis. The final panel consisted of twelve assessors. Sensory DA was undertaken in a series of training and formal sessions. Training was undertaken in three, two hour sessions. During the training sessions, the assessors initially generated descriptive terms by consensus specific for the wines of each season, these attributes were then used to create the proforma and lexicon used in final sessions, in addition to gaining familiarity in recognising and scoring the attributes. The training sessions involved ranking exercises of taste attributes, astringency and identification of aroma standards. Wine samples specific to each vintage were assessed to develop characteristics of aroma, flavour, mouthfeel and aftertaste descriptors. A final list was developed consisting of 8 aroma, 10 flavour, 3 mouthfeel and 4 aftertaste attributes based on panel consensus. Details of all attributes and standards are presented in Table S9.

Final sensory assessment was undertaken in five sessions over two weeks in isolated booths at 22°C under fluorescent lights, as colour was not assessed. Wines were given a three-digit code and presented in randomised order in covered ISO standard glassware containing 50 mL of wine. In each session, the judges assessed 12 samples presented in randomised groups of four with a 30 second break between samples and a forced break of at least 5 minutes after the first two groups. Purified water was provided along with approximately 100 mL of citrus pectin solution (2 g/L, Sigma-Aldrich Co., St Louis, MO) and water crackers for palate cleansing and reference. All treatment replicates were presented in duplicate.

Aroma reference standards were presented at each session to reinforce familiarity of attributes and were available for referral at all times during judging sessions. Reference standards of materials for judging tactile sensations of tannin texture were supplied in the booth for reference and fine tuning. Attributes were separated into groups of aroma, taste and mouthfeel and scored on 15 cm unstructured line scale. Tannin structure was defined by physical sensation, either drying (lack of saliva lubrication) or sensation relating to perceived size. Both of these references to tannin structure and size are physical sensations and not intended to be directly related to actual molecular tannin structure in terms of constitutive elements.

Statistical analysis

One way ANOVA and PCA were undertaken using XLSTAT Version 2015.4.01.20116 (Addinsoft SARL, Paris, France). Repeated measures ANOVA was performed using Genstat version 15 (VSN international, Hemel Hempstead, UK) means were separated via Fishers LSD. Repeated measures ANOVA considered data at successive times including replication and random effects. Sensory data were subjected to mixed models ANOVA on all attributes from DA panels via XLSTAT sensory data analysis plugin. The product characterisation function with assessors (judge) as random effects was used (Y=P+J+P*J) to identify attributes with significant discriminating power which were used for further analysis.

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Tables

Table 1. Site details of vineyards located within the Barossa zone. Location includes nearest township and regional designation E=Eden Valley and B=Barossa Valley, vine establishment dates, rainfall, MJT and relative age gap between old and young vines for all sites.

Table 2. Grape and wine composition at harvest results following repeated measures ANOVA showing interactions and significance.

Table 3. Relative response of discriminating molecular features detected by non-targeted metabolomic analysis of 2015 Shiraz wines. Unknown compounds were unable to be matched with in house libraries. Identified compounds were matched based on fragmentation pattern. Site number followed by a letter designates age where O=Old and Y=Young.

Table 1. Site details of vineyards located within the Barossa zone. Location includes nearest township and regional designation E=Eden Valley and B=Barossa Valley, vine establishment dates, rainfall, MJT and relative age gap between old and young vines for all sites.

Site	Location (region)	Year of establishment (vine age)[†]		Relative age gap[†]	Rainfall[‡] (GS mm)	MJT[§] (°C)	GDD[§]
		Old	Young				
Site 1	Angaston (E)	1908 (105)	1964 (49)	56	391	19.0	1309
Site 2	Eden Valley (E)	1906 (107)	1985 (28)	79	391	19.0	1309
Site 3	Nuriootpa (B)	1885 (128)	2001 (12)	116	203	21.5	1487
Site 4	Tanunda (B)	1845 (168)	1996 (17)	151	203	21.5	1487
Site 5	Rowland flat (B)	1920 (93)	2007 (6)	87	203	21.5	1487

[†]Vine age in years since establishment, at commencement of study in 2013.

[‡]Growing season (GS) rainfall accessed from Bureau of Meteorology [100]

[§]MJT and Growing degree days (GDD) October - April inclusively taken from Gladstones [47].

Table 2. Grape and wine composition at harvest results following repeated measures ANOVA showing interactions and significance.

	Site	Age	Season	Site.Age	Season.Site	Season.Age	Season.Site.Age
Grape measures							
Harvest Brix	<0.001	0.00	<0.001	0.218	0.004	0.364	0.039
Harvest pH	<0.001	0.50	<0.001	<0.001	0.006	0.17	<0.001
Harvest TA (g/L)	<0.001	0.06	<0.001	<0.001	<0.001	<0.001	<0.001
Anthocyanin (mg/g)	<0.001	0.05	<0.001	0.153	0.018	0.434	0.817
Total phenolics§ (mg/g)	<0.001	0.93	<0.001	0.056	0.013	0.099	0.392
Tannin† (mg/g)	0.019	0.56	<0.001	0.900	<0.001	0.912	0.333
Berry mass (g)	<0.001	0.61	<0.001	<0.001	<0.001	0.058	<0.001
Wine Measures							
Wine Alcohol %	<0.001	<0.001	<0.001	0.012	<0.001	0.436	<0.001
Wine pH	<0.001	<0.001	<0.001	0.002	<0.001	0.217	0.303
Wine TA	<0.001	0.037	<0.001	0.236	<0.001	0.052	0.005
Anthocyanins‡ (mg/L)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Hue (no units)	<0.001	<0.001	<0.001	0.738	<0.001	0.04	<0.001
Total phenolics§ (au)	<0.001	<0.001	<0.001	<0.001	<0.001	0.478	0.021
Tannin† (mg/L)	<0.001	0.003	<0.001	0.01	<0.001	0.631	0.444

† Tannins determined by the MCP tannin assay (Mercurio et al. 2007) expressed as milligrams of epicatechin equivalents per gram of fresh grape or mg/L of wine.
‡ Anthocyanin content determined spectrophotometrically at 520nm (Iland at al. 2004), expressed as malvidin equivalent units.
§ Phenolic substances determined from absorbance at 280nm (Iland et al. 2004), expressed as absorbance units (au) per berry or per gram of fresh berry mass or mg/L of wine.

Table 3. Relative response of discriminating molecular features detected by non-targeted metabolomic analysis of 2015 Shiraz wines. Unknown compounds were unable to be matched with in house libraries. Identified compounds were matched based on fragmentation pattern. Site number followed by a letter designates age where O=Old and Y=Young.

ID (rt_mz pair)	11.64_399.0	12.14_399.0	39.21_401.14	90.95_455.06	Quercetin	Kaempferol
rt	11.64	12.14	39.21	90.95	108.86	113.57
mz	399.0024	399.0025	401.1445	455.0636	301.0346	285.0399
Site 1 O	150289	210128	83259	39247	382975	123547
Site 2 O	178534	232781	77346	41147	425130	177716
Site 3 O	191291	262382	58645	27017	447269	222803
Site 4 O	236555	304859	70707	24163	590979	404238
Site 5 O	90712	160218	74234	10867	414872	160515
Site 1 Y	49617	91305	87827	13975	395933	189027
Site 2 Y	132749	166946	63997	64723	312475	61732
Site 3 Y	206339	302698	62339	28529	344419	151593
Site 4 Y	218970	294116	73312	24938	556355	360524
Site 5 Y	113904	170833	73278	12035	547386	323655

Figures

Figure 1. Combined PCA of mean values for basic chemical measures for wine over three seasons. 2013 (◊) 2014 (□) and 2015 (○). Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. *Labels marked are vine age in years.

Figure 2. Combined PCA bi-plot of mean values for tannin composition in finished wine harvested in 2014 and 2015 from Table S5 symbols indicate vintage 2014 (□) and 2015 (○). Circles identify regional grouping, dashed line contains vineyards in Barossa Valley region and dotted line contains vineyards in Eden Valley region. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲= old and ● = young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. GC = epigallocatechin, MC = mass conversion, MM = molecular mass, mDP = mean degree of polymerisation, ECG = epicatechin gallate, Epicat Conc = Epicatechin concentration.

Figure 3. PCA biplot presenting combined intensity scores of LC-MS/MS data (green) and tannin compositional data (blue) from 2015 Shiraz wines overlaid with mean sensory DA results (purple) of mouthfeel attributes for the same wines. Wines are from five sites, two ages and two regions within the Barossa zone, Eden Valley (site 1 and 2) dotted elipse and Barossa Valley (sites 3,4,5) dashed circle. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲= old and ● = young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. Sensory prefix indicates modality: P = palate upon tasting, M = mouthfeel, AT = after taste following expectoration. Tannin abbreviations are; GC = epigallocatechin, MC = mass conversion, MM = molecular mass, mDP = mean degree of polymerisation, ECG = epicatechin gallate, Epicat Conc = Epicatechin concentration. The unknown molecular features labelled as their rt_mz pairs.

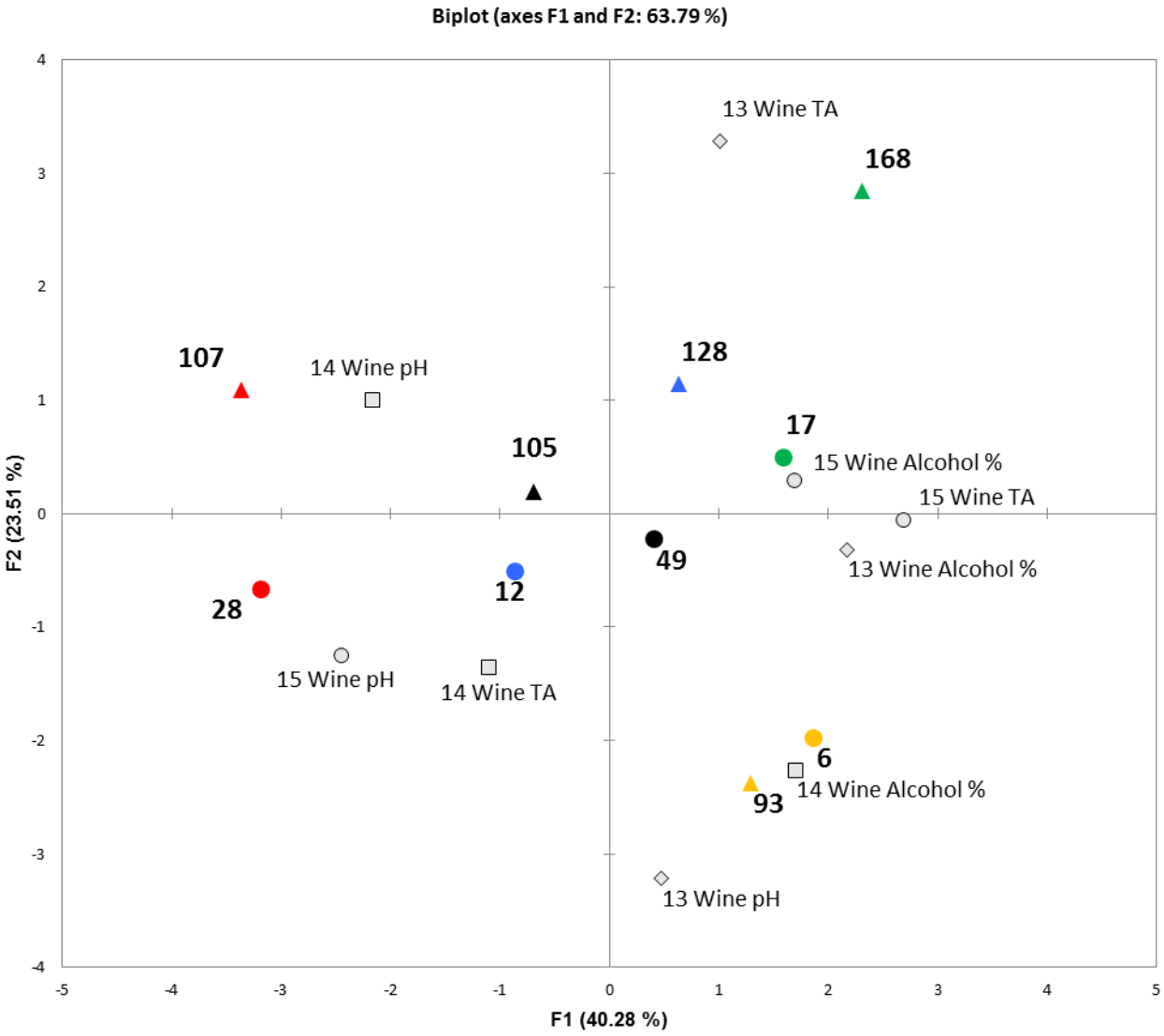
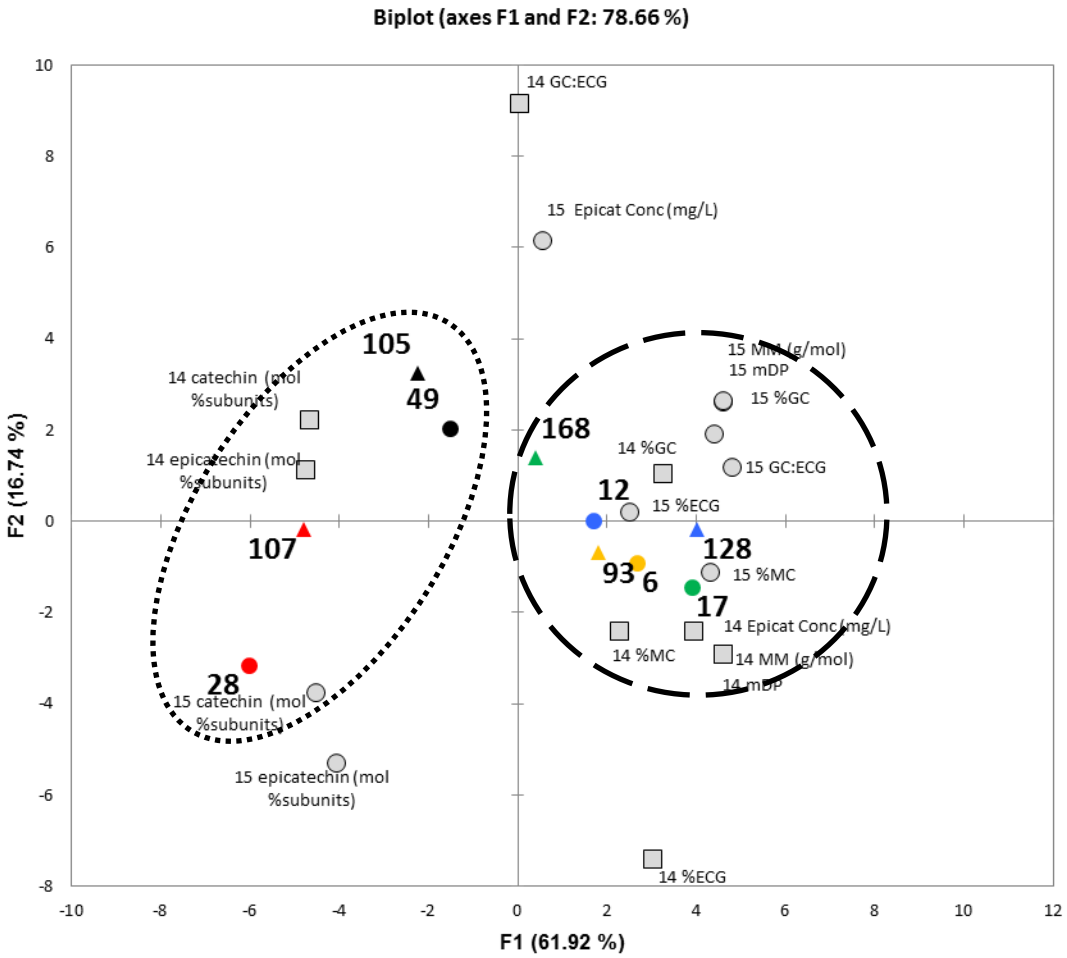


Figure 1. Combined PCA of mean values for basic chemical measures for wine over three seasons. 2013 (◊) 2014 (□) and 2015 (○). Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. *Labels marked are vine age in years.



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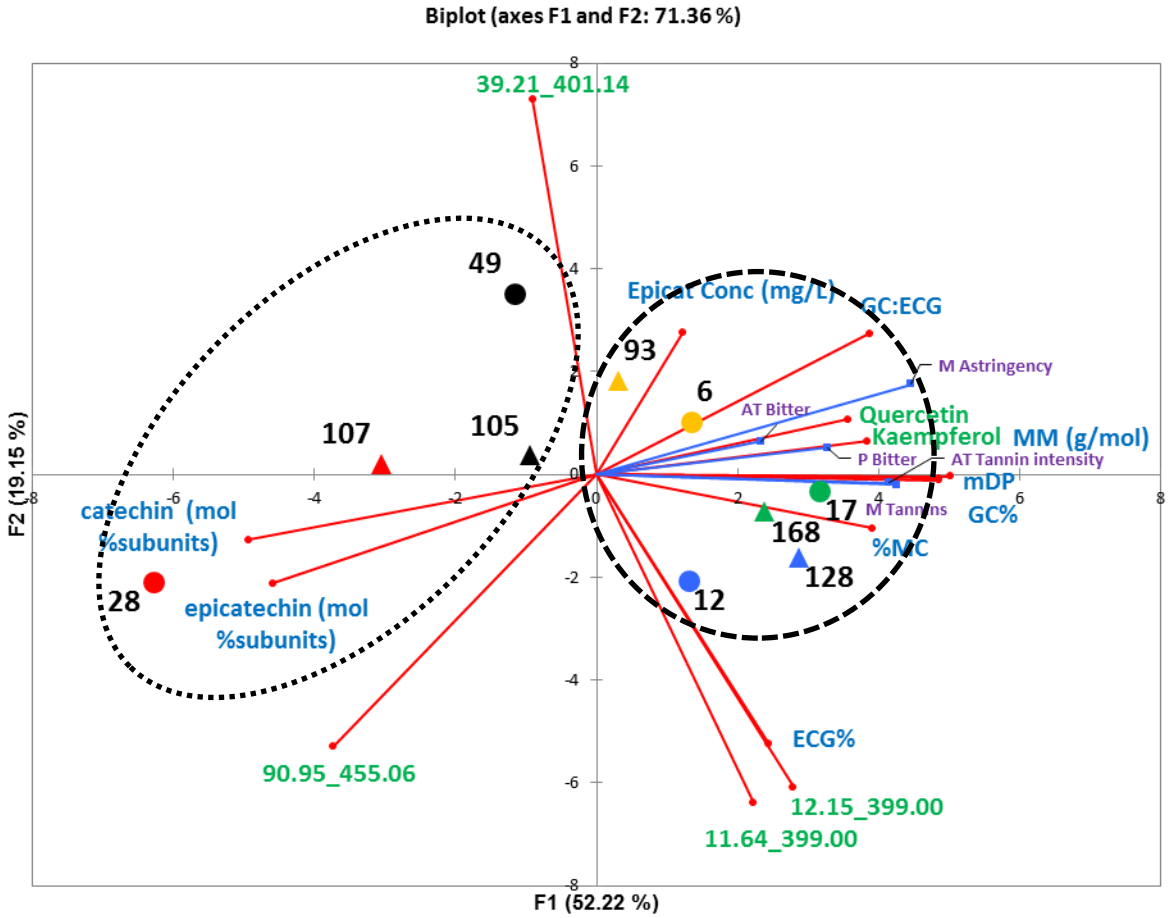

Figure 3. PCA biplot presenting combined intensity scores of LC-MS/MS data (green) and tannin compositional data (blue) from 2015 Shiraz wines overlaid with mean sensory DA results (purple) of mouthfeel attributes for the same wines. Wines are from five sites, two ages and two regions within the Barossa zone, Eden Valley (site 1 and 2) dotted elipse and Barossa Valley (sites 3,4,5) dashed circle. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲= old and ● = young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. Sensory prefix indicates modality: P = palate upon tasting, M = mouthfeel, AT = after taste following expectoration. Tannin abbreviations are; GC = epigallocatechin, MC = mass conversion, MM = molecular mass, mDP = mean degree of polymerisation, ECG = epicatechin gallate, Epicat Conc = Epicatechin concentration. The unknown molecular features labelled as their rt_mz pairs.

Supplementary material

Supplementary Tables

Table S1. Climatic data for Nuriootpa, Barossa Valley, South Australia. For growing seasons 2013 – 2015, October – April

Table S2. Grape composition of Barossa Valley Shiraz fruit at harvest in 2013, 2014 and 2015

Table S3. Vineyard and management details for each Barossa Valley vineyard for growing seasons 2012 – 2015, October - April.

Table S4. Wine composition of Barossa zone Shiraz harvested in 2013, 2014 and 2015 from five sites each with two age classes**.**

Table S5. Tannin composition of Barossa zone Shiraz wines for vintage years 2013/14 and 2014/15

Table S6. Mean sensory scores of mouthfeel related attributes following sensory DA of 2015 Shiraz wines from the Barossa zone. Attributes are divided by sensory modality: palate (P) mouthfeel (M) and after taste (AT)

Table S7. Eigenvectors resulting from PCA of wine compositional measures for finished Shiraz wines from five sites and three growing seasons harvested from vines of contrasting planted age.

Table S8. SPE and HPLC-MS conditions for non-targeted metabolomic analysis

Table S9 Sensory standards and attributes provided during tasting sessions or in prior training for wine sensory DA in classes of modality: aroma, flavour, mouthfeel and aftertaste.

Supplementary Figures

Figure S1. PCA Score and loadings plots for selected measures of grape at harvest (a) and wine at bottling (b) from Shiraz in the Barossa zone, South Australia over growing seasons 2012/13 2013/14 and 2014/15. Year of harvest is indicated and coloured in each plot.

Figure S2. Summary of seasonal climatic data for each season, approximate growth stages indicated.

Bureau of meteorology site 23373 in Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m.

Figure S3 Combined PCA of mean values for basic chemical maturity measures of grapes over three seasons. 2013 (◊) 2014 (□) and 2015 (○). Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5.

Table S1. Climatic data for Nuriootpa, Barossa Valley, South Australia. For growing seasons 2013 – 2015, October – April.

Growing season	Mean daily maximum	Mean daily minimum	E°days**	Rainfall (mm)	MJT (°C)	MFT (°C)
Season 1 2012-2013	27.0	12.2	1686	110	21.8	22.5
Season 2 2013-2014	26.0	11.5	1536	255	23.3	21.8
Season 3 2014-2015	26.0	11.3	1520	145	20.8	23.6
*Long term average**	*25.2*	*11.3*	*1545*	*203*	*21.5*	*21.4*

*Long term average from 1952- 2015, Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m (Bureau Of Meteorology 2015). **Calculated as per Gladstones (2011).

MJT = mean January temperature. MFT = mean February temperature.

Table S2. Grape composition of Barossa Valley Shiraz fruit at harvest in 2013, 2014 and 2015 from five sites each with two age classes.

	2013				2014				2015			
SITE 1	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	0.9	0.7	ns	ns	1.2	1.0	ns	ns	1.3	1.3	ns	ns
Harvest Brix	27.7	28.1	ns	ns	24.0	24.6	ns	ns	26.6	27.2	ns	ns
Harvest pH	3.59	3.60	ns	ns	3.90[b]	4.39[a]	0.017	0.47	4.01	4.08	ns	ns
Harvest TA (g/L)	6.7	6.3	ns	ns	6.8[a]	5.6[b]	0.011	0.80	4.2	3.8	ns	ns
Anthocyanins (mg/g)‡	1.65	1.82	ns	ns	1.44	1.74	ns	ns	1.46	1.61	ns	ns
Total phenolics (mg/g)§	1.75	1.73	ns	ns	1.48	1.56	ns	ns	1.36	1.43	ns	ns
Tannins (mg/g)†	3.9	4.1	ns	ns	6.9	6.5	ns	ns	5.9	6.3	ns	ns
SITE 2												
Berry mass (g)	0.8	0.9	ns	ns	1.2	1.3	ns	ns	1.4	1.6	ns	ns
Harvest Brix	24.4	26.9	ns	ns	23.5	23.3	ns	ns	24.1	24.7	ns	ns
Harvest pH	3.49	3.45	ns	ns	4.01	3.51	ns	ns	3.88	3.60	ns	ns
Harvest TA (g/L)	6.02	6.18	ns	ns	6.47[b]	7.55[a]	0.003	0.88	5.46[b]	9.33[a]	0.007	4.48
Anthocyanins (mg/g)‡	2.05	1.99	ns	ns	1.36	1.45	ns	ns	1.37	1.45	ns	ns
Total phenolics (mg/g)§	2.03	1.92	ns	ns	1.26	1.34	ns	ns	1.26	1.22	ns	ns
Tannins (mg/g)†	4.8	4.4	ns	ns	5.7	6.1	ns	ns	4.8	4.3	ns	ns
SITE 3												
Berry mass (g)	1.1[a]	0.9[b]	0.033	0.17	1.1	1.0	ns	ns	1.4	1.4	ns	ns
Harvest Brix	27.2	27.7	ns	ns	24.6	24.6	ns	ns	26.1	25.8	ns	ns
Harvest pH	3.56	3.687	ns	ns	4.02	4.13	ns	ns	3.81	3.87	ns	ns
Harvest TA (g/L)	6.1[a]	5.0[b]	0.025	1.52	5.67	5.48	ns	ns	4.41	4.55	ns	ns
Anthocyanins (mg/g)‡	1.95	1.99	ns	ns	1.96	1.95	ns	ns	1.83	1.90	ns	ns
Total phenolics (mg/g)§	1.83	1.78	ns	ns	1.64	1.66	ns	ns	1.44	1.49	ns	ns
Tannins (mg/g)†	3.9	4.0	ns	ns	6.8	7.5	ns	ns	5.9	5.5	ns	ns
SITE 4												
Berry mass (g)	0.7	0.8	ns	ns	0.9[a]	0.7[b]	0.016	0.21	1.0	1.1	ns	ns
Harvest Brix	28.6	27.6	ns	ns	24.0[b]	25.3[a]	0.001	0.75	25.5[b]	27.6[a]	0.002	1.50
Harvest pH	3.35[b]	3.61[a]	0.002	0.15	3.98	3.90	ns	ns	3.56[b]	3.83[a]	0.003	0.20
Harvest TA (g/L)	7.75[a]	4.99[b]	0.000	0.35	5.67	5.23	ns	ns	4.86[a]	3.91[b]	0.001	0.43
Anthocyanins (mg/g)‡	2.42	2.25	ns	ns	1.77	1.92	ns	ns	1.96	1.76	ns	ns
Total phenolics (mg/g)§	2.49	2.04	ns	ns	1.71	1.82	ns	ns	1.47[a]	1.25[b]	0.016	0.13
Tannins (mg/g)†	3.3	3.2	ns	ns	5.4	4.5	ns	ns	6.1	6.5	ns	ns
SITE 5												
Berry mass (g)	1.2	1.1	ns	ns	1.2	1.2	ns	ns	1.4	1.3	ns	ns
Harvest Brix	27.3	27.5	ns	ns	25.7	25.1	ns	ns	24.0	25.0	ns	ns
Harvest pH	3.70	3.73	ns	ns	4.23	4.11	ns	ns	3.88	3.75	ns	ns
Harvest TA (g/L)	5.24	5.03	ns	ns	4.75	5.18	ns	ns	4.79[a]	4.38[b]	0.034	0.75
Anthocyanins (mg/g)‡	1.84	1.92	ns	ns	1.41	1.58	ns	ns	1.13	1.45	ns	ns
Total phenolics (mg/g)§	1.76	1.83	ns	ns	1.40	1.38	ns	ns	1.25	1.49	ns	ns
Tannins (mg/g)†	4.4	3.8	ns	ns	6.4	6.0	ns	ns	6.2	6.2	ns	ns

† Tannins determined by the MCP tannin assay (Mercurio et al. 2007) expressed as milligrams of epicatechin equivalents per gram fresh berry mass.

‡Anthocyanin content determined spectrophotometrically at 520nm (Iland at al. 2004), expressed as malvidin equivalent units.

§Phenolic substances determined from absorbance at 280nm (Iland et al. 2004), expressed as absorbance units (au) per berry or per gram of fresh berry mass.

Table S3. Vineyard and management details for each Barossa Valley vineyard for growing seasons 2012 – 2015, October - April.

Site	Age	Pruning	Cane or cordon #	Cordon height (m)	Trellis configuration	Management		Soil group-Sub group†	Average Irrigation volume‡
						Floor	Undervine		
Site 1	Old	Cane	2-3	1.0	Single cordon wire	Cultivated	Mulch	B-B4	0.4ML/ha
	Young	Cane	2-3	1.1,1.3	Two wires 200mm apart	Cultivated	Mulch	B-B4	" "
Site 2	Old	Cane	2-3	1.0	Single wire	Sward	Mulch	D-D2	0.3ML/ha
	Young	Cane	2	1.0	Single wire pus four catch wires	Sward	Mulch	D-D2	" "
Site 3	Old	Spur bilateral cordon	2	1.2	Single wire + roll wire 250mm above	Cultivated	Cultivated	G-G3	0.48ML/ha
	Young	Spur bilateral cordon	2	1.2	Single wire + roll wire 250mm above	Cultivated	Cultivated	G-G3	" "
Site 4	Old	Cane	2-4	1.0,1.3	Two wires	Sward	Cultivated	D-D2	**
	Young	Cane	2-4	1.1,1.4	Two wires	Sward	Cultivated	D-D2	0.2ML/ha
Site 5	Old	Cane	2	1.0	Single wire	Sward	Herbicide	D-D2	0.2ML/ha
	Young	Cane	2	1.0	Single wire	Sward	Herbicide	D-D2	" "

†Australian soil classification subgroup codes [101-103] represent:
B-Shallow soil on calcrete or limestone
B4-Shallow red loam on limestone
D-Hard red-brown texture-contrast soils with alkaline subsoil
D2-Loam over red clay
G-Sand over clay soils
G3-Thick sand over clay
‡October – April inclusive
**Only young vines at site 4 irrigated.

Table S4. Wine composition of Barossa zone Shiraz harvested in 2013, 2014 and 2015 from five sites each with two age classes**.**

	2013				2014				2015			
Site 1	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Wine Alcohol %	16.15	17.17	ns	ns	13.91	14.19	ns	ns	16.27	17.22	ns	ns
Wine pH	3.58	3.66	ns	ns	3.41[b]	3.52[a]	0.044	0.11	3.79	3.80	ns	ns
Wine TA	7.04	7.12	ns	ns	11.70	11.60	ns	ns	6.58	7.05	ns	ns
Hue†	0.70	0.73	ns	ns	0.65	0.66	ns	ns	0.62[b]	0.64[a]	0.009	0.01
Colour density (au) †	14.44	17.66	ns	ns	10.08	10.15	ns	ns	9.52[b]	14.05[a]	0.018	3.273
Phenolics (au)†	43.5[b]	54.5[a]	0.03	9.55	36.9[b]	44.8[a]	0.004	3.67	43.2	51.8	ns	ns
Anthocyanins (mg/L)†	311[b]	379[a]	0.00	33	367[b]	436[a]	0.012	44	561	585	ns	ns
Tannins (mg/L) ‡	1861	2278	ns	ns	737	921	ns	ns	1493	1602	ns	ns
Site 2												
Wine Alcohol %	14.35[b]	16.25[a]	0.04	1.69	13.69	13.59	ns	ns	13.59	14.14	ns	ns
Wine pH	3.50[b]	3.69[a]	0.01	0.13	3.74[b]	4.06[a]	0.001	0.10	3.86	3.95	ns	ns
Wine TA	7.03	6.67	ns	ns	10.4[a]	9.81[b]	0.002	0.24	6.22	6.17	ns	ns
Colour density (au) †	12.04	12.86	ns	ns	7.55	8.09	ns	ns	6.69	7.29	ns	ns
Hue†	0.70[b]	0.77[a]	0.02	0.05	0.68	0.67	ns	ns	0.68	0.68	ns	ns
Phenolics (au)†	43.3	48.6	ns	ns	35.5	36.5	ns	ns	32.7	35.2	ns	ns
Anthocyanins (mg/L)†	273	286	ns	ns	348[b]	447[a]	0.002	37	412[b]	500[a]	0.035	77
Tannins (mg/L) ‡	1704	1868	ns	ns	638	608	ns	ns	723	774	ns	ns
Site 3												
Wine Alcohol %	16.23	16.55	ns	ns	14.37[a]	14.05[b]	0.049	0.32	16.23	15.89	ns	ns
Wine pH	3.64	3.79	ns	ns	3.49[b]	3.70[a]	0.006	0.11	3.73[b]	3.92[a]	0.009	0.11
Wine TA	7.44	7.02	ns	ns	7.41[a]	6.52[b]	0.003	0.39	7.02	6.37	ns	ns
Colour density (au) †	16.36	16.88	ns	ns	12.91	12.98	ns	ns	12.28	11.71	ns	ns
Hue†	0.67	0.70	ns	ns	0.61	0.63	ns	ns	0.60	0.62	ns	ns
Phenolics (au)†	51.7	49.8	ns	ns	50.8	51.4	ns	ns	50.2	48.9	ns	ns
Anthocyanins (mg/L)†	399	387	ns	ns	555[b]	585[a]	0.047	29	630	649	ns	ns
Tannins (mg/L) ‡	2228	2359	ns	ns	1140	1278	ns	ns	1554	1478	ns	ns
Site 4												
Wine Alcohol %	17.51[a]	16.50[b]	0.02	0.71	13.53[b]	15.61[a]	0.001	0.60	15.40[b]	16.90[a]	0.002	0.60
Wine pH	3.51[b]	3.61[a]	0.04	0.10	3.50	3.63	ns	ns	3.41[b]	3.66[a]	0.000	0.07
Wine TA	7.47	7.24	ns	ns	6.74	7.20	ns	ns	8.04[a]	7.48[b]	0.002	0.23
Colour density (au) †	14.99[b]	17.82[a]	0.005	1.454	12.36	13.25	ns	ns	11.37	11.49	ns	ns
Hue†	0.73	0.70	0.00	0.01	0.67	0.68	ns	ns	0.58[b]	0.65[a]	0.006	0.04
Phenolics (au)†	50.5	48.1	ns	ns	44.3[b]	55.9[a]	0.008	6.46	46.8	44.5	ns	ns
Anthocyanins (mg/L)†	333	361	ns	ns	395[b]	507[a]	0.008	64	642[a]	500[b]	0.002	55
Tannins (mg/L) ‡	2029[a]	2284[b]	0.04	236	1148[b]	1757[a]	0.021	456	1254	1501	ns	ns
Site 5												
Wine Alcohol %	16.88	16.61	ns	ns	15.65	15.31	ns	ns	14.92	15.38	ns	ns
Wine pH	3.77	3.77	ns	ns	3.24	3.27	ns	ns	3.67	3.63	ns	ns
Wine TA	6.74	6.68	ns	ns	10.30	8.76	ns	ns	7.42[b]	8.16[a]	0.002	0.29
Colour density (au) †	15.16	14.63	ns	ns	15.47	14.21	ns	ns	7.53	8.67	ns	ns
Hue†	0.73	0.72	ns	ns	0.65	0.67	ns	ns	0.65	0.68	ns	ns
Phenolics (au)†	48.4	47.3	ns	ns	51.6	49.3	ns	ns	36.9	39.2	ns	ns
Anthocyanins (mg/L)†	346	332	ns	ns	383	378	ns	ns	394	376	ns	ns
Tannins (mg/L) ‡	2207	2014	ns	ns	2007	1885	ns	ns	1375	1437	ns	ns

† Determined by the modified Somers assay (Mercurio et al. 2007) expressed as absorbance units (au).

‡Determined by the MCP tannin assay (Mercurio et al. 2007) expressed as milligrams of epicatechin units per litre.

Table S5. Tannin composition of Barossa zone Shiraz wines for vintage years 2013/14 and 2014/15.

	2014				2015			
Site 1	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
MM (g/mol) †	1677[b]	2031[a]	0.014	234	2603	2498	ns	ns
mDP‡	5.65[b]	6.83[a]	0.014	0.779	8.69	8.34	ns	ns
GC (%)§	30.49	31.29	ns	ns	28.29	30.33	ns	ns
ECG (%)¶	1.10	1.28	ns	ns	3.05	2.76	ns	ns
GC:ECG††	28.01	24.48	ns	ns	9.30	11.09	ns	ns
%MC‡‡	21.28	26.45	ns	ns	22.32	16.45	ns	ns
catechin (mol %subunits)	13.94[b]	10.76[a]	0.005	0.016	8.75	8.88	ns	ns
epicatechin (mol %subunits)	3.81	3.90	ns	ns	2.60	2.90	ns	ns
Site 2								
MM (g/mol) †	1555	1662	ns	ns	1708[a]	1370[b]	0.050	333
mDP‡	5.24	5.59	ns	ns	5.72[a]	4.59[b]	0.049	1.122
GC (%)§	24.53	24.50	ns	ns	26.62[b]	23.19[a]	0.036	0.030
ECG (%)¶	1.45[b]	1.87[a]	0.04	0.004	2.63	2.98	ns	ns
GC:ECG††	17.12[a]	13.11[b]	0.044	3.841	10.27	7.85	ns	ns
%MC‡‡	28.47	25.77	ns	ns	19.55	15.20	ns	ns
catechin (mol %subunits)	14.67	13.72	ns	ns	13.04[b]	15.89[a]	0.032	0.024
epicatechin (mol %subunits)	4.43	4.45	ns	ns	4.33	5.64	ns	ns
Site 3								
MM (g/mol) †	3012[a]	2508[b]	0.009	293	3365	2954	ns	ns
mDP‡	10.06[a]	8.39[b]	0.009	0.983	11.18	9.83	ns	ns
GC (%)§	36.79[a]	35.13[b]	0.048	0.016	34.78	33.26	ns	ns
ECG (%)¶	2.07[a]	1.94[b]	0.011	0.000	3.35	3.31	ns	ns
GC:ECG††	17.76	18.13	ns	ns	10.38	10.08	ns	ns
%MC‡‡	37.85[a]	27.22[b]	0.009	0.061	32.81	28.35	ns	ns
catechin (mol %subunits)	7.44[a]	8.57[b]	0.034	0.010	6.49	7.35	ns	ns
epicatechin (mol %subunits)	2.52[b]	3.35[a]	0.001	0.003	2.31	2.75	ns	ns
Site 4								
MM (g/mol) †	2134[b]	3168[a]	0.005	513	2620[b]	3209[a]	0.024	463
mDP‡	7.15[b]	10.58[a]	0.005	1.705	8.71[b]	10.68[a]	0.022	1.503
GC (%)§	35.40	36.76	ns	ns	36.95	35.42	ns	ns
ECG (%)¶	1.61[b]	2.13[a]	0.011	0.003	3.10	2.88	ns	ns
GC:ECG††	22.08	17.33	ns	ns	11.94	13.49	ns	ns
%MC‡‡	28.34	31.27	ns	ns	22.36	28.31	ns	ns
catechin (mol %subunits)	10.14[a]	6.79[b]	0.003	0.014	8.24[b]	6.58[a]	0.013	0.011
epicatechin (mol %subunits)	3.89[b]	2.71[b]	0.027	0.010	3.05	2.54	ns	ns
Site 5								
MM (g/mol) †	2865[b]	3238[a]	0.050	372	2511[b]	2754[a]	0.008	140
mDP‡	9.59	10.83	ns	ns	8.38[b]	9.18[a]	0.009	0.474
GC (%)§	34.20[b]	36.81[a]	0.018	0.019	32.37	32.50	ns	ns
ECG (%)¶	1.94	1.95	ns	ns	2.84[b]	3.10[a]	0.030	0.002
GC:ECG††	17.65	18.87	ns	ns	11.40[a]	10.49[b]	0.050	0.911
%MC‡‡	23.82	25.49	ns	ns	32.84	28.32	ns	ns
catechin (mol %subunits)	7.36[b]	6.34[a]	0.029	0.009	8.73[a]	7.74[b]	0.002	0.004
epicatechin (mol %subunits)	3.10	2.91	ns	ns	3.03	2.96	ns	ns

† MM molecular mass by phloroglucinolysis method, based on measured subunits and their proportional molar contribution.

‡ mDP mean degree of tannin polymerisation, based on ratio of measured extension to terminal subunits.

§ percentage of epigallocatechin as extension subunit of total subunits by molar proportion.

¶ percentage epicatechin gallate of total subunits by molar proportion.

†† ratio of epigallocatechin subunits to epicatechin subunits.

‡‡ percentage of tannin converted to subunits, based on total tannin concentration measured by MCP tannin assay.

Table S6. Mean sensory scores of mouthfeel related attributes following sensory DA of 2015 Shiraz wines from the Barossa zone. Attributes are divided by sensory modality: palate (P) mouthfeel (M) and after taste (AT).

Site	AT Bitter	AT Tannin intensity	P Bitter	M Astringency	M Tannins
Site 1 Old	4.7	6.7	4.1	7.0	5.4
Site 1 Young	5.8	7.4	4.9	8.2	6.8
Site 2 Old	3.7	4.9	3.8	5.3	5.1
Site 2 Young	3.8	5.2	2.9	4.9	5.1
Site 3 Old	5.5	9.2	5.5	9.6	8.7
Site 3 Young	5.3	7.5	4.6	7.9	7.1
Site 4 Old	4.1	6.8	4.3	7.2	6.9
Site 4 Young	5.1	8.1	4.2	8.8	7.9
Site 5 Old	3.5	6.1	3.4	7.8	6.4
Site 5 Young	4.5	7.9	4.1	8.7	8.4

Table S7. Eigenvectors resulting from PCA of wine compositional measures for finished Shiraz wines from five sites and three growing seasons harvested from vines of contrasting planted age.

Eigenvectors	F1	F2	F3
13 Wine Alcohol %	0.390	-0.057	-0.340
13 Wine pH	0.084	-0.580	-0.410
13 Wine TA	0.181	0.592	-0.162
14 Wine Alcohol %	0.306	-0.407	0.078
14 Wine pH	-0.391	0.180	-0.373
14 Wine TA	-0.199	-0.244	0.447
15 Wine Alcohol %	0.304	0.053	-0.438
15 Wine pH	-0.441	-0.224	-0.324
15 Wine TA	0.484	-0.010	0.220

Table S8. SPE and HPLC-MS conditions for non-targeted metabolomic analysis.

SPE conditions	
Cartridge conditioning	1mL methanol followed by 1mL MilliQ water
Sample loading	10 mL (2 mL of sample + 8 mL Milli-Q water)
(Sample was resuspended – 0.100 mL sample + 9.9mL water)	
Washing	1mL 2% methanol
Dried at full vacuum	5min
Elution solvent	1mL methanol
Evaporated methanol to dryness using the TurboVap	
Redissolved in 30uL Solvent B followed by 20uL of Solvent A	
HPLC conditions	
Injection volume	1μL
Flow rate	0.22mL/min
Solvent A	2% formic acid, 0.5% methanol in miliQ-water
Solvent B	2% formic acid, 2% Milli-Q water, 40% acetonitrile in methanol
Column	Phenomenex Kinetex PFP 2.7um 2.1 x 150mm
Column temperature	30°C
DAD acquisition range	200-600 nm

HPLC timetable		
Time (min)	Flow Rate	% Solvent B
0	0.22 ml/min	0
25		1
100		10
130		20
140		35
152		90
155		90
155.5		0
156		90
156.5		0
157		90
157.5		0
167		0

MS conditions ESI Negative	
Source temperature	200 °C
Gas flow	7 L/min
Nebuliser pressure	2 Bar
Capillary Voltage	+ 2500 V
End Plate Offset	- 500 V
Scan type	Scan from 50 to 1650 m/z
Acquisition rate	2.0 Hz
Calibration Solution	5mM sodium hydroxide, 0.2% formic acid in 50% 2-propanol

Table S9. Sensory standards and attributes provided during tasting sessions or in prior training for wine sensory DA in classes of modality: aroma, flavour, mouthfeel and aftertaste.

	Season	**Reference standards if supplied or training solution.**
Class	**2014/15**	
A	Red fruit	Raspberry, red cherry, strawberry
A	Dark fruit	Black-berry -currant, satsuma plum
A	Dried fruit	Dried pitted prune, fig, date
A	Jammy character	Mixture of red and dark fruit jams
A	Herbaceous	Fresh cut grass
A	Fruit character	**Redskin™ candy
A	Alcohol	No standard
	Complexity	No standard
F	Dark fruit	as above
F	Fresh Fruit Character	combination of previous
F	Savoury	Black olives, beef jerky
F	Green - Herbaceous	as above
F	Floral	Several fresh violet flowers
F	Confectionary	Redskin™ candy
F	Mocha - Chocolate	Generic mocha drink powder
T	Acid	Training : Tartaric acid solution
T	Bitter	Training : Quinine sulphate solutions
T	Salty	Training : Sodium chloride solutions
M	Alcohol	No standard
M	Body	No standard
M	Astringency	Feeling of mouth surfaces adhering or sticking together*
M	Tannins	Training via tannin solutions
AT	Length	Overall lingering flavour sensation
AT	Alcohol	Heat and warmth after expectorating
AT	Tannin intensity	Intensity and length of tannin sensation
AT	Bitter	Bitterness after expectorating

*Physical touch standards provided: silk, velvet, fine sandpaper and coarse sandpaper

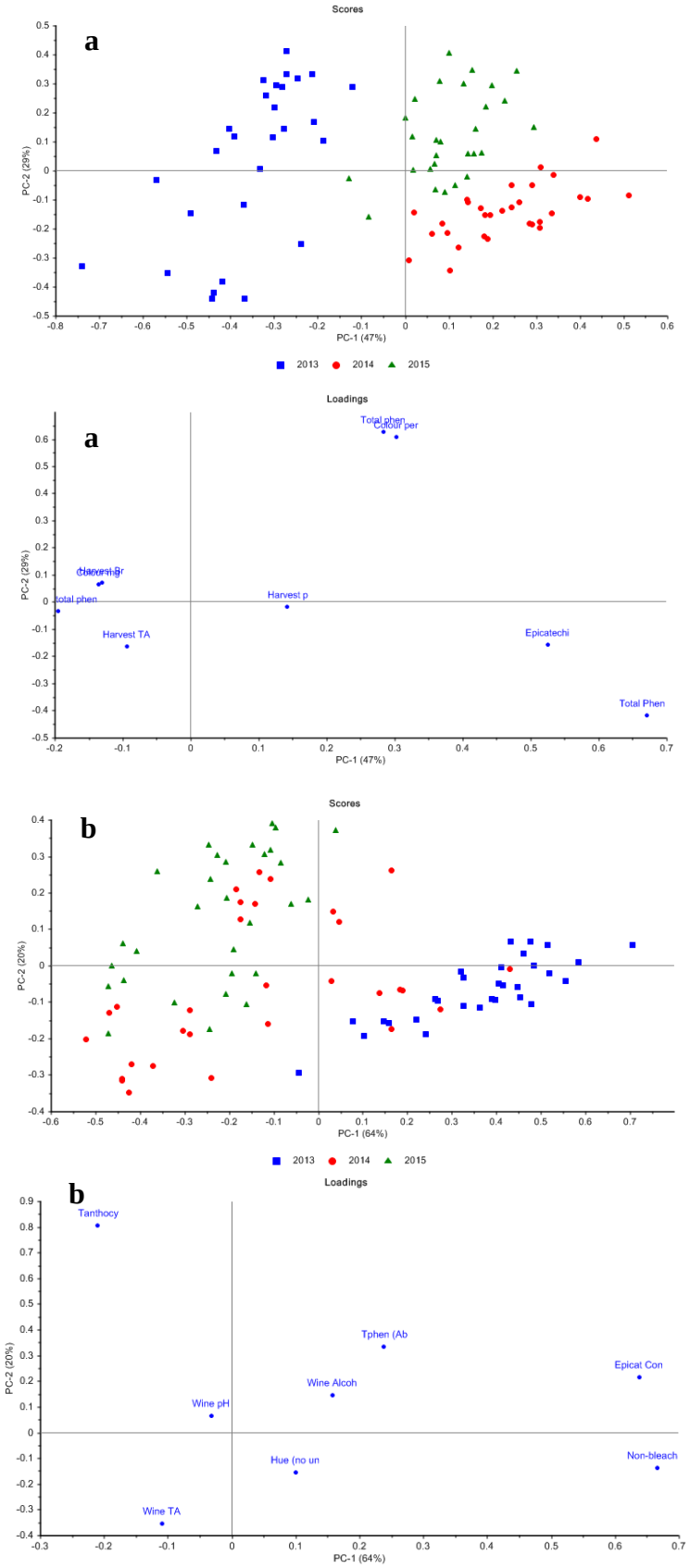

Figure S1. PCA Score and loadings plots for selected measures of grape at harvest (a) and wine at bottling (b) from Shiraz in the Barossa zone, South Australia over growing seasons 2012/13 2013/14 and 2014/15. Year of harvest is indicated and coloured in each plot.

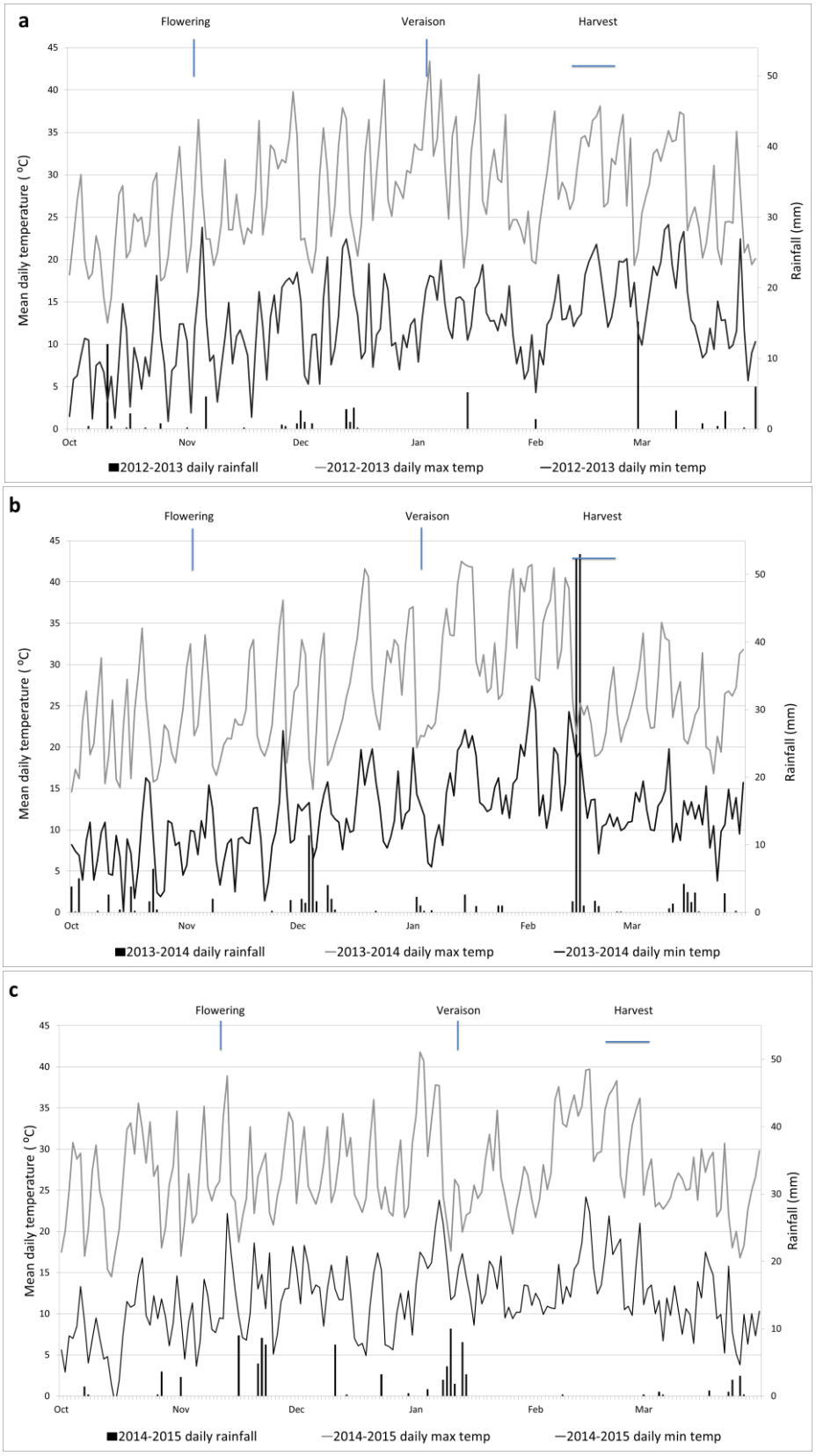

Figure S2. Summary of seasonal climatic data for each season, approximate growth stages indicated. Bureau of meteorology site 23373 in Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m.

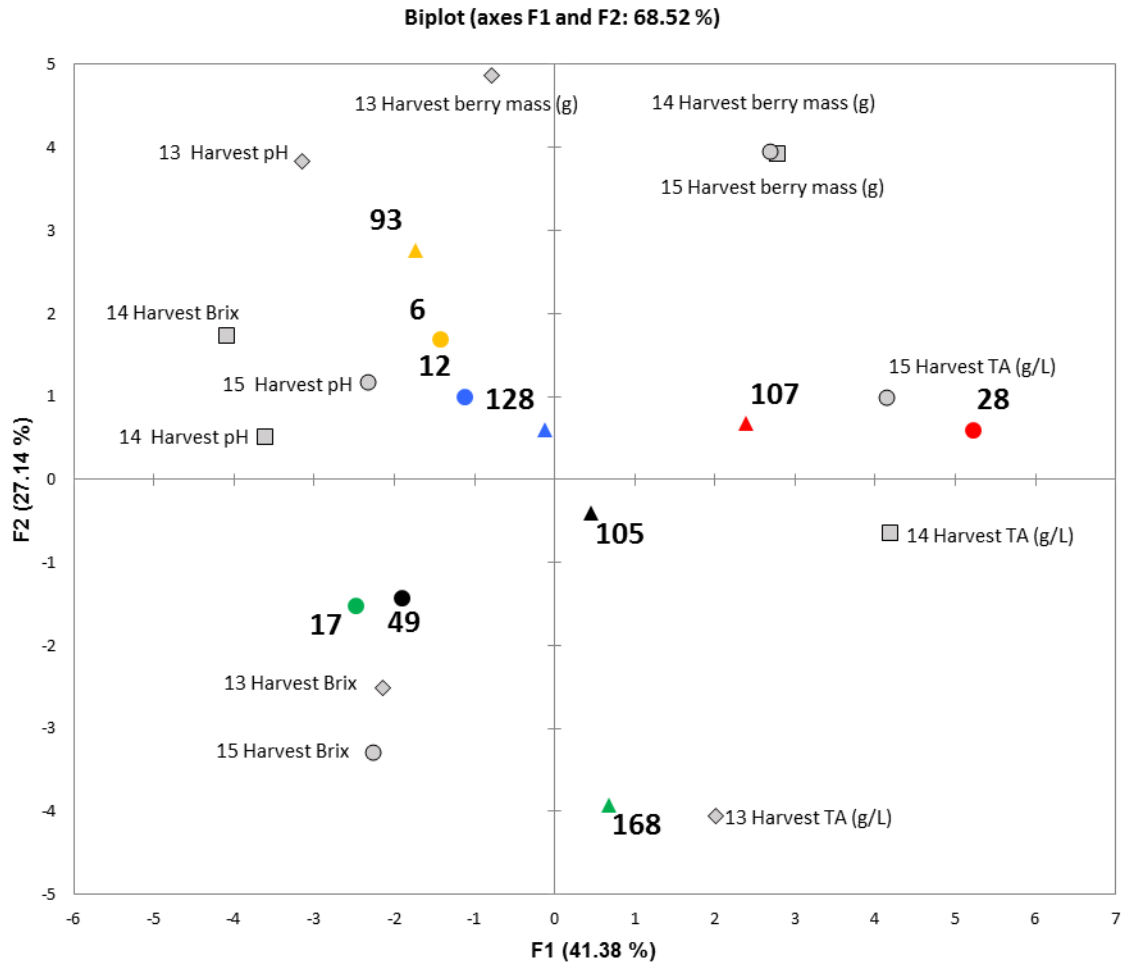

Figure S3. Combined PCA of mean values for basic chemical maturity measures of grapes over three seasons. 2013 (◊) 2014 (□) and 2015 (○). Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5.

Chapter 5. Prepared Manuscript 3: The effect of vine age on the sensory properties of grapes and wine volatile metabolites in Shiraz.

Statement of Authorship

Title of Paper	The effect of vine age on the sensory properties of grapes, wine and volatile metabolites in Shiraz.
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Principal Author

Name of Principal Author (Candidate)	Dylan Grigg
Contribution to the Paper	Designed and conducted the research experiments, analysed the data and drafted and constructed the manuscript.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 24/05/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The effect of vine age on the sensory properties of grapes, wine and volatile metabolites in Shiraz

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Abstract

A well-entrenched belief worldwide is that older vines produce wines of greater quality than younger vines. To date, this is largely un-tested scientifically. This study investigated the effect of vine age on chemical and sensory properties of fruit and wine using some of the worlds oldest vineyards in the Barossa Valley, Australia. Five commercial Shiraz vineyards were selected and contained vines planted at two distinct times (‘old’ or ‘young’; average difference was more than 97 years). At each site, vines of both age groups are planted in close proximity with similar conditions and management. Small batch wines were made from fruit harvested from old and young vines. Assessment of the sensory aspects associated with vine age found minor differences in fresh grapes. Differences were mainly related to berry acidity perception, despite sugar levels being similar. Wine sensory descriptive analysis consistently revealed differences in aroma and flavour attributes. Older vines were perceived to have greater flavour and aroma particularly fresh red fruit, floral or confectionary characters. Wines from younger vines were higher in perceived alcohol sensation with a darker fruit profile. Analysis of volatile compounds confirmed differences observed via sensory descriptive analysis and the differences appear to be related to vine age.

Keywords: *sensory analysis, wine, grapes, Shiraz, volatiles, vine age*

Introduction

Grapevines *Vitis vinifera. L.* are perennial plants that can yield fruit for decades and even centuries. Frequently, across a broad range of media (industry journals, blogs, wine writers etc.) and in the scientific community greater vine age is suggested to produce wines of better quality [1-6]. Despite the frequency of this suggestion, few scientific studies have empirically investigated the influence of vine age on grape or wine quality.

The analysis of the effect of vine age on fruit and wine sensory attributes has yielded a wide range of results. Zufferey and Maigre [7] reported that Syrah (syn. Shiraz) wines from 34 year old vines had greater bouquet and greater general expression of quality and finesse than wines from 5 year old vines. Heymann and Noble [8] reported that sensory characters of Cabernet Sauvignon wines from older vines (20 years) had greater berry aroma and fruit flavour than younger vine's (5 years) wines which were perceived as more vegetative and green. Reynolds, *et al.* [3] explored the effect of vine age and vintage between vines aged 4 and 14 years with mixed results; young vines were judged as more vegetal in one season but not in the other.

The term quality in relation to both grapes and wine is complex; the search for objective measures and indicators of quality is both broad and ongoing [9-11]. Often, basic chemical measures are used to measure grape and wine quality, which include: pH, acidity, sugar and anthocyanin and tannin composition. These objective measures are often aligned with sensory studies to find correlations or indicators that might assist the identification of quality. One method used in sensory studies is descriptive analysis (DA) which uses a trained panel to assess attributes within presented samples (i.e. grapes or wine) [12]. When applied to fresh grapes this is known as berry sensory assessment (BSA). BSA has been demonstrated practically for use in industry [13] and also in a range of research applications, including: canopy manipulation, water stress and elevated temperature [14-17].

Wine sensory DA, like BSA, is a method for measuring physical responses to products via several sensory modalities including taste, smell and touch [18]. There are numerous examples of sensory DA which have been applied to wine research, such as profiling cultivars [19-23], to broader questions such as to define the terroir of a region [24] or viticultural manipulation such as sunlight exclusion [25]. The sensory profile of wine is a complex mixture of volatile and non-volatile compounds derived from numerous sources during the winemaking process [26, 27].

Studies have identified a large number of volatile compounds present in wine via the combination of gas chromatography and mass spectroscopy (GC-MS). The detection of compounds via GC-MS have been found to be indicative or specific for a wide range of variables such as: cultivar [28], season [29] or terroir [30]. The use of GC-MS has been widely implemented in wine aroma studies [22, 31-33] providing both quantification and identification of analytes making it ideal to validate DA and uncover unique profiles that may be associated with vine age.

The combination of sensory and chemical compositional data can enhance our understanding of the drivers of wine quality [22, 23]. Data from sensory studies is often combined with multivariate statistical techniques such as principal component analysis (PCA) to determine relationships [20, 34-36] that may be otherwise hidden [37]. Use of PCA to make statistical evaluations of sensory data is well established and widespread, including comparisons such as soil texture and vine size [24], sodium chloride concentration [38] and volatile composition of grapes and wine [11, 29, 36].

The main objective of this study was to investigate if vine age was associated with differences in grape and wine sensory analysis. In addition to sensory assessments, the volatile composition of wines produced was also examined in the final season to further validate the findings. The aim of this study was to determine the sensory outcome and volatile composition relating to vine age. Further viticultural and compositional data is presented in other manuscripts. Here we report a comparison of a single cultivar, Shiraz, across five sites, with age differences ranging from 56 to 151 years between old and young vines.

Materials and methods

Sites, Experimental Design and Plant Material.

Five commercial Shiraz (*Vitis vinifera* L.) vineyards were selected in 2012 within the Barossa wine zone of South Australia (Table S1). General climatic statistics for the region are presented in Table S2. Specific climatic observations for the growing seasons 2012/13, 2013/14 and 2014/15 are presented in Supplementary Figure S1. The greater Barossa wine zone is divided into subregions: i) the Barossa Valley where sites 3, 4 and 5 are found, which is generally considered drier and warmer than ii) the Eden Valley region which contains sites 1 and 2. Sites were chosen with the assistance of local industry members based on health of vines of two different ages in each vineyard. Vine health is defined as vines that are free of visible symptoms and still productive. Consideration was given to relative homogeneity of site and vine

characteristics between vine age groups within each site such as soil type, row orientation, pruning, trellis system and vineyard floor management. Each site has had vines established at different times, older vines aged 93, 105, 107, 128 and 168 years and comparatively younger vines aged 5, 12, 17, 28 and 49 years (at the inception of the experimental period). Younger vines at each site were vegetatively propagated via hardwood cuttings taken from older vines with the exception of site 5. Site 5 was propagated via layering and severed from the mother vine after five years. All vines are growing on own roots. Clonal status of vine material is unknown, therefore all vines propagated via cuttings are also to be considered quasi clones. At each vineyard, three replicates containing 4 or 5 vines in adjacent rows were selected resulting in 12-15 vines per age treatment. Due to propagation technique at site 5, old and young vines are interspersed resulting in two adjacent rows of vines being selected and grouped to form treatment replicates. No interference was made with respect to vine management at any time, which was left to the respective site managers.

Winemaking

Fruit was harvested by hand from each replicate into 20 kg vented crates and transported to the University of Adelaide, South Australia, Waite Campus for processing. Field replicate identity was maintained during the winemaking process. Fruit was stored overnight in a 4°C cool room to allow uniformity of inoculation conditions. Fruit was crushed and de-stemmed using a small combination crusher de-stemmer (Grifo Machine Enologiche, Piadena. Italy) into 20 L plastic fermentation vessels and allowed to reach room temperature (22°C). At the crusher 50 ppm of potassium metabisulphite was added to each replicate.

Post crushing, ferments were inoculated with 200 ppm of commercial dried yeast (AWRI 796, Laffort, Bordeaux, France) and 200 ppm of nutrient (Dynastart, Laffort, Bordeaux, France). Following an initial drop of 2° Baume 200 ppm diammonium phosphate (DAP) was added along with malo-lactic bacteria culture (Lalvin VP41, Lallemand, France) at 0.2 ppm to complete primary and secondary fermentation simultaneously.

Must was fermented on skins under controlled conditions at 22°C, plunged twice per day with a stainless steel plunger to break and wet the cap only. Baume (measures using a Hydrometer) and temperature were monitored and recorded daily. When Baume was ≤2° or after 5 days of skin contact wines were pressed off using a 20 L hydraulic water bag press (300 KPa max). Pressed wine was transferred into 10 L glass demijohns to complete malo-lactic fermentation (MLF). MLF was monitored approximately weekly using an L-Malic acid enzymatic test kit

(Vintessential, Dromana, Australia). Following completion of MLF (≤0.05 g/L malic acid) wines were sulphured to a free level of 30 ppm. Wines were bottled directly, into 750 ml glass claret bottles and sealed under screwcap. The wines were then stored at a constant 15°C for future wine sensory and chemical evaluations.

Sensory Evaluation

All participants were actively involved in the wine industry at the time of analysis and gave their informed consent for inclusion before commencement. The study was conducted in accordance with local laws and regulation and was approved by the Ethics Committee of the University of Adelaide Office of Research Ethics, Compliance and Integrity: H-2017-054.

Both wine and berry sensory analyses were undertaken using DA techniques at the University of Adelaide's Waite campus using dedicated sensory facilities. Sensory evaluation of both grapes and wine was divided into two stages: initial training was undertaken in an open plan room to facilitate discussion and agreement on descriptors, while formal assessments were undertaken in individual booths under fluorescent light. In each booth a citrus pectin solution (2 g/L, Sigma-Aldrich Co,. St Louis, MO) and plain water crackers were made available to panellists to alleviate palate fatigue as described by Mantilla, *et al.* [39].

Berry Sensory Descriptive Analysis

A random sample of 100 berries was selected from bunches prior to crushing for winemaking. Berries were carefully cut from bunches to retain the pedicel-berry connection. Three berries were placed into labelled bags in triplicate and stored at -20^{o}C in preparation for sensory analysis. Prior to sensory analysis, grape samples were placed into labelled individual containers to defrost at room temperature for 20 minutes.

Training: assessors were selected for the BSA panel based on their experience in sensory evaluation, availability and familiarity with general grape and wine assessment principles. Each panel consisted of assessors twelve, six or eight assessors in each season 2012/13, 2013/14 and 2014/15, respectively. Assessor training was undertaken as described by Mantilla, *et al.* [39] to determine an assessor's familiarity with basic taste sensations such as sweetness, acidity, bitterness and mouthfeel characters. Training sessions were then conducted to establish appropriate descriptors via group consensus using a subset of berries from each season.

Formal BSA was conducted over four sessions in two weeks, at the conclusion of harvest each season, as all samples were frozen for convenience. Assessors evaluated 22 attributes (Table

S2) in randomly presented samples each given a unique three digit code and consisting of three berries, each sample was presented in duplicate.

Wine Sensory Descriptive Analysis

Prior to formal sensory analysis each wine was assessed informally by 3-4 experienced wine assessors to determine if wines were sound and to derive suitable descriptors for sensory analysis.

Each season, a panel of assessors was selected consisting of both male and female staff and students from the University of Adelaide. All assessors were highly experienced in sensory DA, and familiar with wine sensory analysis. Panels consisted of ten, nine and twelve assessors for each season 2012/13, 2013/14 and 2014/15 respectively. Sensory DA was undertaken in a series of training and formal sessions. During the training sessions, the assessors initially generated descriptive terms via consensus specific for the wines of each season, these attributes were used to create the proforma and lexicon used in final sessions, in addition to gaining familiarity in recognising and scoring the attributes. The training sessions involved ranking exercises of taste attributes, astringency and identification of aroma standards. Wine samples specific to each vintage were assessed to develop characteristics of aroma, flavour, mouthfeel and aftertaste descriptors. Descriptors were agreed upon by consensus and a final list was developed consisting of 16 aroma, 12 flavour, 3 mouthfeel and 3 aftertaste descriptors for 2012/13 samples. For 2013/14 samples attributes tested were reduced to 8 aroma, 10 flavour, 3 mouthfeel and 4 aftertaste attributes based on panel consensus and the same attributes were assessed for 2014/15 (detail of all attributes and standards is presented in Table S3).

Sensory assessment was undertaken in five sessions over two weeks, immediately after training, in isolated booths at 22^{o}C under fluorescent lights as colour was not assessed. All wines were presented in randomised order in covered ISO standard glassware containing 50 mL of wine. In each session, the judges assessed 14 samples presented in randomised groups of four with a 30 second break between samples and a forced break of at least 5 minutes after the first two groups. Purified water was provided along with approximately 100 mL of citrus pectin solution (2 g/L, Sigma-Aldrich Co., St Louis, MO) and water crackers for palate cleansing and reference. All treatment replicates were presented in duplicate.

Aroma reference standards were presented at each session for re-enforcement of attributes and were available for referral at all times during judging sessions. Reference standards of materials for judging tactile sensations of tannin texture were supplied in the booth for reference and fine

tuning. Attributes were separated into groups of aroma, taste and mouthfeel and scored on 15 cm unstructured line scale. Tannin structure was defined by physical sensation, either drying (lack of saliva lubrication) or sensation relating to perceived size. Both of these references to tannin structure and size are physical sensations and not intended to be directly related to actual molecular tannin structure in terms of constitutive elements.

Quantification of volatile aroma compounds by SPME GCMS

Targeted analysis of 28 fermentation-derived volatile compounds by solid phase microextraction gas chromatography (SPME-GC-MS) was undertaken on finished bottled wines soon after sensory analysis in the final season (see Table 1 for compound list). Retrospective analysis was not undertaken due to the time elapsed since previous seasons bottlings. The analysis was undertaken by the SA Metabolomics Australia node at the Australian Wine Research Institute, Waite Campus Urrbrae. The quantification was performed using SIDA techniques as described by Siebert, *et al.* [40] with the following modifications.

The gas chromatograph was fitted with a 60 m x 0.25 mm wax column with 0.25 um film thickness. The vial and its contents were heated to 35°C for 5 minutes with agitation. The SPME fibre (polyacrylate) was exposed to the headspace in the sample for 10 minutes and was then desorbed in the injector (splitless mode) for 10 minutes. The injector temperature was set at 260°C. Oven temperature was started at 35°C, held at this temperature for 2 minutes then increased to 150°C at 55°C/min, and held at this temperature for 2 minutes, then increased to 150°C at 5°C/min, and held for 2 minutes, and finally increased to 230°C and held for 5 minutes. The total run time was 42 minutes.

Authentic reference standards were synthesised in-house or purchased from Sigma-Aldrich [40]. Raw data from Agilents' ChemStation software (v E.02.02.1431) were converted into Mass Hunter data files and processed using Mass Hunter Workstation Software for Quantitative Analysis (v B.0600).

Statistical Analysis

ANOVA and repeated measures ANOVA analyses were performed using Genstat version 15 (VSN international, Hemel Hempstead, UK), and means were separated using Fishers least significant difference (LSD) test ($p<0.05$). Analysis of sensory properties for both grapes and wine was conducted individually for each season 2012/13, 2012/14 and 2014/15. ANOVA was undertaken to determine which sensory attributes were significantly different between treatments using assessors as random effects, reducing the number of attributes to allow further

investigation of relationships via PCA. Sensory data were subjected to mixed models ANOVA on all attributes from DA panels via XLSTAT Version 2015.4.01.20116 (Addinsoft SARL, Paris, France) sensory data analysis plugin. The product characterisation function with assessors (judge) as random effects was used (Y=P+J+P*J) to identify attributes with significant discriminating power for further PCA analysis. The Pearson correlation matrix was calculated and inspected for significantly correlated attributes at α=0.05. Individual sensory or aroma volatiles were grouped if found to be highly correlated (r >0.90).

Results and discussion

Berry Sensory Analysis

PCA of the all significantly different BSA attributes revealed 81% of the variability is explained by the first two PCs (Figure 1). These attributes were found to be consistent in at least 2 seasons with the exception of astringency and acidity measures which were consistent in all 3 seasons and the specific 'red fruit' and 'dried fruit' which were significantly different in 2013 only. The influence of the first two PCs is similar, with PC1 explaining 44% of the variation and 36% on PC2. The data separates vine age on PC2, while site is represented by PC1. All of the old vine BSA results are found in the positive space of PC2, the only young vine sample to appear in this region of the biplot is from site 5. Even though this young vine data point shares the old vine PCA space, the magnitude and direction of separation between vine age classes at site 5 follows the same trend as all other sites. The relative proximity of the data points from site 5, could possibly be due to propagation technique, which is unique to this site. Site 5 is propagated via layering, therefore young and old vines are inter-planted in the same row. This results in reduced spatial distance between age classes compared to the other sites.

The attributes associated with young vine fruit along PC2 are greater pulp sweetness, in addition to dried fruit flavour. While the relationships are unclear here these differences may be due to differences in canopy architecture between old and young vines presented in chapter 3. Younger vines were found to be associated with lower pruning mass (chapter 3). In the positive space of PC2, greater skin and pulp acidity and red fruit flavour are associated with old vine fruit. Again chapter 3 presented older vines having greater fruit mass (kg/m) via greater bunch mass and berry number, this may have had some relationship to the sensory properties due to well understood correlations between vine yield and maturity interactions. Despite seasonal differences in intensity, sensory attributes present consistent trends relating to vine age. Grouping is observed according to vintage when analysed, the sensory profile of 2014

wines is found in the positive space of both PCs while 2012 and 2013 are loosely clustered in the top left of the biplot. Attributes relating to acidity and skin disintegration are consistently presented in the positive space of PC2 irrespective of the growing season.

Panellists consistently perceived acidity in both skin and pulp in each season. However, the common determinants of maturity in relation to acid, such as pH and TA (titratable acidity) did not vary significantly each season (Table S1). This was also observed in the study of Olarte Mantilla, *et al.* [35] who found skin and pulp acidity to differ significantly (2010, skin, and 2011, pulp). These differences observed with BSA might suggest that the sensory perception of acidity is not wholly captured by the common methods of pH and TA measurement in this case. Not in grapes but wine, sourness shows a stronger correlation with TA than pH [41].

Berry ripening involves many changes, some of which have been previously characterised by BSA [42]. The study of Le Moigne, *et al.* [42] found seed astringency was significant in determining ripening stage: reducing in astringency over time. Uncrushed seeds from older vines were found to be more astringent (via the physical sensation of tongue movement) than fruit from young vines. In addition, the measure of skin disintegration or the ease of which the skin can be broken to pieces via chewing was found to be more difficult in older vine fruit. As grapes mature synthesis of phenols and condensed tannins in the skins and seeds decreases [43] combined with an accumulation of flavanols and anthocyanins [44]. Skin composition and cell wall structure are known to change with ripening resulting in a loss of firmness via disassembly of the mesocarp cell walls [45]. Key changes during ripening have been shown to be cell wall composition and enzyme activity [46]. More recent research into cell death during ripening has found that riper characters in Shiraz berries were associated with greater berry cell death [17]. In addition, Cabernet Franc assessed via BSA found attributes relating to increased sweetness and lower acidity were related to ripeness progression [42]. This suggests that the fruit from younger vines, according to BSA, may be showing riper characters than the older vine fruit.

Relative ‘ripeness’, measured as Brix, was similar amongst age groups each year; however, the sensory profile was not. Harvested Brix levels were not significantly different between vine ages except for site 4 in 2014 and 2015 (Table S1). The Brix level in grape berries is known to contribute to the perception of sweetness [14]. This was only statistically identified via BSA in the 2015 season. Sadras, *et al.* [15] observed that sensory profiles of fruit could differ at the same Brix level due to external environmental changes. The relative environmental constant of these vines suggests that differences seen here are possibly a result of another driver. Observed

differences in the perception of sweetness may be due to the perception of differing levels of acidity or acid composition. Astringency while different in this study isn't known to affect sweetness however, it has been observed to decrease during ripening [42]. Based on these results, increased vine age produces sensory attributes which associate with red fruit flavours and acidity perception, past studies into vine age have not incorporated BSA age based comparisons.

Wine Sensory Analysis

It is well established that wine sensory properties are influenced by the composition of the grapes used to produce them [36]; however, direct correlations with wine are often not strong [47]. Individual volatile compounds may be either suppressive or amplifying in combination [48]. In addition, non-volatile compounds (polyphenols, tannins, glycerols and organic acids) can also significantly impact the perception of wine aroma qualities, both positively and negatively [49]. Sensory DA is subject to these influences, therefore consistency in characters between seasons can give us a clear signal that repeated attributes are representative.

Following wine sensory, DA attributes that were consistently different in all three seasons via ANOVA ($p<0.05$) were subjected to PCA. The PCA of these attributes explains 61% of the variability within the dataset in the first two principal components (Figure 2). Attributes that were found to be significant and consistently different between age groups include fresh fruit flavours and aromas, aroma of red fruit, bitterness and persistence of aftertaste. The alignment of sites on PC1 indicates this axis represents site; largely driven by attributes of length of aftertaste, bitter taste and aftertaste. This is demonstrated clearly in this figure by site 5 where both vine age categories are presented in close proximity on PC1; these vines are physically the closest due to propagation technique i.e. they are in the same row. Interestingly this same feature was also observed in the BSA data (Figure 1).

Supplementary variables present attributes that showed consistent differences in two out of three seasons. The majority of these attributes are found in the bottom right quadrant of the PCA. In relation to age the variables with the greatest relationship to the young vine samples are savoury, complexity, dark fruit and alcohol. These attributes are in contrast to the fresh fruit and red fruit characters that appear to define old vine samples more consistently.

Site 2 shows a distinct site influence with old and young vine wines displaying the most negative positions along PC1. This indicates that both old and young vine wines present unique sensory attributes compared to all other sites, most notably reduced length. The same

observation was made following BSA analysis regarding the site influence according to PCA. Despite the observed strong grouping of Site 2 in both grape and wine sensory characters, in each case, the physical distance between old and young samples in the PCA space follows the same trend as other sites, with old vine samples having a more positive score along PC2. Moreover, all wines produced from older vines consistently achieved higher (more positive) values along PC2 than their younger equivalents. While the site by site scale of differences between old and young differs, the direction in trend is consistent towards common sensory characters.

These findings that vine age is associated with characteristics such as red berry and fruity characters concur with previous reports of Zufferey and Maigre [7] and Heymann and Noble [8]. This suggests that vine age may influence the sensory perception of wines produced from vines of differing ages.

Volatile compounds in old and young vine wines

To further investigate sensory trends, the underlying volatile composition of the 2015 vintage wines was explored. Quantitative analysis of 28 fermentation-derived products was performed on each winemaking replicate. The concentration of fermentation-derived volatiles was analysed along with the 2015 DA results. The complete dataset was subjected to PCA to visualise uniformity of the fermentation replicates (Figure S1). Grouping of replicates by site was clear, giving a good indication of consistency in fermentation dynamics and vineyard replication.

All 28 fermentation products were subjected to ANOVA resulting in 12 significantly different products ($p<0.05$) between age classes (Table 1). PCA was undertaken on all significantly different products to explore the volatile space of the studied wines (Figure 3). Following PCA the first two components were selected and displayed 81% of the total variance in the dataset. With the exception of acetic acid and hexanol, all significantly different values were quantitatively greater in the wines classed as old (Table 1).

The group of compounds with the greatest variability in the dataset were acetates, while alcohols and esters had the least presented the least variability. Ethyl esters and fusel alcohol acetates are known to be essential for a wines aromatic profile, specifically fruitiness [50]. This is notwithstanding the non-linear, additive or competitive effects that individual compounds may exert on sensory perception [50, 51]. Changes in compound abundance below the aroma active threshold can still affect the sensory outcome [51]. For example, very small changes, as

low as 1.3%, of the olfactory threshold of certain ethyl esters in de-aromatised wine has been found to affect red and black-berry aromas [50]. In addition, reconstitution experiments with individual compounds have highlighted the cumulative effects whereby alteration of single compounds did not have a great effect on sensory perception [31]. This is further complicated by cross modal perception, where sub threshold odorants combined with subthreshold flavour, perception can still occur [52].

The individual separation of samples along PC1 (57%) presents a site by site relationship with vine age. While clustering is not observed, all samples from young vines present more negative values on PC2 than old vine wines from the same site. This feature is mainly driven by acetates (2-methylpropyl acetate, 2-methylbutyl acetate, 3-methylbutyl acetate, hexyl acetate and 2-phenyl ethyl acetate). A very close correlation between the acetates 2 & 3-methylbutyl acetate and hexyl acetate was observed as shown in Figure 3 by the acute angle of the vectors in the biplot and supported by Pearson correlation values of r=0.978 (p<0.0001) and r=0.977 (p<0.0001) respectively. Fruit flavour (berry) has been found to be positively correlated with levels of hexyl acetate in Cabernet Sauvignon and negatively correlated with spicy aroma [36]. These findings are supported by this study even though different cultivars were investigated. The relative abundance of compounds that may become acetate ester precursors in fruit appear to be influenced by both harvest time and Brix [53]. In this study harvest time and Brix were no different for all but one site each, leading to the conclusion that differences presented here are not due to maturity but related to vine age.

Site 2 shows greater difference between old and young vines along PC1 as compared to PC2; this component displays the greatest discrimination of vine age categories. The second dimension, PC2, explains an additional 23% of the total variance. Vector strength and direction on PC2 favour older vine wines in a similar trend to the sensory data previously presented. In addition, compounds associated with negative sensory characters (acetic acid and hexanol) were significantly greater in young vine wines. These findings show that differences in metabolite composition were detected in wines produced from vines of differing age.

In addition to acetates, fatty acids also have been shown to be important contributors to red wine quality [54]. Esters have been noted to be an especially important class of compound in the volatile aroma composition of young wine [55]. Fruitiness is not the result of a single compound and may be enhanced or suppressed by the presence of other compounds [54]. Younger vine wines from 2015 were judged as having lower fruity characters. They were also

quantitatively greater in acetic acid, which can suppress fruity characters [54]. Moreover, these wines were greater in hexanol which has been related to herbaceous or non-fruit characters [54].

In our study old vine wines recorded mean values of hexanoic and octanoic acid, significantly greater than those of young vine wines, decanoic acid was also greater but not significant (Table 1). Hexanoic, octanoic and decanoic acids have been found to be important contributors to the aroma of high sensory evaluation scores in wine [56]. Vilanova, *et al.* [22] also found that aroma "quality" (as they defined it) was predicted by hexanoic and octanoic acid (and phenylethyl acetate). In addition to the acids already mentioned, phenyl ethyl acetate was also found to be consistently higher in wines from old vines in our study. The presence of these compounds imply a greater potential 'quality' in older vine wines from a compositional point of view.

To examine the consensus between the sensory space and volatile composition, the correlation matrix of variables with statistical significance was analysed by PCA (Figure 4). The combined data set revealed that 33% and 27% of the variation was associated with PC1 and PC2 respectively. All old vine wines presented positive loadings on PC2. The only young vine wine to appear in the positive space of PC2 was from site 3. Pooling of the volatile composition and the sensory scores revealed a common trend of differentiation between age classes. The spatial position of the data points particularly along PC2 favour older vine wines in a similar trend to the sensory data. This analysis presents a range of attributes that are of greater influence on the PC2 in older vine wines, such as; fresh fruit, red fruit and herbaceous characters, along with an absence of heavy savoury or alcoholic characters.

The strong relationship between the old and young vines at site 3 is an interesting and recurrent feature despite their 116 year difference in planted age. This is the only site where spur pruning is applied (short bearers of 2 nodes per bearer) resulting in a permanent woody framework being retained each season. All other sites are cane pruned (long bearers ~10 nodes per bearer). Spur pruning results in fruit on the first and second node only. In contrast, the remaining sites that retain longer canes may bear fruit on any or all retained nodes. While not directly studied here, these management differences may be one explanation for the relative uniformity between old and young in this data set. Vines with a greater volume of old wood have been found to yield higher but not differ markedly in acidity or sugar level at harvest compared to vines with

less old wood [57]. Old cordons (i.e. greater perennial wood volume) have previously been associated with higher yields via bunch mass, and increased TA [58].

Carbohydrate reserves have been reported to influence floral differentiation and inferred to affect inflorescence branching, thereby influencing yield and seasonal variation in fruiting [59]. These carbohydrate reserves may buffer against biotic and abiotic seasonal conditions [60, 61]. Increased yield and enhanced fruit maturity measured as TSS was suggested to be due to the presence of additional wood retained via the vine training system [3]. In addition the study of Reynolds, *et al.* [62] suggested that other benefits were also due to greater volumes of old wood, specifically citing increased monoterpene levels in Riesling. These findings are of interest and this study concurs that the greater potential carbohydrate storage inherent in older vines may be a factor in subtle compositional differences. What is unclear is if the increased perennial wood volume in the young vines at site 3 is equivalent to that of the old vines given the significantly different trunk circumferences (628 mm vs 230 mm).

While final wine composition is mediated through yeast metabolism, studies suggest that grape composition via precursors can influence or modulate products of yeast metabolism, thereby influencing wine volatile composition [53, 63, 64]. In this study we observed differences in volatile composition which are considered to originate in the grape berry due to controlled winemaking. However, further work is required to validate this link via instrumental and sensory characterisation of fresh grape composition at the metabolomic level.

Sensory characteristics of fresh grapes from younger vines were found to be more associated with dark fruit, dried fruit and sweetness. In contrast sensory analysis found grapes from older vines presented greater red fruit characters and acidity perception. In addition mouthfeel characters of astringency and bitterness were found to be greater in older vine grape seeds and skins. Sensory analysis of wine produced from old vines found more intense red fruit and fresh fruit characters, while more intense dark fruit and alcohol were typical for younger vine wines. This was supported by analysis of the chemical compounds that are characteristic for these attributes.

Grape quality (as gauged by price) is suggested to be more dependent on extrinsic factors than the basic composition of the fruit itself [65]. The authors state that if high priced fruit is better for winemaking than lower priced fruit, it is due to other flavour compounds or fruit parameters not measured [65]. Crop price in Cabernet Sauvignon was not related to differences in Brix, pH, anthocyanin concentration, berry mass or number of berries per cluster [65]. This study

supports these findings, because in three seasons of BSA characteristics that had a greater association with older vine fruit were related to mouthfeel and acidity, but not clearly identified by common chemical measures.

In this study, no treatment in a traditional sense was applied to measure a response. In contrast the treatment was the time elapsed since the vines were planted. The hypothesis that greater vine age will result in alteration of intrinsic characters of fruit or wine quality was tested via three methods and interrogated statistically. Sensory perception is highly specific and complex and in this study identified differences in grapes and wine based on vine age. Similarly highly sensitive targeted metabolomic data was able to separate attributes that were unique to vine age categories. Vine age appears to influence certain properties of sensory and volatile composition of wine. Further research using increasingly popular 'omics' technology over a greater number of seasons will potentially identify seasonally-specific traits and stable metabolites providing greater clarity to the quantitative parameters that are valued in wine from older vines. This could potentially assist in improving grape and wine quality through targeted production techniques.

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Tables

Table 1. Mean concentrations (µg/L) of fermentation derived volatile compounds via GC-MS for Barossa Shiraz wines harvested in 2015. For each age group means ±SD (treatment replicates and sites) different letters between each column are significantly different ($p \leq 0.05$) according to ANOVA using Fishers (LSD) for pairwise comparisons.

Table 1. Mean concentrations (µg/L) of fermentation derived volatile compounds via GC-MS for Barossa Shiraz wines harvested in 2015. For each age group means ±SD (treatment replicates and sites) different letters between each column are significantly different ($p \leq 0.05$) according to ANOVA using Fishers (LSD) for pairwise comparisons.

	Old	**Young**	***p*-value**
Acetates			
ethyl acetate	92403 ± 20452	87697 ± 16668	ns
2-methylpropyl acetate	101 ± 33a	72 ± 29b	0.021
2-methylbutyl acetate	1088 ± 411a	669 ± 365b	0.008
3-methylbutyl acetate	4089 ± 1578a	2470 ± 1422b	0.007
hexyl acetate	80 ± 33a	49 ± 29b	0.011
2-phenyl ethyl acetate	1677 ± 753a	1100 ± 661b	0.038
Alcohols			
2-methylpropanol	43611 ± 4791	43270 ± 6378	ns
butanol	2149 ± 551	2167 ± 476	ns
2&3-methylbutanol	151003 ± 12006	149704 ± 11302	ns
hexanol	3034 ± 482b	3369 ± 345a	0.042
2-phenyl ethyl ethanol	256198 ± 51360	297380 ± 90903	ns
Acids			
acetic acid	470289 ± 94443b	578785 ± 77866a	0.002
propanoic acid	3687 ± 822	3423 ± 1159	ns
2-methyl propanoic acid	1398 ± 376	1758 ± 584	ns
butanoic acid	3762 ± 417a	3441 ± 350b	0.033
3-methyl butanoic acid	1011 ± 161	1206 ± 332	ns
2-methyl butanoic acid	2656 ± 647	2539 ± 589	ns
hexanoic acid	2716 ± 548a	2252 ± 300b	0.009
octanoic acid	1998 ± 277a	1706 ± 309b	0.012
decanoic acid	520 ± 71	467 ± 69	ns
Esters			
ethyl propanoate	630 ± 126a	519 ± 84b	0.010
ethyl 2-methylpropanoate	92 ± 30	108 ± 48	ns
ethyl butanoate	404 ± 75a	347 ± 69b	0.044
ethyl 2-methylbutanoate	11 ± 5.5	14 ± 8.2	ns
ethyl-3-methylbutanoate	19 ± 9.2	22 ± 10	ns
ethyl hexanoate	497 ± 79	446 ± 55	ns
ethyl octanoate	284 ± 38	253 ± 47	ns
ethyl decanoate	143 ± 12	128 ± 35	ns

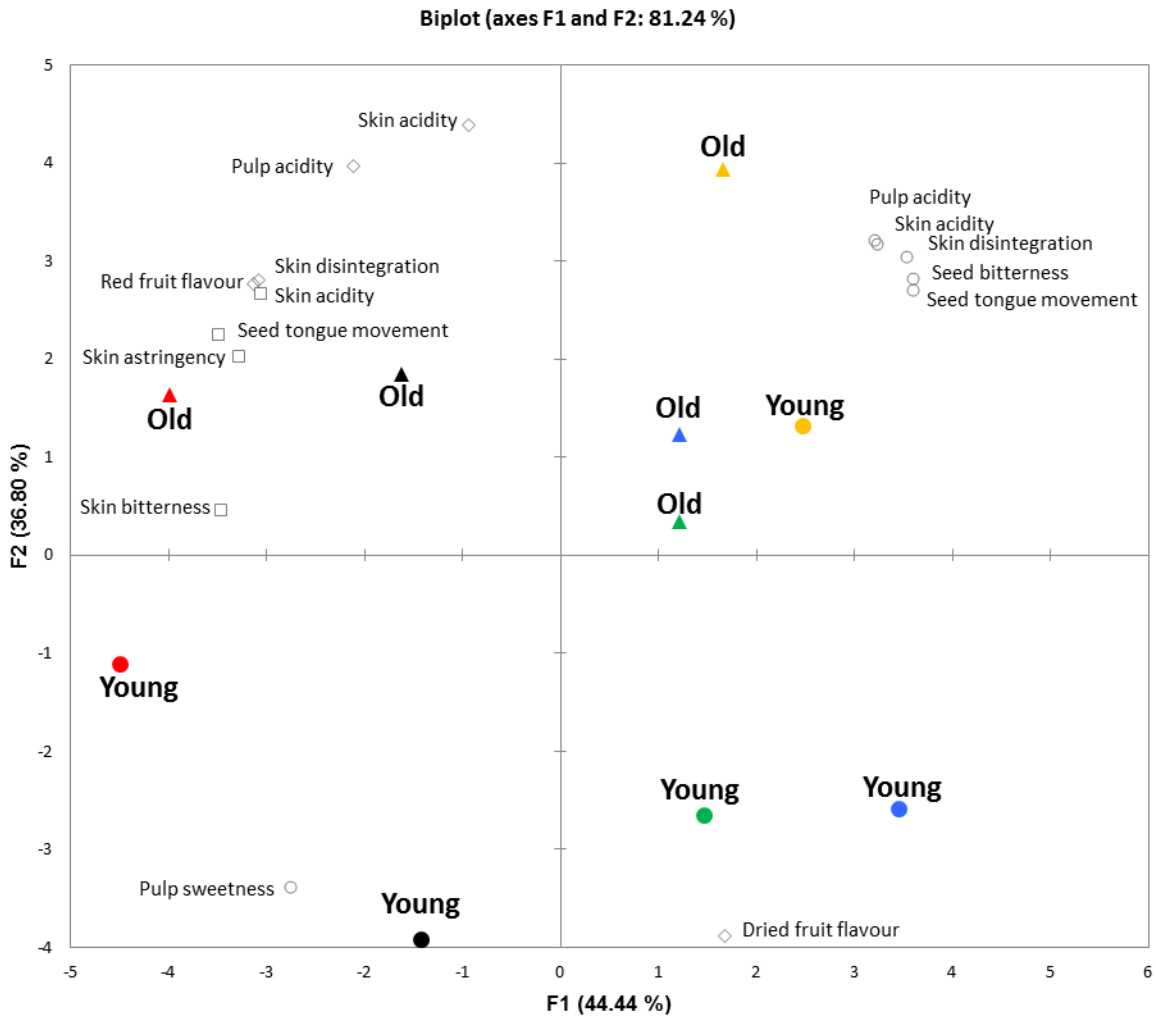
Figures

Figure 1. BSA PCA biplot showing scores and loadings of the significantly different ($p<0.05$) sensory attributes from BSA of Shiraz berries harvested in 2013 (◊) 2014 (□) and 2015 (○). Labels indicate vine age class at each site with common colours representing site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5.

Figure 2. Wine PCA of mean sensory scores for attributes that were significantly discriminating ($p<0.05$) and appeared in all three years. Supplementary variables are attributes that were significantly different ($p<0.05$) in any two years only. Attributes are labelled per vintage year 2013 (◊) 2014 (□) and 2015 (○). Labels with common colours represent site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5. Sensory DA attribute prefix signifies: A-Aroma, F-flavour (palate), T-Taste sensation, M-mouthfeel (physical), AT-After taste (following expectoration). *

Figure 3. PCA biplot showing scores and loadings of the standardised means of significantly different ($p<0.05$) volatile compounds by GC-MS analysis of Barossa Shiraz wines from 2015. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Metabolites labelled in italics are the only values that were greater in intensity in young vine wines, all others were greater in old vine wines.

Figure 4. PCA biplot showing scores and loadings of standardised means for significant ($p<0.05$) sensory attributes from DA (open markers + solid vectors) and volatile compounds (closed markers + broken vectors) determined for Shiraz wines from differing ages in the Barossa valley. Labels indicate site and age category, markers represent nominal age classification where ▲=old and ●=young and shared colours are from the same location.



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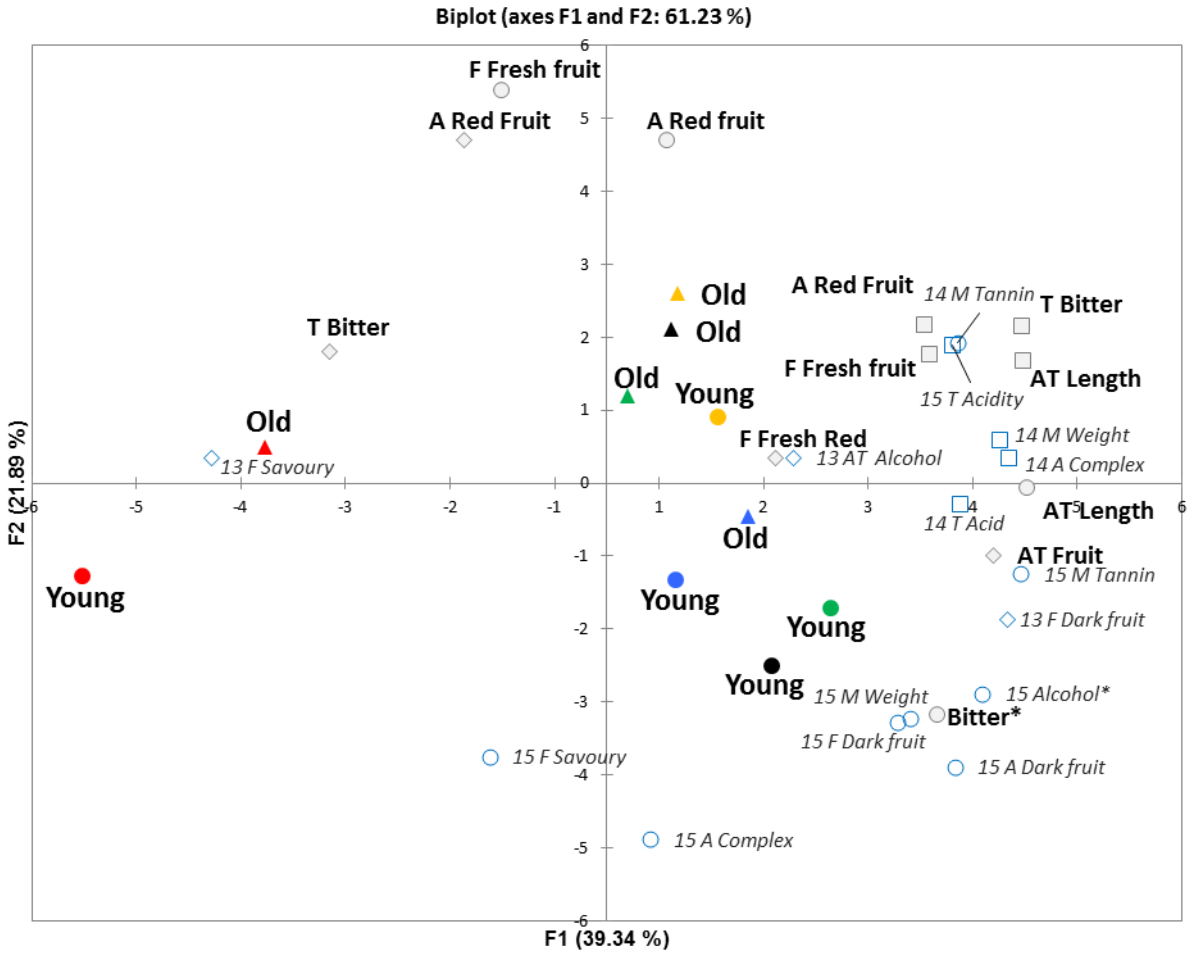

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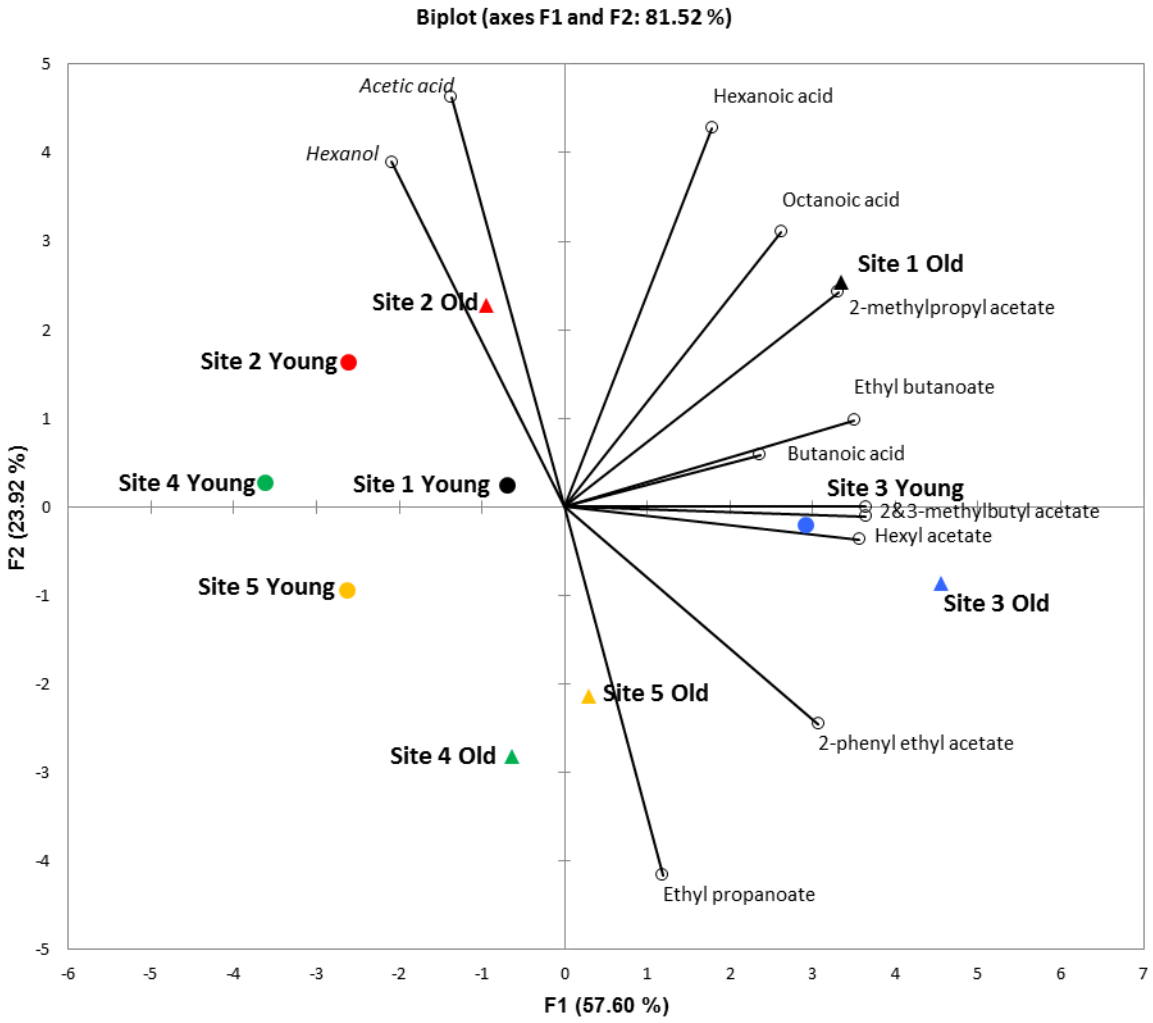

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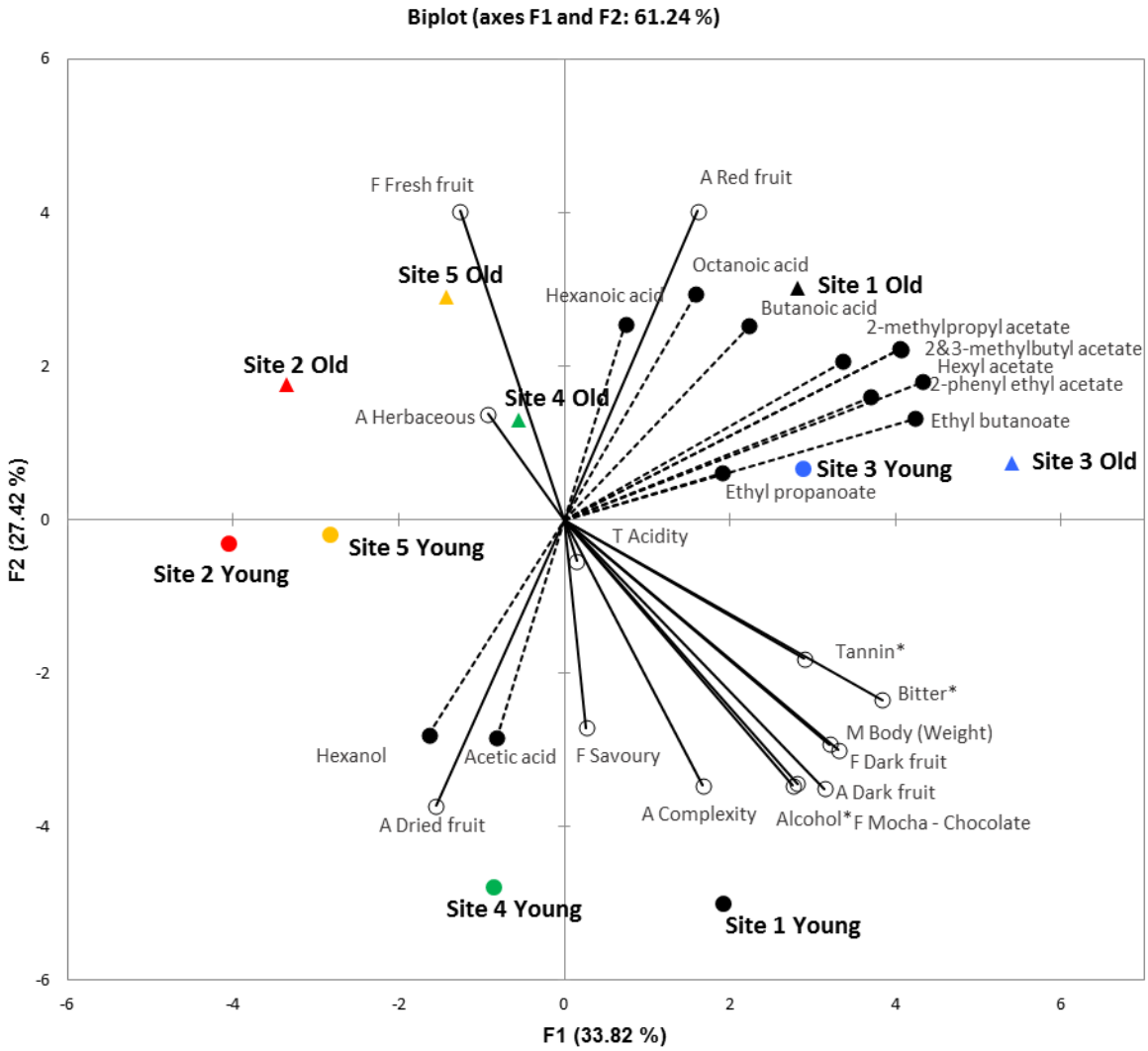

Figure 4. PCA biplot showing scores and loadings of standardised means for significant ($p<0.05$) sensory attributes from DA (open markers + solid vectors) and volatile compounds (closed markers + broken vectors) determined for Shiraz wines from differing ages in the Barossa valley. Labels indicate site and age category, markers represent nominal age classification where ▲=old and ●=young and shared colours are from the same location. Sensory DA attribute prefix signifies: A-Aroma, F-flavour (palate), T-Taste sensation, M-mouthfeel (physical), AT-After taste (following expectoration). *denotes grouping of highly correlated attribute modalities of taste and aftertaste.

Supporting material

Tables

Table S1. Grape berry parameters at harvest in 2013, 2014 and 2015 seasons from five Barossa Valley Shiraz vineyards with vines of contrasting age.

Table S2. Pulp, skin and seed sensory attributes of Shiraz berries harvested from the Barossa Valley in 2012/13, 2013/14 and 2014/15 used in BSA including word anchors.

Table S3. Sensory standards and attributes provided during tasting sessions or in prior training for wine sensory DA in classes of modality: aroma, flavour, mouthfeel and aftertaste.

Table S4. Mean concentrations of fermentation-derived products via GC-MS for Barossa Shiraz wines from harvest in 2015. For each age group means ±SD for treatment replicates. *indicates significance between vine age classes within site only via direct pairwise comparison ($p \leq 0.05$) using Fishers LSD.

Table S5. Mean values of the sensory ratings following DA of small batch Shiraz wines produced in 2013, 2014, 2015 from the Barossa Valley. P value represents discriminating power of variables observed between old and young samples for each attribute.

Table S6. Wine composition of Barossa zone Shiraz harvested in 2013, 2014 and 2015 from five sites each with two age classes**.**

Figures

Figure S1. PCA biplot showing scores and loadings of volatile compounds by winemaking replicate from 2015 Barossa Shiraz wines. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5.

Table S1. Grape berry parameters at harvest in 2013, 2014 and 2015 seasons from five Barossa Valley Shiraz vineyards with vines of contrasting age.

	2013				2014				2015			
SITE 1	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	0.9	0.7	ns	ns	1.2	1.0	ns	ns	1.3	1.3	ns	ns
Harvest Brix	27.7	28.1	ns	ns	24.0	24.6	ns	ns	26.6	27.2	ns	ns
Harvest pH	3.59	3.60	ns	ns	3.90[b]	4.39[a]	0.017	0.47	4.01	4.08	ns	ns
Harvest TA (g/L)	6.7	6.3	ns	ns	6.8[a]	5.6[b]	0.011	0.80	4.2	3.8	ns	ns
	2013				**2014**				**2015**			
SITE 2	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	0.4	0.4	ns	ns	1.2	1.3	ns	ns	1.4	1.6	ns	ns
Harvest Brix	24.4	26.9	ns	ns	23.5	23.3	ns	ns	24.1	24.7	ns	ns
Harvest pH	3.49	3.45	ns	ns	4.01	3.51	ns	ns	3.88	3.60	ns	ns
Harvest TA (g/L)	6.02	6.18	ns	ns	6.47[b]	7.55[a]	0.003	0.88	5.46[b]	9.33[a]	0.007	4.48
	2013				**2014**				**2015**			
SITE 3	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	1.1 [a]	0.9 [b]	0.033	0.17	1.1	1.0	ns	ns	1.4	1.4	ns	ns
Harvest Brix	27.2	27.7	ns	ns	24.6	24.6	ns	ns	26.1	25.8	ns	ns
Harvest pH	3.56	3.69	ns	ns	4.02	4.13	ns	ns	3.81	3.87	ns	ns
Harvest TA (g/L)	6.1[a]	5.0[b]	0.025	1.52	5.67	5.48	ns	ns	4.41	4.55	ns	ns
	2013				**2014**				**2015**			
SITE 4	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	0.32[b]	0.81[a]	0.000	0.09	0.86[a]	0.72[b]	0.016	0.21	1.0	1.1	ns	ns
Harvest Brix	28.6	27.6	ns	ns	24.0[b]	25.3[a]	0.001	0.75	25.5[b]	27.6[a]	0.002	1.50
Harvest pH	3.35[b]	3.61[a]	0.002	0.15	3.98	3.90	ns	ns	3.56[b]	3.83[a]	0.003	0.20
Harvest TA (g/L)	7.75[a]	4.99[b]	0.000	0.35	5.67	5.23	ns	ns	4.86[a]	3.91[b]	0.001	0.43
	2013				**2014**				**2015**			
SITE 5	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Berry mass (g)	1.2	1.1	ns	ns	1.2	1.2	ns	ns	1.4	1.3	ns	ns
Harvest Brix	27.3	27.5	ns	ns	25.7	25.1	ns	ns	24.0	25.0	ns	ns
Harvest pH	3.70	3.73	ns	ns	4.23	4.11	ns	ns	3.88	3.75	ns	ns
Harvest TA (g/L)	5.24	5.03	ns	ns	4.75	5.18	ns	ns	4.79[a]	4.38[b]	0.034	0.75

Table S2. Pulp, skin and seed sensory attributes of Shiraz berries harvested from the Barossa Valley in 2012/13, 2013/14 and 2014/15 used in BSA including word anchors.

Component	Attribute	Anchors on scale
Pulp	Fresh dark fruit	Absent to intense
	Dried fruit	Absent to intense
	Red fruit	Absent to intense
	Detachment of pulp from the skin	Easy to separate, 50% attached/separated, firmly attached
	Juiciness in the pulp	All gelatinous, 50% gelatinous/juicy, all juicy
	Sweetness	Not very sweet to very sweet
	Acidity	Low to very acidic
Skin	Disintegration	Easy (homogenous mixture), Fairly easy (remains in small pieces), Difficult (remains in large pieces)
	Acidity	Low to very acidic
	Dark grape flavour	Absent to intense
	Bitterness	Absent to intense
	Astringency	Absent to intense
	Tannic intensity	Tongue slides effortlessly, sticks slightly, slides with difficulty, with great difficulty
	Grain size of tannins	Fine (silk), Medium (velvet), Coarse (high grade sand paper)
	Astringency (Time needed to re-salivate)	Easy to re-salivate or difficult to re-salivate after more than 5 seconds
Seed	Colour	Green, yellow-green to dark brown
	Astringency (uncrushed)	Absent to intense
	Crushability	Soft (like Pumpkin seeds), brittle (like roasted Hazel nut, Crunchy (Like Passionfruit seeds)
	Flavour	No flavour, herbaceous to toasted
	Astringency	Absent to intense
	Bitterness	Absent to intense
	Ease of tongue movement	Tongue slides effortlessly, sticks slightly, slides with difficulty, with great difficulty over roof of mouth

Table S3. Sensory standards and attributes provided during tasting sessions or in prior training for wine sensory DA in classes of modality: aroma, flavour, mouthfeel and aftertaste.

	Season	**Season**	**Season**	**Reference standards if supplied or training solution.**
Class	**2012/13**	**2013/14**	**2014/15**	
A	Red fruit	Red fruit	Red fruit	Raspberry, red cherry, strawberry
A	Dark Fruit	Dark Fruit	Dark fruit	Black-berry -currant, satsuma plum
A	Dried Fruit	Dried Fruit	Dried fruit	Dried pitted prune, fig, date
A	Jammy character	Jammy character	Jammy character	Mixture of red and dark fruit jams
A	Herbaceous	Herbaceous	Herbaceous	Fresh cut grass
A	Confectionary	Fruit character	Fruit character	**Redskin™ candy
A	Alcohol	Alcohol	Alcohol	No standard
		Complexity	Complexity	No standard
F	Fresh Red			as above
F	Dark Fruit	Dark Fruit	Dark fruit	as above
F	Dried Fruit	Fresh Fruit Character	Fresh Fruit Character	combination of previous
F	Savoury	Savoury	Savoury	Black olives, beef jerky
F	Green - Herbaceous	Green - Herbaceous	Green - Herbaceous	as above
F		Floral	Floral	Several fresh violet flowers
F	Confectionary	Confectionary	Confectionary	Redskin™ candy
F		Mocha - Chocolate	Mocha - Chocolate	Generic mocha drink powder
T	Acid	Acid	Acid	Training : Tartaric acid solution
T	Bitter	Bitter	Bitter	Training : Quinine sulphate solutions
T	Salty	Salty	Salty	Training : Sodium chloride solutions
M		Alcohol	Alcohol	No standard
M	Body	Body	Body	No standard
M	Astringency	Astringency	Astringency	Feeling of mouth surfaces adhering or sticking together***
M	Tannins	Tannins	Tannins	Training via tannin solutions
AT		Length	Length	Overall lingering flavour sensation
AT	Alcohol	Alcohol	Alcohol	Heat and warmth after expectorating
AT	Fruit	Tannin intensity	Tannin intensity	Intensity and length of tannin sensation
AT	Non-Fruit	Bitter	Bitter	Bitterness after expectorating

*Variation in alignment is due to assessor consensus of attributes applicable to each season. 2012/13 included the following additional attributes not listed: A-Savoury, floral, spice, liquorice, pepper, olives, earthy, tobacco, leather and F-Spice, Sweet
**2012/13 only
*** Physical touch standards provided: silk, velvet, fine sandpaper and coarse sandpaper

Table S4. Mean concentrations of fermentation-derived products via GC-MS for Barossa Shiraz wines from harvest in 2015. For each age group means ±SD for treatment replicates. *indicates significance between vine age classes within site only via direct pairwise comparison ($p \leq 0.05$) using Fishers LSD.

	Site 1		**Site 2**		**Site 3**		**Site 4**		**Site 5**	
	Young	**Old**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**
Acetates										
ethyl acetate	98824 ± 6856*	115257 ± 3306*	79799 ± 5554	74595 ± 9554	84615 ± 1536	76790 ± 2029	115892 ± 13882	116349 ± 6664	68755 ± 1616	79022 ± 4878
2-methylpropyl acetate	90 ± 2.6*	147 ± 2.7*	76 ± 3.4	92 ± 8.8	51 ± 3.8	61 ± 3	123 ± 2.2	126 ± 3.9	39 ± 1.3	77 ± 4.3
2-methylbutyl acetate	722 ± 44*	1420 ± 74*	471 ± 35	679 ± 92	503 ± 47	747 ± 89	1490 ± 59	1677 ± 25	434 ± 11	917 ± 78
3-methylbutyl acetate	2681 ± 203*	5282 ± 196*	1723 ± 166	2469 ± 378	1819 ± 184	2854 ± 376	5649 ± 415	6398 ± 89	1539 ± 58	3442 ± 342
hexyl acetate	61 ± 8.6*	111 ± 11*	19 ± 1	41 ± 8.5	41 ± 6.3	61 ± 9.2	106 ± 13	124 ± 3.8	34 ± 2.9	65 ± 7.8
2-phenyl ethyl acetate	1046 ± 87*	1394 ± 147*	413 ± 16	779 ± 155	999 ± 86	1403 ± 174	2539 ± 62	2932 ± 144	981 ± 53	1877 ± 95
Alcohols										
2-methylpropanol	37594 ± 1924*	42979 ± 1334*	42487 ± 2368	51769 ± 1708	53037 ± 1839	43769 ± 2214	35955 ± 1674	39103 ± 1470	44838 ± 861	40436 ± 1689
butanol	2809 ± 51*	2216 ± 104*	1445 ± 96	1125 ± 83	2185 ± 105	2608 ± 98	2214 ± 166	2422 ± 109	2196 ± 182	2372 ± 55
2&3-methylbutanol	147422 ± 2822*	137095 ± 1286*	138187 ± 3761	162836 ± 8302	161396 ± 7905	159953 ± 6343	140439 ± 5360	140497 ± 7156	157989 ± 11432	154632 ± 6429
hexanol	3478 ± 55	3413 ± 229	3282 ± 184	3653 ± 302	3864 ± 66	2900 ± 252	2873 ± 227	2614 ± 100	3181 ± 107	2587 ± 159
2-phenyl ethyl ethanol	213372 ± 12813*	175163 ± 7534*	209817 ± 15816	260236 ± 17740	399737 ± 12389	294997 ± 18337	258891 ± 24032	242072 ± 34567	392255 ± 8347	308519 ± 4884
Acids										
acetic acid	571028 ± 21590	579938 ± 14703	646828 ± 20117	558785 ± 30832	662711 ± 32767	373669 ± 30121	485428 ± 46214	470955 ± 10900	496810 ± 1625	368099 ± 28176
propanoic acid	4966 ± 137	4642 ± 207	3939 ± 40	4081 ± 145	2237 ± 170	2385 ± 108	3961 ± 71	4054 ± 185	2194 ± 187	3273 ± 115
2-methyl propanoic acid	1224 ± 70	1356 ± 71	1611 ± 181	2086 ± 183	2642 ± 100	1281 ± 21	1091 ± 110	1158 ± 24	1998 ± 41	1109 ± 67
butanoic acid	3492 ± 211*	4341 ± 55*	3286 ± 118	3401 ± 160	3045 ± 195	3345 ± 100	3399 ± 98	4104 ± 79	3967 ± 55	3617 ± 59
3-methyl butanoic acid	943 ± 44*	826 ± 84*	968 ± 39	1222 ± 44	1595 ± 80	1126 ± 80	870 ± 69	882 ± 55	1543 ± 85	998 ± 10
2-methyl butanoic acid	2262 ± 216	1964 ± 143	1775 ± 21	2091 ± 133	3068 ± 169	3658 ± 261	2299 ± 214	2673 ± 102	3212 ± 76	2896 ± 162
hexanoic acid	2403 ± 32*	3370 ± 193*	2589 ± 234	3267 ± 118	1864 ± 64	2137 ± 112	2416 ± 46	2619 ± 50	2045 ± 34	2189 ± 97
octanoic acid	1783 ± 48*	2254 ± 86*	1951 ± 155	2244 ± 89	1320 ± 37	1609 ± 134	2141 ± 52	2089 ± 64	1478 ± 52	1791 ± 81
decanoic acid	569 ± 16*	623 ± 11*	477 ± 52	526 ± 19	415 ± 6.4	447 ± 21	487 ± 4.3	557 ± 17	396 ± 21	446 ± 18
Esters										
ethyl propanoate	652 ± 30*	534 ± 18*	420 ± 14	490 ± 22	481 ± 21	833 ± 56	535 ± 11	669 ± 18	512 ± 13	622 ± 15
ethyl 2-methylpropanoate	76 ± 2.5*	65 ± 1.2*	66 ± 0.9	92 ± 6.2	172 ± 1.3	145 ± 22	65 ± 5.4	76 ± 6.9	149 ± 4.4	84 ± 5
ethyl butanoate	408.6 ± 19*	486 ± 8.9*	311 ± 18	348 ± 16	305 ± 21	348 ± 24	466 ± 13	495 ± 6.8	285 ± 10	342 ± 22
ethyl 2-methylbutanoate	8.23 ± 0.49*	5.01 ± 0.33*	6.46 ± 0.13	9.11 ± 0.87	23.69 ± 0.51	20.22 ± 3.67	7.47 ± 1.44	8.57 ± 1.23	22.91 ± 1	11.31 ± 1.37
ethyl-3-methylbutanoate	15.85 ± 0.99*	10.86 ± 0.57*	10.55 ± 0.58	15.19 ± 1.52	34.29 ± 0.88	35.55 ± 5.73	13.63 ± 1.32	15.54 ± 2.24	31.46 ± 1.11	18.37 ± 1.86
ethyl hexanoate	513 ± 14*	608 ± 11*	446 ± 40	537 ± 30	394 ± 18	422 ± 31	501 ± 2.1	509 ± 17	396 ± 9.4	411 ± 25
ethyl octanoate	284 ± 7*	333 ± 9.1*	265 ± 28	280 ± 17	205 ± 10	241 ± 16	326 ± 4.7	313 ± 13	209 ± 15	252 ± 13
ethyl decanoate	154 ± 3	140 ± 17	164 ± 36	153 ± 11	101 ± 7.4	131 ± 7.3	132 ± 0.5	152 ± 5.4	89 ± 1	140 ± 4.4

Table S5. Mean values of the sensory ratings following DA of small batch Shiraz wines produced in 2013, 2014, 2015 from the Barossa Valley. P value represents discriminating power of variables observed between old and young samples for each attribute.

Season 2012/13													
Site + age class	A-Red Fruit[a]	T-Bitter[a]	T-Fresh Red[a]	AT - Length[a]	F-Dark fruit[b]	F - Savoury[b]	AT - Alcohol[b]	A - Savoury	A - Leather	T - Sweet	A - Tobacco	P - Confection	A - Confection
Site 1 Old	5.964	6.188	7.493	6.055	6.817	2.294	8.913	3.004	2.149	3.871	1.641	4.191	4.060
Site 1 Young	4.252	6.502	5.981	5.852	7.367	2.789	8.916	3.582	3.136	3.884	2.912	3.306	2.929
Site 2 Old	6.303	6.244	5.880	5.113	5.701	3.031	7.441	3.089	1.886	2.646	1.552	3.403	4.554
Site 2 Young	5.172	6.816	5.595	5.028	6.149	3.543	8.838	3.963	3.180	3.438	3.104	2.933	3.479
Site 3 Old	5.333	5.587	6.196	6.192	7.266	2.430	8.553	2.745	1.789	4.182	2.232	4.141	3.691
Site 3 Young	4.940	5.491	6.254	6.445	7.980	2.124	9.003	3.196	1.877	4.879	1.693	4.623	3.722
Site 4 Old	5.167	5.946	5.622	6.131	7.243	2.676	9.054	4.521	2.697	4.202	2.506	3.024	2.431
Site 4 Young	5.089	4.776	6.392	7.668	7.651	2.051	8.488	2.309	1.533	4.740	1.817	4.218	3.517
Site 5 Old	5.696	6.607	5.134	5.872	6.780	2.929	8.677	3.597	2.145	3.764	1.703	3.787	4.375
Site 5 Young	5.920	6.005	6.785	6.643	7.502	2.486	9.775	3.105	2.135	3.571	1.326	4.073	4.577
p	0.022	0.050	0.014	0.001	0.007	0.001	0.001	0.005	0.003	0.003	0.014	0.012	0.026

Season 2013/14											
Site + age class	A-Red Fruit[a]	T-Bitt[a]	T-Fresh fruit[a]	AT-Length[a]	T-Acid[b]	M-Weight[b]	A-Complex[b]	Tannin[b]	F-Hrb	A-Fresh fruit	F-Flor
Site 1 Old	8.455	8.834	7.936	8.842	8.545	8.345	8.133	8.960	5.276	7.427	6.821
Site 1 Young	8.934	8.967	8.091	9.304	8.945	8.826	8.780	9.014	4.587	8.922	7.375
Site 2 Old	8.556	7.451	6.731	7.908	8.594	7.447	7.478	7.920	6.072	7.614	6.675
Site 2 Young	6.725	6.972	5.848	6.765	6.893	7.757	7.325	7.787	8.264	5.426	4.814
Site 3 Old	7.903	8.616	9.011	8.859	8.846	9.272	9.318	8.877	5.072	8.016	7.119
Site 3 Young	8.311	8.413	7.809	8.425	7.889	8.314	8.062	8.458	4.964	7.887	7.949
Site 4 Old	8.495	8.720	7.988	8.375	7.914	8.822	8.462	8.789	6.031	8.036	7.214
Site 4 Young	8.745	9.238	6.558	9.419	9.881	9.700	9.768	10.007	5.068	7.052	6.574
Site 5 Old	9.511	9.951	8.233	10.056	8.698	9.528	9.452	10.324	5.593	8.581	8.521
Site 5 Young	8.571	9.656	7.409	9.716	8.804	9.570	9.308	10.332	4.891	7.516	7.559
p	0.032	0.001	0.003	0.001	0.001	0.040	0.012	0.001	0.001	0.017	0.035

Season 2014/15

Site + age class	A-Red fruit[a]	Bitter*[a]	T-Fresh fruit[a]	AT-Length[a]	F-Dark fruit[b]	A-Dark fruit[b]	F-Savoury[b]	T-Acid[b]	M-Weight[b]	Alcohol*[b]	A-Complex[b]	Tannin*[b]	A-Herb	A-Dried fruit	F Mocha-Chocolate
Site 1 Old	8.520	4.406	9.353	8.295	9.585	8.665	5.012	6.352	8.698	8.799	6.523	6.376	5.606	3.688	4.026
Site 1 Young	5.876	5.380	6.239	8.489	10.123	10.441	7.209	7.114	10.356	9.953	8.835	7.493	4.258	6.623	4.795
Site 2 Old	6.359	3.753	8.623	5.483	7.509	7.626	6.111	5.967	6.788	5.866	6.239	5.090	5.723	5.242	3.356
Site 2 Young	6.155	3.343	8.618	6.326	8.862	8.271	5.897	5.550	7.189	6.615	7.186	5.062	6.733	6.247	3.338
Site 3 Old	7.061	5.542	8.439	8.389	10.445	10.029	5.512	7.189	9.392	8.512	7.352	9.171	5.391	4.927	4.338
Site 3 Young	6.933	4.922	7.661	7.723	9.374	9.489	4.724	6.602	8.477	8.219	6.868	7.467	4.323	5.421	3.952
Site 4 Old	8.009	4.173	9.679	8.544	8.886	8.405	5.239	7.995	9.024	7.996	7.015	6.960	6.109	4.376	3.211
Site 4 Young	5.491	4.627	8.059	8.323	10.695	9.948	5.438	7.520	9.344	10.070	6.855	8.248	4.376	5.598	4.806
Site 5 Old	7.159	3.416	9.492	7.873	8.558	8.659	4.694	8.023	6.947	6.889	4.611	6.779	3.629	4.868	2.642
Site 5 Young	6.739	4.322	8.665	7.533	8.262	8.705	4.082	8.529	7.177	7.699	6.403	8.384	4.432	5.918	2.771
p	0.038	0.022	0.008	0.001	0.005	0.007	0.012	0.001	0.001	0.001	0.011	0.001	0.001	0.01	0.014

Table S6. Wine composition of Barossa zone Shiraz harvested in 2013, 2014 and 2015 from five sites each with two age classes**.**

	2013				2014				2015			
Site 1	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD	Old	Young	P-value	5% LSD
Wine Alcohol %	16.15	17.17	ns	ns	13.91	14.19	ns	ns	16.27	17.22	ns	ns
Wine pH	3.58	3.66	ns	ns	3.41[b]	3.52[a]	0.044	0.11	3.79	3.80	ns	ns
Wine TA	7.04	7.12	ns	ns	11.70	11.60	ns	ns	6.58	7.05	ns	ns
Site 2												
Wine Alcohol %	14.35[b]	16.25[a]	0.04	1.69	13.69	13.59	ns	ns	13.59	14.14	ns	ns
Wine pH	3.50[b]	3.69[a]	0.01	0.13	3.74[b]	4.06[a]	0.001	0.10	3.86	3.95	ns	ns
Wine TA	7.03	6.67	ns	ns	10.4[a]	9.81[b]	0.002	0.24	6.22	6.17	ns	ns
Site 3												
Wine Alcohol %	16.23	16.55	ns	ns	14.37[a]	14.05[b]	0.049	0.32	16.23	15.89	ns	ns
Wine pH	3.64	3.79	ns	ns	3.49[b]	3.70[a]	0.006	0.11	3.73[b]	3.92[a]	0.009	0.11
Wine TA	7.44	7.02	ns	ns	7.41[a]	6.52[b]	0.003	0.39	7.02	6.37	ns	ns
Site 4												
Wine Alcohol %	17.51[a]	16.50[b]	0.02	0.71	13.53[b]	15.61[a]	0.001	0.60	15.40[b]	16.90[a]	0.002	0.60
Wine pH	3.51[b]	3.61[a]	0.04	0.10	3.50	3.63	ns	ns	3.41[b]	3.66[a]	0.000	0.07
Wine TA	7.47	7.24	ns	ns	6.74	7.20	ns	ns	8.04[a]	7.48[b]	0.002	0.23
Site 5												
Wine Alcohol %	16.88	16.61	ns	ns	15.65	15.31	ns	ns	14.92	15.38	ns	ns
Wine pH	3.77	3.77	ns	ns	3.24	3.27	ns	ns	3.67	3.63	ns	ns
Wine TA	6.74	6.68	ns	ns	10.30	8.76	ns	ns	7.42[b]	8.16[a]	0.002	0.29

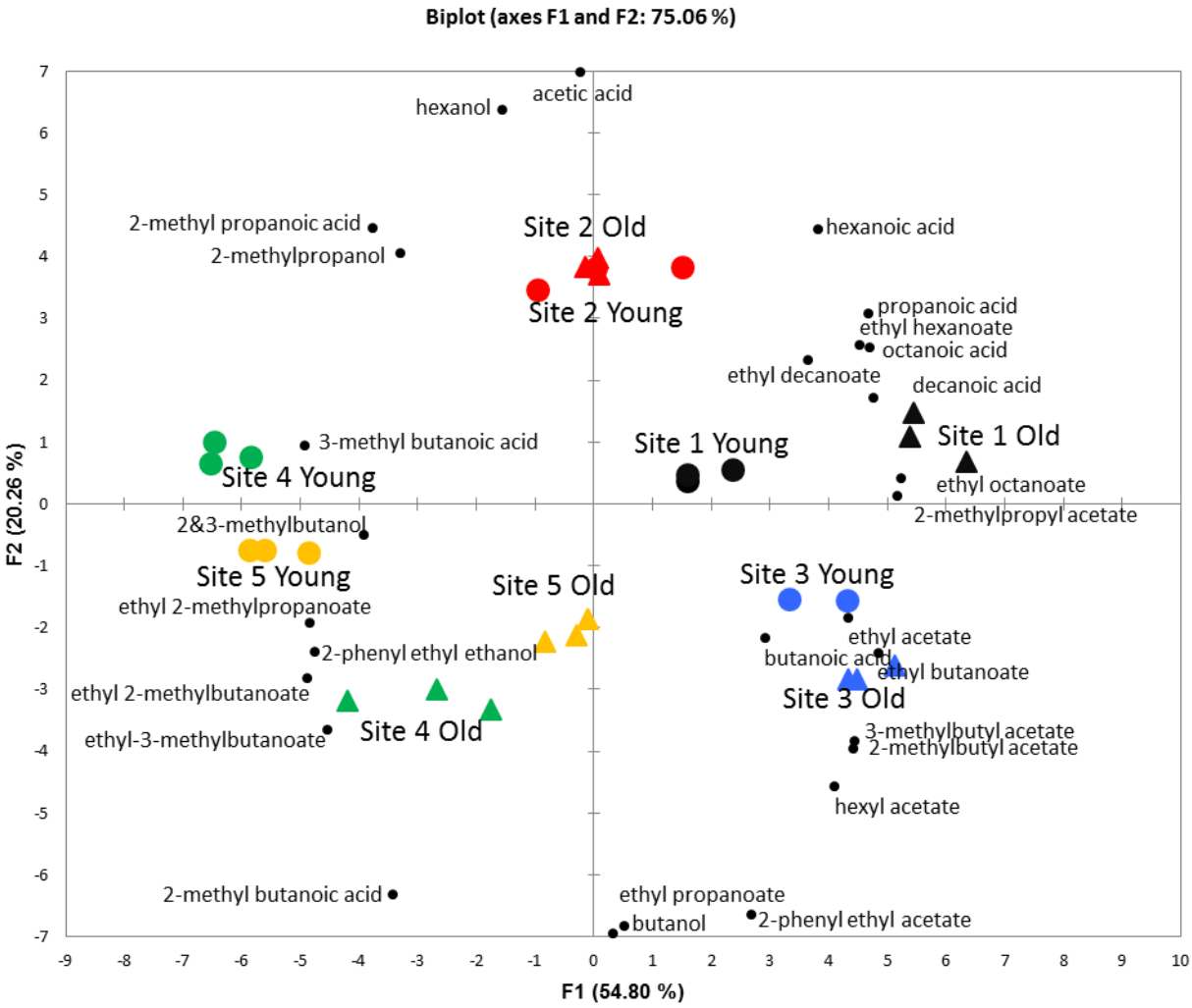

Figure S1. PCA biplot showing scores and loadings of volatile compounds by winemaking replicate from 2015 Barossa Shiraz wines. Labels indicate vine age at each site with common colours representing each site, marker shape represents nominal age classification where ▲=old and ●=young. Black = Site 1, Red = Site 2, Blue = Site 3, Green = Site 4, Orange = Site 5.

Chapter 6 Prepared Manuscript 4: The multiple influences of DNA methylation in *Vitis Vinifera* L. cv. Shiraz; chronological age, environment and propagation technique.

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Name of Principal Author (Candidate)	Dylan Grigg
Contribution to the Paper	Designed and conducted the research experiments, analysed the data and edited and constructed the manuscript.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The multiple influences of DNA methylation in grapevine *Vitis vinifera* L. cv. Shiraz; chronological age, environment and propagation technique.

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Abstract

Fruit traits affecting wine quality are largely reliant on the location or 'terroir' of the particular vineyard of origin. However, plant age since establishment is also suggested to impart increased quality to wines. In other long living plants, phenotypic differences between plants at different developmental stages have been associated with the accumulation of epigenetic markers acquired through rounds of mitotic cycles. Although geographic location has been shown to influence the epigenome of plants, to date no investigation into the planted age of the perennial grapevine has included genetic, or epigenetic analysis which could have impacts on viticultural and oenological quality traits. Here we used methylation sensitive Genotyping by Sequencing to study the combined effect of plant age, vineyard location and propagation system on the DNA methylation profile of five commercial Shiraz vineyards planted with a unique cohort of vines ranging in age from 6 to 168 years. Differences in plant methylation profiles were found to be mainly driven by geographic distance between vineyards. The second driver of epigenetic differentiation was positively associated to differences in vine age. Our results also indicate that while vegetative propagation via dormant cuttings induces alteration of DNA methylation, layering does not seem to affect the DNA methylation profile of the new propagules. We speculate that DNA methylation differences (or the absence of differences) between the mother (or old plant) and young plants propagated with each system might be related to differences in quality traits of fruit and wine produced from plants of different ages. We finally discuss the possible implications of our results on the improvement of environmental adaption which may result in increased quality or production outputs.

Introduction

The cultivated grapevine (*Vitis vinifera* L.) is an economically important crop throughout the world. The perennial nature of its cultivation means that following establishment, individual plants may survive for many decades or even centuries [1]. *V.vinifera* readily outcrosses and therefore does not breed true from seed [2]. In order to preserve desirable phenotypic attributes, elite cultivars have been selected and maintained over time via vegetative propagation [3], eliminating genetic recombination via meiosis [4]. For this reason it is difficult to establish when the ancestral seedling first germinated [3] to establish true biological age. Since meiosis is avoided vine age must be referred to as the chronological age (time) since planting. Increasing chronological age following establishment is an unavoidable attribute to which every vine is subjected. The length of time since planting has been shown to have an impact on both fruit yield and wine quality [5,6]. Several studies into the effect of age on vine performance or wine quality have been undertaken, however none presented significant vine ages (i.e. >100 years) [5-10]. Our recent findings indicate that vine age has a positive effect on yield (chapter 3). Moreover, sensory analysis of fruit and wine over three seasons found consistent differences in berry and wine sensory attributes, this association with vine age was supported by metabolomic analysis of volatile compounds (chapter 5). However, our understanding of the molecular processes involved in the regulation of grapevine's agronomic performance with age is minimal.

Plants being sessile have developed strategies to adapt to their environment, specifically via epigenetic modification of their genome [11,12]. Epigenetic mechanisms allow an organism to respond to its environment through changes in gene expression, without corresponding changes in the underlying genome. One of the most well characterised epigenetic mechanisms is DNA methylation. In plants, methylation of cytosine bases can occur in three contexts CG, CHG or CHH, where H is any nucleotide other than G [13,14]. DNA methylation has been identified in a large range of plants and been implicated in a wide variety of phenotypic variation, or plasticity [15-20].

There are many examples in the literature of perennial plants displaying epigenetic adaption to local environments or pressures. Specific examples can be found in relation to; salinity in mangrove- *Laguncularia racemose* [21], drought in Holm oak- *Quercus ilex* [18], herbivory in Aquifoliaceae- *Ilex aquifolium* [17] and white clover- *Trifolium repens* [22] and environment in

White poplar- *Populus alba* [23]. Epigenetic adaption to environment has even very recently been reported in grapevine- *Vitis vinifera* [24].

In general DNA methylation levels have been reported to increase with increasing chronological age of plants or plant organs [25-29] and is a sign of morphogenetic ability loss (such as ability to root) in mature plants [30,31]. While this is relatively consistent across species, several studies have reported reduced methylation or de-methylation with age [32-34]. In other clonally-propagated heterozygous perennials, DNA methylation has been associated with specific phenotypes. For example in *Prunus dulcis* (almond), non-pathogenic decline has been associated with DNA (de) methylation [35] while in *Pinus radiata* DNA demethylation has been found to be a strong indicator of plant rejuvenation [25].

Since this study used closely related plants from the same cultivar and cultivated populations, their age since sexual reproduction is assumed to be identical, while it is known chronological age since planting is not. Next generation sequencing (NGS) and the methylation sensitive genotyping by sequencing technique (ms-GBS) were used to describe genome wide methylation profiles between cohorts of young and old vines with age differences ranging from 56 to 151 years since propagation.

The vineyards used in this study are renowned for producing highly priced premium wines, which in most cases wines from the older plants are valued higher than those from younger plants (Table S1). Grape and wine quality is a complex topic subject to a broad range of influences, the sum of these many influences are known as 'terroir' [36]. Probably the most obvious driver of terroir is the grapevine variety or genotype. However, genotypic similarity does not guarantee phenotypic similarity due to plasticity resulting from gene regulation under different environmental conditions [37]. Other drivers of terroir have been shown to be related to vineyard location (chapter 3), non-volatile composition (chapter 4) and age (chapter 5). Here we aim to test the hypothesis that plant age, vineyard location and propagation system have a measurable effect on grapevine DNA methylation profile.

Materials and methods

Plant material selection and collection

While there is no universally agreed definition of when a vine is 'old', for the purpose of this research older vines at each site will be referred to as 'old' while younger vines will be referred to as 'young'.

Five commercial Shiraz (*Vitis vinifera* L.) vineyards containing vines of contrasting establishment ages were selected within two regions of the Barossa Valley zone of South Australia (Table 1). Sites were chosen with the assistance of local industry members and vineyard owners to ensure an accurate record of vine age, and to minimise differences between vines within each site. Consideration was given to homogeneity of site and vine characteristics between vine age groups and within each site such as: soil type, row orientation, pruning method, trellis system and vineyard floor management. Vines on the end and outside rows were excluded, each replicate row of vines was chosen to avoid missing vines internally preventing reduced competition effects. Each selected site contains vines established at two unique times, old vines ranging from 93 to 168 years, and for comparison, younger vines aged from 6 to 49 years (Table 1). All young vines with exception of site 5 were vegetatively propagated via calloused hardwood cuttings from older vines from the same site. Site 5 was propagated by layering a long cane from the mother plant underground creating a new trunk, the connection was severed after 5 years. All vines were growing on own roots. Virus status is unknown, vine health was assessed visually based on uniformity, representative growth and appearance with the assistance of each vineyard manager / owner. Clonal status of vine material is unknown but, as all vines were propagated via cuttings from the same site they are considered to be a mass selection or quasi-clones*.

At each vineyard, three replicate blocks containing 4 vines in 3 separate rows were selected resulting in 12 vines per age treatment per site. At sites 1-4 each vineyard block was a discrete unit surrounded by vines of the same age. The younger plants at each site were planted in land specifically prepared for establishment with new trellis infrastructure. Due to the propagation technique at site 5, old and young vines are interspersed resulting in two adjacent rows of vines being selected and grouped to form treatment replicates.

Samples from sites 1-5 were all collected in the spring of 2014 during the pre-dawn hours of 2-5am at the standardised grapevine growth stage defined as budburst (E-L stage 4-7) [38]. Leaf samples were collected into 2mL Eppendorf tubes and immediately snap frozen in liquid nitrogen in the field. Samples were transported to the University of Adelaide Waite campus for storage at -80°C until DNA extraction. A total of 120 leaf samples were collected from five sites and two age groups representing 12 vine replicates per site of each age designation.

* Quasi-clone refers to vines that are propagated from a known population of vines NOT one specific vine as a true clone would be.

Plant propagation

To allow the direct comparison of the epigenetic profiles of mother vines, dormant cuttings and layered vines at the time of establishment, new plants were propagated from the same mother plants using both propagation systems. The vineyard manager selected 7 mother vines that had adequate growth to replace a missing vine directly adjacent to it. Selected vines were pruned on the 16th of June 2015 leaving one long cane which was layered approximately 10-15 cm under-ground before being brought above ground and supported by string and a protective guard. From the same vine a selection of approximately 10 canes was made; these were trimmed in the field, bundled and taken to The University of Adelaide Waite campus where they were briefly placed in water before being re-trimmed to 300 mm in length and planted in a sand filled heat bed set to 21°C. Upon callus formation (approximately 6 weeks later) vines were transplanted into neutral potting media in preparation for transportation to the vineyard and re-planting alongside layered canes. Prior to budburst one random cutting per mother plant was selected and planted in an identical protective guard as the layered vine. Both layered vines and cuttings were watered weekly with equal quantities of water to ensure adequate soil moisture. The first formed leaf was collected from each plant type on the 23rd September 2015 at budburst (E-L stage 4-7). Leaf samples were taken in triplicate from each plant and immediately snap frozen in liquid nitrogen in the field. Samples were transported to the University of Adelaide Waite campus for storage at -80°C until DNA extraction.

DNA extraction

Leaf tissue with a fresh frozen mass of 60mg was transferred to 1.1mL microtubes containing one single tungsten ball, samples were cryogenically ground using a Geno/Grinder© 2010 Automated cell lyser, (SPEX sample prep, Metuchen, NJ, USA). DNA extraction was undertaken utilising the LGC genomics oKtopure™ liquid handling robot and sbeadex™ chemistry (LGC limited, Berlin, Germany) at the Australian Centre for Plant Functional Genomics (University of Adelaide, Waite campus Urrbrae). The manufacturer's protocol was followed using an LGC 'maxi-prep' DNA extraction kit with the following modifications; addition of 2% w/v PVP-40 (Sigma-Aldrich, Steinheim, Germany) to the proprietary lysis buffer prior to incubation, plus the addition of 7 units of RNase per mL of lysis buffer prior to extraction (Sigma-Aldrich, Steinheim, Germany).

Following extraction, DNA concentration was measured using PicoGreen© quantification reagents (Thermo Fisher scientific, Wilmington, DE, USA) further concentration determination was made using Thermo Scientific NanoDrop™ 1000 spectrophotometer

(Thermo Fisher scientific, Wilmington, DE, USA). All DNA samples were diluted to a working concentration of 20ng/µl.

Methylation sensitive genotyping by sequencing (ms-GBS)

Library preparation for ms-GBS was performed using a modification to the original Genotyping By Sequencing (GBS) protocol of Elshire, et al. [39] as described by Kitimu, et al. [40]. In short; two sequencing libraries with independently barcoded samples were prepared using 93 samples and 1 blank control per library by genomic DNA restriction using a combination of two restriction enzymes, followed by ligation of sequencing adapters containing co-adhesive ends for both restriction enzymes and by PCR amplification of the ligation products.

Restriction of 200 ng of genomic DNA was carried out in a reaction volume of 20µl containing 2µl of 10X NEB smart cut buffer and 8U of restriction enzymes *Msp*I and HF-*Eco*RI (New England BioLabs Inc., Ipswich, MA, USA) by incubating the reaction mix at 37°C for 2 hours, followed by an inactivation step of 65°C for 20 minutes in a Bio-rad T-100 Themal cycler. A set of 96 barcoded adapters with an *Msp*I overhang and a common Y adapter with an *Eco*RI overhang were designed for the ligation reaction and annealed prior to ligation as described by Elshire, et al. [39]. The ligation reaction (40µl in total) was carried out on the same PCR plate adding T4 ligase (200U), 10X NEB T4 ligase buffer, 0.1pmol of the barcoded *MspI* adapter and 15 pmol of the common *Eco*RI Y adapter to each of the restriction products.

Ligation was completed at 22°C for 2h followed by an enzyme inactivation step of 65°C for 20min. In order to remove non-ligated adapters ligation products were purified by mixing a 1 volume of ligation product with 0.85 volumes of AMPure XP magnetic beads (Agencourt Bioscience Corporation, Beckman Coulter) as per the manufacturers guidelines. Concentration of purified samples was determined via NanoDrop™ 1000 spectrophotometer. Samples were equimolarly pooled to a uniform concentration of 40ng/uL into two libraries each containing 93 DNA samples and one water control.

Each library was then divided into eight PCR tubes for PCR amplification. Each 25µl PCR reaction consisted of 10µl DNA library, 5µl of Q5 high fidelity reaction buffer, 2µl of 10µM forward and reverse primers, and 0.5µl dNTP. PCR Reactions were performed in a Bio-Rad T-100 Thermal cycler for 16 cycles of 95°C (30 sec), 62°C (30 sec), 68°C (30 sec), with an additional step at 72°C for 5 min. All eight PCR products from each library were pooled into a single volume and size selected for fragments bigger than 200bp using AMPure XP magnetic beads as described above. Captured fragments were eluted in 30ul of water prior to

measurement of final concentration using Qubit (ThermoFisher Scientific). Library fragment size range in each library was confirmed using a Bioanalyser High Sensitivity DNA assay (Agilent Genomics). Each individual library was 75 bp paired-end sequenced on the Illumina NextSeq V4 platform at the Australian Genome Research Facility Ltd (AGRF), Adelaide, South Australia.

Genetic and Epigenetic analysis

The raw reads from the Illumina NextSeq platform in fastq format were first de-multiplexed into respective samples using the barcode sequences. GBSX v1.1 [41] was used allowing for zero mismatches in the barcode or enzyme cut site with hammings algorithm to detect mismatches or indels. Paired end reads were merged using bbtools package [42] prior to being aligned to the 12x grapevine reference genome (http://plants.ensembl.org/Vitis_vinifera/) using Bowtie2 and sorted using samtools.

Single nucleotide polymorphism (SNP) calling was undertaken using the Tassel 5.0 GBSv2 pipeline (http://www.maizegenetics.net/tassel). Sequencing reads containing identified SNPs were mapped to the *V.vinifera* 12x genome and filtered using a quality score of 30 and minimum read depth of 10. Further filtering was undertaken to discard SNPs that were present in less than 90% of samples, in addition SNPs with a minimum allele frequency of 0.001 and a maximum allele frequency of 1.0 were discarded. SNPs within water control wells were discarded. Low quality or non-call (NC) loci accounted for 0.03% of total SNP loci, NC loci less than 10% were accepted and remaining NC loci were removed. Remaining SNPs were imported into Tassel for analysis subjected to Principal Coordinate Analysis (PCoA) to determine if any underlying genetic structure was present within the sample cohort. Further K-means clustering and discriminant analysis of principal components (DAPC) [43] using the adgenet (http://adegenet.r-forge.r-project.org/) package for R software [44] was undertaken to further validate if any inherent genetic structure was present.

Differences in DNA methylation between populations (here individuals of the same age within a vineyard are considered a population) was inferred by quantitative analysis of sequencing coverage generated for each loci (i.e. the higher the sequencing coverage in a given locus the lower the methylation on that locus) as shown before for the analysis of methylation sensitive amplified polymorphisms (MSAPs) [45] and for ms-GBS data [40]. In this case DNA methylation analysis was undertaken using a combination of in-house scripts and freely available packages in the R software environment [44]. Filtered reads were mapped to determine the density of ms-

GBS markers across the *V. vinifera* genome using a 500kb sliding window approach. Both GC% and gene density statistics were exported from the biomart tool within ensemble plant online database (http://plants.ensembl.org).

Differential methylation analysis was performed using the ms-gbsR package created by Benjamin Mayne (http://github.com/BenjaminAdelaide/ms-gbsR). Read counts from sorted and indexed bam files are read into the R environment to detect differential methylation using the diffmeth.R function and edgeR package [46]. The diffmeth function within the ms-gbsR package removes cut sites with low coverage (i.e. <1cpm), following this library size is recomputed using the default trimmed mean of M-values (TMM) before estimating dispersion parameters and fitting a gene wise negative binomial GLMs output. Similarity between groups was estimated using Mahalanobis distance [47] which was then used to build unrooted Neighbour Joining (NJ) trees. The significance of the observed differences in locus coverage was inferred via a coefficient contrast analysis utilising the Benjamini Hochburg method for p-values, adjusted for multiple comparisons using the false discovery rate (FDR). Loci presenting a FDR lower than 0.05 were deemed differentially methylated markers (DMMs). Variable thresholds for each specific comparison were used to adjust for stringency and ensure the number of DMMs were found in an adequate number of plant samples.

Further analysis was carried out using the FIEmspro package (https://github.com/wilsontom/FIEmspro) to compare grouping factors via principal component linear discriminant analysis (PCLDA) and hierarchical clustering analysis (HCA).

Samples were then grouped into different hierarchical levels by geographic origin, age and propagation system. Pairwise comparisons between all groups were carried out to generate matrices for geographic distances (in Km) (calculated using GenAlEx 6.5 [48], age differences (in years since planting) and number of DMMs. Finally, the lists of detected DMMs for each comparison were interrogated via the program bedtools 'closest' feature (Unix environment) to determine the distance of DMMs to annotated genes on the *V. vinifera* 12x reference genome. Genes found within 5kb of a DMM were considered as differentially methylated between the compared populations.

Results

Effect of plant location, age and propagation system on epigenetic variation

Mapping of ms-GBS markers

One of the key advantages of using the ms-GBS technique is that it simultaneously provides data that can be used for the analysis of DNA methylation and also SNP based genotyping. A total number of 539,373,059 raw reads remained after quality filtering and alignment. Sliding window analysis of the density of restriction sites that generated sequencing reads that passed filtering revealed the ms-GBS protocol generated a similar number of markers per 500kb window (Figure 1 Track 2) across all chromosomes. A total of four windows in chromosomes 8, 9, 12 and 18 did not contain any ms-GBS markers. All four windows mapped to high CG and gene poor regions (Figure 1 Tracks 3 and 6).

Analysis of genetic variability

To determine if any genetic structure is present within the five vineyards surveyed a SNP analysis was undertaken. With all samples being field labelled as the same cultivar this was undertaken to determine if any underlying relationships might be present or out-groups identified as mislabelled cultivars. A total of 47533 SNPs were identified which were reduced to 14208 SNPs after quality filtering.

Principal co-ordinate analysis (PCoA) was carried out using a distance matrix of the filtered SNP dataset, this showed no evident genetic groups within the vines in this study (Figure S1). More stringent analysis was undertaken by applying Discriminant analysis of principal components (DAPC) [43]. Firstly, unsupervised K-means clustering was performed to partition variation into within group and between group components while attempting to minimise the latter. To determine if there is any structure within the data set the maximum possible numbers of PC's (120) were chosen. Figure S2A displays the cumulated variance explained by the eigen values whereby the smooth curve indicates no clear groups are present. Following this the Bayesian Information Criterion (BIC) value (i.e. the best number of clusters that is less than the number of individuals and explains the observed genetic variability) was determined to be one (Figure S2 B), indicating that there is no genetic structure in the dataset. This validates the observation of the PCoA analysis which also presents no structural grouping within the SNP matrix.

Analysis of epigenetic variability

Analysis of the relative effect of vineyard location, propagation system and vine age on plant DNA methylation profiles

Similarity analysis of the ms-GBS profiles using NJ trees based on Mahalanobis distance between groups showed two main clades (Figure 2A). These clades separated young and old vineyards in site 5 from the rest of the samples. On the clade containing sites 1 to 4, vineyards were separated into two sub-clades according to their region of origin (i.e. sites 1 and 2 Eden Valley and sites 3 and 4 Barossa Valley). Finally, vineyards on the Eden Valley clade grouped by site, while the Barossa Valley clade grouped by age. HCA presents only one cluster that separated by age which contained the two oldest sites in this study. Individually these sites each present the greatest chronological age difference between young and old vines in the study; at 116 and 151 years respectively. Similarly, PCLDA analysis of the ms-GBS generated DNA methylation profiles on the full dataset grouping by site shows that 28% of the variation results from one site alone (site 5) (Figure 2B). The remaining 15% of the variation is likely to be environmental as sites of both ages are spread along DF2. More precisely, this may be displaying subregional effects. Sites 1 and 2 are both found in the cooler and more elevated Eden Valley and display more positive loadings on DF2. In contrast sites 3 and 4 present more negative loadings and are found in the warmer Barossa Valley region. Minor separation between plants of different ages within the same site on DF2 (15%) presents the influence of age differences between samples.

The number of significant (FDR <0.05) differentially methylated markers (DMMs) was calculated on pairwise comparisons based on age at all sites (all old v all young) and then individually at each site. The majority of DMMs were able to be mapped to resolved positions on the 12x grapevine reference genome (Table 2). Markers mapped to unresolved genomic regions are indicated on Table 2 but were excluded from subsequent analysis unless noted.

Following calculation of the number DMMs within sites, all pairwise inter- and intra-site comparisons were also computed (Site 1 Old vs Site 1 Young, Site 1 Old vs Site 2 Old and so on) (Table S4). This DMM matrix was combined with distance and age matrices to determine how age and geographic differences affect the number of detected DMMs by plotting all three matrices as a contour plot (Figure 2C). The presence of a high number of DMMs on the diagonal indicates that both geographic distance and age difference contribute to epigenetic differentiation between samples. However, the high number of DMMs detected at large geographic distances irrespective of age difference indicates that geographic distance is a major

contributor to epigenetic variability. Finally, the general pattern of increased epigenetic differentiation with plant age difference was distorted by the absence of DMMs detected between old and young vines from site 5 (Table 2).

We then attempted to find DMMs purely associated to plant age differences and not influenced by vineyard location or propagation system. To achieve this, two groups were created containing plants from all vineyards separated into old and young this resulted in zero DMMs. This finding was surprising since the effect of methylation differences was observed earlier in the matrix and in the contour plot in Figure 2C.

Effect of propagation system on plant DNA methylation profiles

In order to individually examine the effect of the propagation system, we first compared the epigenetic profiles of all plants from sites 1 to 5 grouped by propagation system (layering or dormant cuttings) and age (young or old). PCLDA of ms-GBS generated DNA methylation profiles shows that the propagation system used to generate new plants in each vineyard and plant age explain 83% of the observed epigenetic variability (Figure 3A). Plants propagated using layering (old_lay and young_lay (at Site 5)) separated from the rest of the sites on Discriminant Factor 1 (DF1) which presents 63% of the total variation in the dataset. DF 2 (20% of the total variability) separates plants by age irrespective of propagation technique. The young plants, from all locations are consistently located on the positive quadrants of DF 2, and old plants are located on the negative quadrants of DF 2.

In order to better determine the effect of different propagation systems on the epigenetic differentiation between mother plants and propagules an *in situ* propagation experiment was undertaken. Based on 7 old plants from site 5, we analysed the epigenetic differentiation between plants propagated by dormant cuttings and layering from the same mother plants during 2015. NJ tree based on Mahalanobis distance revealed that while layered plants are practically identical to their mother plants, dormant cutting propagation induces large scale epigenetic differentiation in leaf tissue (Figure 3B). Moreover, the number of DMMs detected between mother plants and cuttings was 3950 DMMs, where zero DMMs were detected between the same mother plants and layered propagules (Table 2).

Effect of location and plant age on plant DNA methylation profiles

Due to the strong influence that propagation system showed on DNA methylation profiles, we next selected only vineyards propagated via dormant cuttings (sites 1-4) to investigate the potential influence of vineyard geographic origin and age. The ms-GBS profiles derived from

these four sites were analysed by PCLDA (Figure 4) which shows that 25% of the variation (DF1) appears to be associated with environment (site). More precisely, this may be displaying subregional effects. Sites 1 and 2 are both found in the cooler and more elevated Eden Valley region of the Barossa zone and display more negative loadings on DF2. In contrast sites 3 and 4 present more positive loadings and are found in the warmer Barossa Valley region. Furthermore, minor separation on DF2 (15%) presents the influence of age differences between samples in addition to the regional site separation.

Initially DMM analysis was undertaken comparing all old vine samples to all young vine samples to test common markers associated with plant age. This resulted in zero DMMs being found. This finding was surprising since the effect of methylation differences was observed earlier in the matrix and presented in the contour plot in Figure 2C. The strong influence of propagation technique uncovered via previous PCLDA was a possible explanation for overriding differential pairwise comparisons of age across all sites.

The observation of the influence of age over the number of detected DMMs in vineyards propagated by dormant cuttings but not by layering prompted further analysis to determine the effect of age between each related site. We then calculated DMMs in vineyards 1 to 4. Sites 1 and 2 (with age differences of 56 and 79 years respectively) did not present any DMMs when the ms-GBS profiles of young and old vines were compared independently. However, sites 3 and 4 (with age differences of 116 and 151 years respectively) showed 1564 and 1883 DMMs respectively. Interestingly, the distribution and density of these DMMs across the *V. vinifera* genome follows similar patterns independently of the vineyard analysed (Figure 1 tracks 7 and 9). Analysis at a chromosome level of the directionality of methylation change (i.e. loss versus gain of methylation (hypomethylation and hypermethylation respectively) in young plants compared to old plants on sites 3 and 4, showed that both types of changes occur across the genome (Figure 1 tracks 7 and 9).

Finally, as described above, we interrogated our ms-GBS results for DMMs purely associated to plant age differences by separation of plants with consistent propagation technique from sites 1-4 into old and young. This analysis yielded 135 unique loci that were found to be differentially methylated between old and young plants (Table 2). Most of these DMMs were found to regionally cluster across all chromosomes (Figure 1 track 5). The analysis of directionality of methylation change in young plants compared to old plants from sites 1 to 4

combined, showed similar patterns to those observed for sites 3 and 4 analysed independently (Figure 1 track 4, 7 and 9).

Analysis of the effect of dormant cutting propagation on gene methylation

To determine the potential functional changes induced by the observed flux in DNA methylation between old and young plants, we searched for genes within 5Kb of the detected DMMs (deemed as differentially methylated genes (DMgs) hereafter) for sites 3, and 4 and also for plants propagated via dormant cuttings on site 5 (MvC) during 2015. Analysis of the distribution of DMMs around genes shows the majority of the observed changes in DNA methylation between old and young plants happen in the body of genes (i.e. between the Transcription Start Site (TSS) and the Transcription End Site (TES)) (Figure 5). The number of detected DMMs then decreases with distance from the gene body. This decrease is symmetrical in both directions (i.e. both from the TSS and the TES).

We then counted the number of genes deemed as differentially methylated in each of the three comparisons described in the previous paragraph. This analysis yielded a total of 5597 DMgs (For a complete list see Appendix file 1). Of these, 1980, 1966 and 2835 corresponded to sites 3, 4 and site 5 MvC respectively (Table 2 and Appendix file 1). The majority of the DMgs identified (80%) were vineyard specific (.i.e. were detected only in one vineyard), while 18% were common to two vineyards and 2% or 90 genes were common to all vineyards (Figure 6A-B). The number of DMgs shared by any two vineyards ranged from 370 (vineyards 3 and 5) to 465 (vineyards 3 and 4) (Figure 6B). Finally, the search for DMgs associated to the 135 unique loci found to be significantly differentially methylated between old and young plants when sites 1 to 4 where analysed together, yielded a total of 40 genes (Table S3); that were considered to be purely associated to plant age differences. DMgs were found in 12 of the 19 *V. vinifera* chromosomes both in gene poor and rich regions (Figure1 track 6).

Discussion

Grapevine age in terms of chronological time since planting is not a trait that can be replicated. The planted age of a vineyard can only advance with the passing of time. Therefore, time that passes from establishment to the present day creates a potential point of difference in terms of marketing for the final product, wine. This raises the question; does greater chronological age influence wine quality? Certain media and experts suggest this is true [49]. The vines used for this study are known produce wines of different quality levels, as judged by their final bottle

price; ranging from $30 to $699 (at the time of writing). They are also known to be among the oldest examples of their cultivar (Shiraz) in the world. Vine ages in this study range from 6 to 168 years since planting with a mean age difference of 98 years. Moreover, sensory analysis of fruit and wine over three seasons found consistent differences in sensory attributes associated with vine age, these were supported by metabolomic analysis in the final season.

The genetic similarity between clones (or quasi-clones) within sites was expected to be high since according to vineyard documented history, young plants were mass selected from old plants from the same site. Variability between sites was also expected to be low based on previous studies of intra-varietal diversity in grapevine [50-52]. These expectations were supported via the analysis of 14208 high quality SNPs using PCoA and DAPC (Figure S1 and Figure S2). None of the statistical tests applied revealed any genetic structure, either within sites or between them. However, as we have shown previously (see chapters 3, 4, and 5), genotypic similarity does not guarantee phenotypic similarity. This variability is possibly due to differences in gene regulation induced by environmental differences between vineyards, termed phenotypic plasticity [37]. Therefore, the aforementioned fruit and wine quality differences are not likely to be due to variation in the underlying genetic profile of each vineyard or age class.

This plasticity is known to be, at least partially controlled by epigenetic mechanisms such as DNA methylation [16-18]. This supports our findings which show that all tested variables (propagation system, vineyard location and plant age) contribute to the epigenetic differentiation between grapevine plants (Figure 2). According to our results, propagation system appears to be the major contributor to such differentiation (Figure 2A) with 25 to 63% of the total variability (Figures 2B and 3A). Moreover, both types of propagation systems tested here (layering and dormant cuttings) seemed to have a very different effect of the epigenetic separation of propagules from their mother plants. Our results indicate that while dormant cuttings induce large scale global epigenetic variation, layering seems to maintain the epigenetic profiles of mother plants in the generated propagules (Figure 3). This effect was also supported by the lack of DMMs detected between old and young plants at site 5 (Table 2 and Figure 2C). In fact, the lack of differential methylation induced by layering shown by HCA, PCLDA and DMMs was a possible explanation for overriding differential pairwise comparisons of age across all sites (Table 2).

It is possible, therefore, that the strength of the epigenetic similarity between samples of different age from site 5 alone negated finding any DMMs in the other 4 sites when pooled to

include sites 1-5. This is even more evident when removing site 5 from the analysis (retaining sites 1 to 4 only) resulted in a pooled comparison which yielded 135 significantly different DMMs between old and young plants (Table 2). This indicated that propagation technique could be responsible for the inability to identify consistent shared DMMs across all sites 1-5.

To confirm if the propagation technique was associated with the observed separation, data was re-analysed with propagation technique as the grouping variable (Figure 3). The results show a clear distinction between vines propagated via layering and those propagated via cuttings. If this effect resulted from a site-related environmental variable, it would be expected that sites 1-4 would show some separation as in Figure 2B as all sites are in different locations. However, Figure 3A shows sites 1–4 grouping by propagation technique first, then within that by age. This indicates that a unique DNA methylation profile is present due to propagation technique or by an extreme unknown local effect, represented here by DF2 with some variability due to chronological age on DF1. This is consistent with past findings that despite genetic similarity, epigenetic profiles can vary based on differing environments (Gao et al., 2010;Lira-Medeiros et al., 2010;Raj et al., 2011).

To further validate these findings a separate cohort of layered and dormant cuttings vines were propagated in parallel from the same mother vines at site 5. The results of tissue sampling at budburst resulted in a significant number of DMMs being present between mother and clone but not between mother and layered plants. Although at this point the mother and layer are still connected and effectively one plant, despite the presence of adventitious rooting underground, the results observed in the samples propagated in 2015 mimic those observed in plants propagated in the same vineyard during 2007 (Table 2), which were physically severed from their mother plants approximately five years later. The significant difference in DMMs (Table 2) in the vines propagated via dormant cuttings compared to their mother plants suggests that some form of epigenetic rejuvenation has occurred. Although this epigenetic rejuvenation effect induced by vegetative propagation is not unheard of (Fraga et al., 2012), the lack of plants generated via sexual reproduction in our experiment does not allow us to test this.

The second driver of epigenetic differentiation according to our results was geographic distance between vineyards (Figures 2C and 4). Epigenetic variability seemingly driven by differences in vineyard location ranged from 15 to 25% of the total observed variability as measured by PCLDA (Figures 2B and 4). Analysis of the number of DMgs found in all vineyards propagated by dormant cuttings showed that a great majority of these were only present in 1 (80%) or 2

(18%) of the vineyards analysed (Figure 6). This indicates that 98% of changes in DNA methylation around genes can be attributed to exposure to specific environmental conditions and not to aging alone. The effect of geographic distance is not surprising since the environmental effect of methylation profile on plant biodiversity in the same species has been suggested in grapevine [24] and in other species [23]. This effect was evident in the analysis of secondary metabolites in these sites which found a strong environmental effect based on region (chapter 4). However, the diagonal spread of relatively high DMMs on the age axis indicates not only that geographic distance has an influence on the presence of methylation markers but so too does age.

The influence that plant ageing has on DNA methylation has been the subject of past research. DNA methylation has been suggested to be a mechanism for epigenetic regulation of phase change [53] and whole plant maturity [54]. Our results indicate that the directionality of the observed changes in DNA methylation with plant propagation happens in both directions (hypomethylation and hypermethylation) (Figure 1 tracks 4, 7 and 9). Even though multiple studies have found that DNA methylation levels increase with chronological age [25,27-29,53,55], the literature is not unanimous on this subject [32-34]. Further analysis of the results shown here is needed to determine the general directionality of DNA methylation, if any, during vegetative propagation and ageing in grapevine.

A visual summary of DMMs can be seen in the circos plot in Figure 1 specifically in track 5 which presents DMM number in relation to gene density and closest gene feature. This shows that the majority of DMMs are found in regions that are gene rich, while markers are distributed more evenly along the chromosomes. In addition, markers within close proximity (within 5kb) to genes are found on only 12 chromosomes (Figure 1 track 6). The chromosomal regionality of the observed changes in DNA methylation and the correlations with genomic features hinted by Figure 1 indicates that further analysis of the results are needed

The pattern of DMMs found when comparing sites 1 – 4 presents an increase in markers with age difference. One limitation of this study is the number of sites available and therefore the data points are too few to apply a sound regression analysis. The strength of this study is the number of individual plants used per group (n=12) and the selection of t=10 for the methylation analysis, as this requires a differentially methylated marker to be present in a minimum of 10 samples during pairwise analysis in order to be accepted as a DMM. The increase in methylation markers with increasing age is consistent with previous studies [25-29,53,55]. In this

case the increase in chronological age presented the greatest number of markers in addition to the greatest spread of methylation changes in comparison between age classes. Not surprisingly changes in genomic methylation have been suggested to be useful as a marker of ageing [25].

The alteration in methylation profile based on age presents an interesting observation, especially in relation to the plant memory hypothesis [56]. The plant memory hypothesis suggests that response of exposure to a stress, through methylation episodes might lead to higher overall methylation levels, thus constituting a storage mechanism or memory of the response [56]. This would potentially be of interest considering greater chronological time since establishment may result in a higher frequency of epigenetic markers due to increased environmental adaption. In *Trifolium repens* this effect has recently been verified, where stress induced memory altered growth of clonal offspring [22]. If this is faithfully transferred to the progeny during propagation then transgenerational effects across clonal generations may be particularly important [12]. Via this mechanism it is possible that attributes of an emerging clonal plant might be significantly modified by the experience of the maternal plant that are no longer present: the result being potentially greater experience and resilience. However, these discussions are often related to ramets and clones that have adventitiously arisen from the maternal parent. This is not the case in viticultural production whereby a clone is severed from the parent prior to callus and organ formation. Similarly, in our study site 5 is an exception due to the new vines being propagated while still connected to the maternal plant, then being separated only after several growing seasons.

Over the productive life of a long lived species it is possible that many stressful events may occur. It has been shown that a single disturbance to a plant population may be retained as a unique epigenetic profile some 20 years later [57]. Considering this, the potential number of 'events' that a vine 170 years in age may have been subjected to could be numerous and significant. Plant hardening is described as a moderate or mild stress whereby plants are preconditioned for greater stress tolerance [58]. The passing of time could present significant plant 'hardening' events which may be epigenetically transferred to clonally propagated offspring, almost a form of epi-breeding.

Individuals (in this case quasi-clones) can have divergent histories on account of different environmental, biotic and abiotic factors. These histories can result in different levels of gene transcription [59]. These divergent transcriptomes could represent greater diversity within gene expression responses, effectively buffering against the deleterious effects associated with a lack

of genetic diversity [59]. This could imply that, the older a vineyard, the greater the diversity due to epigenetic modification and subsequent gene transcription resulting in differences in phenotypes of oenological importance, even at a molecular or metabolomic level. Raj, et al. [59] used unrooted dormant cuttings of poplar showing that epigenetic induced drought tolerance was conferred from maternal experience to cuttings in new environments. This supports the suggestion that the inheritance of epigenetic traits needs to consider not just the GxE interaction, but also the G x parental E method of adaptive transgenerational plasticity [60].

Therefore it is tempting to speculate that the epigenetic profiles of elite vineyards may be more faithfully replicated in the progeny if propagation is undertaken via layering. The process of callus formation may present a potential 're-setting' of epi-alleles acquired with plant ageing. In fact, previous studies have suggested that loss of morphogenic ability (i.e. the ability of plant regeneration from mature tissues) with plant age might be associated to the hyper methylation of genes needed for the morphogenic response [25]. Exactly how this epigenetic rejuvenation occurs in the callus and is then transmitted to the rest of the propagule is currently unknown. However changes in methylation profile have been proven during clonal propagation of Radiata pine; specifically demethylation [25].

An alternative cause epigenetic re-setting specifically relating to viticulture could be linked to the pruning system applied to plants being propagated. Trees with crown damage (due to browsing, fire or other sources of tissue damage) undergo vegetative phase change (VPC). VPC induces a reversion to a vigorous juvenile vegetative state [61]. Long term defoliation by herbivory has been shown to alter the DNA methylation patterns of predated plants [62]. In the same way seasonal cultural vineyard management (ie. dormant pruning or routine trimming) could lead to the potential alteration of epigenetic markers involved in molecular pathways relating to quality. The importance of these findings for the wine industry will be to determine if these epigenetic profiles are faithfully transferred and retained when growing in an alternate environment. If so, there are implications and opportunities for epi-breeding via layering, effectively skipping whole genome phase change associated with rejuvenation. Further work to identify markers associated with alternative propagation may provide potential for micro-evaluation of ecologically important traits [37]. Recent methylation profiling in almond suggests that currently unexploited opportunities exist in selection of epigenetic-like factors with positive contributions to plant performance and yield [35].

In this study, chronological age since vine establishment was associated with a change in the DNA methylation profile. The two largest age differences presented a large number of DMMs. The effect of plant age on epigenetic differentiation was smaller between layered plants (at site 5, age difference 87 years) than for the rest of the sites (age differences ranging from 56 to 151 years). Vines propagated via layering were statistically inseparable based on differential methylation despite the progeny being severed from the mother some years earlier. Further work to identify if the methylation differences observed result in differences in transcription would shed more light onto the potential benefits that increased age and layer assisted breeding might confer in relation to phenotypic or quality traits in *V. vinifera*.

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Tables

Table 1. Closest township, vine establishment dates and relative age gap (in brackets) between old and young vines for all Barossa Valley sites sampled.

Table 2. Summary of unique differentially methylated markers (DMMs) found following pairwise comparisons of both old and young cohorts and propagation method. Subscript indicates threshold number of samples that markers must be present in to be differentially methylated.

Table 1. Closest township, vine establishment dates and relative age gap (in brackets) between old and young vines for all Barossa zone sites sampled. Regions are indicated as EV=Eden Valley, BV=Barossa Valley.

		Year of establishment (vine age)*					
Site	Location	Old	Young	Relative age gap*	Approximate Coordinates	Degree days**	Region ID
Site 1	Angaston	1908 (107)	1964 (51)	56	-34.47;139.07	1309	EV
Site 2	Eden Valley	1906 (109)	1985 (30)	79	-34.49;139.12	1309	EV
Site 3	Nuriootpa	1885 (130)	2001 (14)	116	-34.41;139.00	1487	BV
Site 4	Tanunda	1845 (170)	1996 (19)	151	-34.50;138.96	1487	BV
Site 5	Rowland	1920 (95)	2007 (8)	87	-34.56;138.94	1487	BV

*Vine age and relative age difference in years since establishment, as at time of sampling in 2014.

**Degree days are raw accumulation as per [63].

Table 2. Summary of unique differentially methylated markers (DMMs) found following pairwise comparisons of both old and young cohorts and propagation methods. Subscript indicates threshold number of samples that markers must be present in to be differentially methylated. *Only DMMs significantly different at FDR<0.05 are reported.

Age based pairwise comparison (Old v Young)	**DMM* count (Mapped)**	**DMM* count (Un-resolved)**	**Diff meth gene count**	**Age difference (years)**
All Sites $_{t60}$	0	0	NA	97.8
Site 1 $_{t10}$	0	0	NA	56
Site 2 $_{t10}$	0	0	NA	79
Site 3 $_{t10}$	1564	187	1980	116
Site 4 $_{t10}$	1883	303	1966	151
Site 5 $_{t10}$	0	0	NA	87
Site 1-4 $_{t48}$	135	42	40	100.5
Site 5 (mother v dormant cuttings)	3950	1845	2835	90
Site 5 (mother v layering)	0	1	NA	90

Figures

Figure 1. Circos plot of key features which characterise the epigenome of *V.vinifera* vines cv. Shiraz of contrasting planted age, data is based on differential methylation analysis resulting from ms-GBS assay.

Figure 2. Effect of vineyard location, propagation system and vine age on plant DNA methylation profiles. A. Mahalanobis distance HCA dendrogram representing epigenetic differentiation between vineyards based on the ms-GBS profiles of 12 plants per vineyard. B. PCLDA results of epigenetic differentiation between Shiraz plants grouped by vineyard age and propagation system (Young plants on site 1 to 4 were propagated using dormant cuttings (green labels) from old plants (black labels), young plants on site 5 were propagated using layering (blue labels) from old plants (red labels). C. Contour plot showing the effect of geographic distance (km) and plant age difference (years) on the number of DMMs per pairwise vineyard comparison. D. HCA dendrogram presenting Mahalanobis distance of global methylation profile based on one seasons propagation experiment using different propagation techniques showing mother, layer and clone based on the ms-GBS profiles of 7 plants per plant type.

Figure 3. Effect of propagation system on plant DNA methylation profiles. A. PCLDA results of epigenetic differentiation between Shiraz plants labelled by vineyard age and grouped by propagation system (Young plants at site 1 to 4 were propagated using dormant cuttings (green labels) from old plants (black labels), young plants at site 5 were propagated using layering (blue labels) from old plants (red labels). B. HCA dendrogram presenting Mahalanobis distance of global methylation profile based on one seasons propagation experiment using different propagation techniques showing mother, layer and clone based on the ms-GBS profiles of 7 plants per plant type.

Figure 4. Effect of vineyard location, and vine age on plant DNA methylation profiles. Results following PCLDA of global methylation profile of sites 1 to 4 which young vines share the common propagation technique of callus cuttings. Colours are unique for each site and labels are descriptive.

Figure 5. Relative proximity of differentially methylated marks in relation to gene location on *V. vinifera* 12x reference genome in 1Kb bins both up and down stream of transcription start site (TSS) and transcription end site (TES).

Figure 6. Representation of the number of differentially methylated genes (DMg) in plants propagated using dormant cuttings. Genes were considered differentially methylated if a ms-GBS generated DMM is in or within 5kb of their coding sequence according the *V. vinifera* 12x reference genome. A- presents total number of unique DMgs unique between samples (red) and common amongst at least two samples (yellow) and common amongst all samples (green). B- Venn diagram displaying the breakdown of unique and common DMgs amongst samples and their number of DMgs in common.

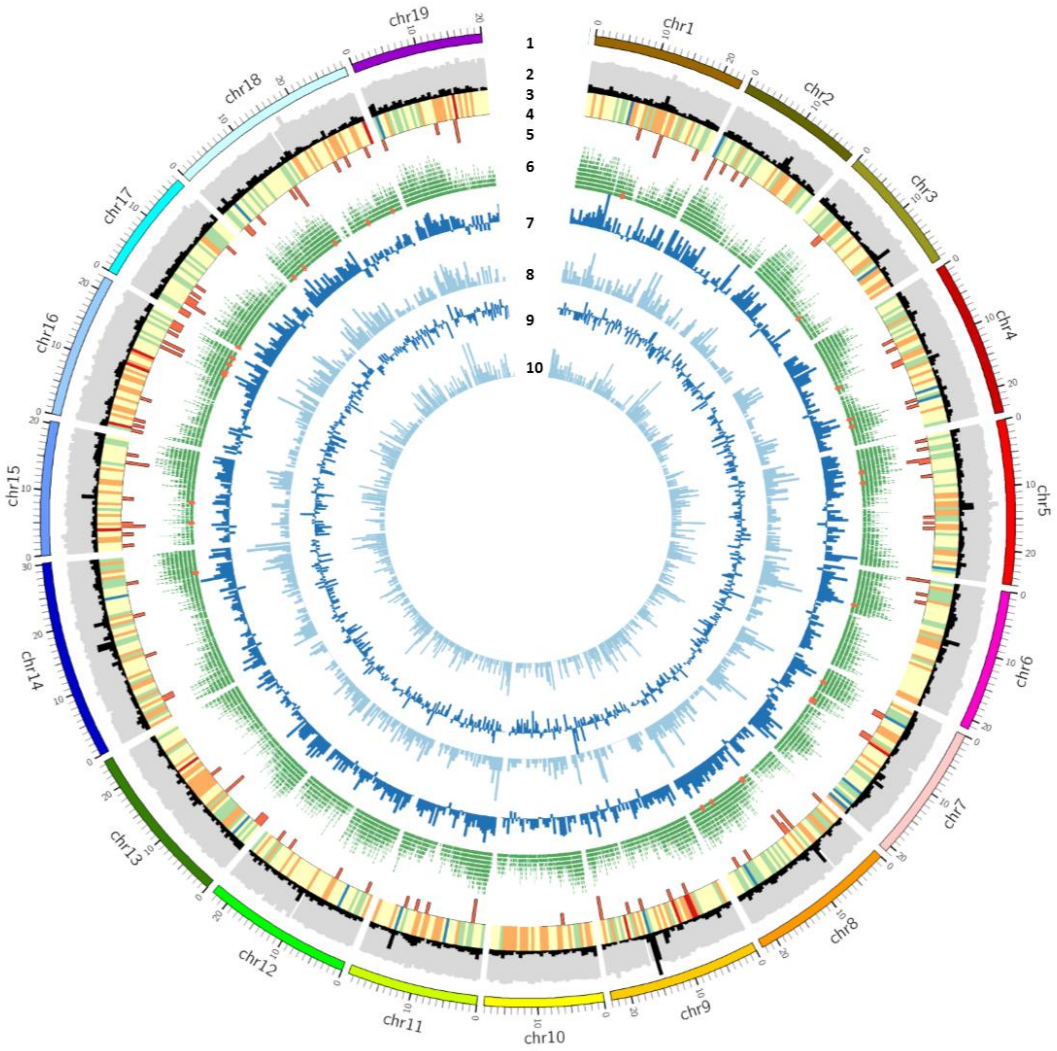
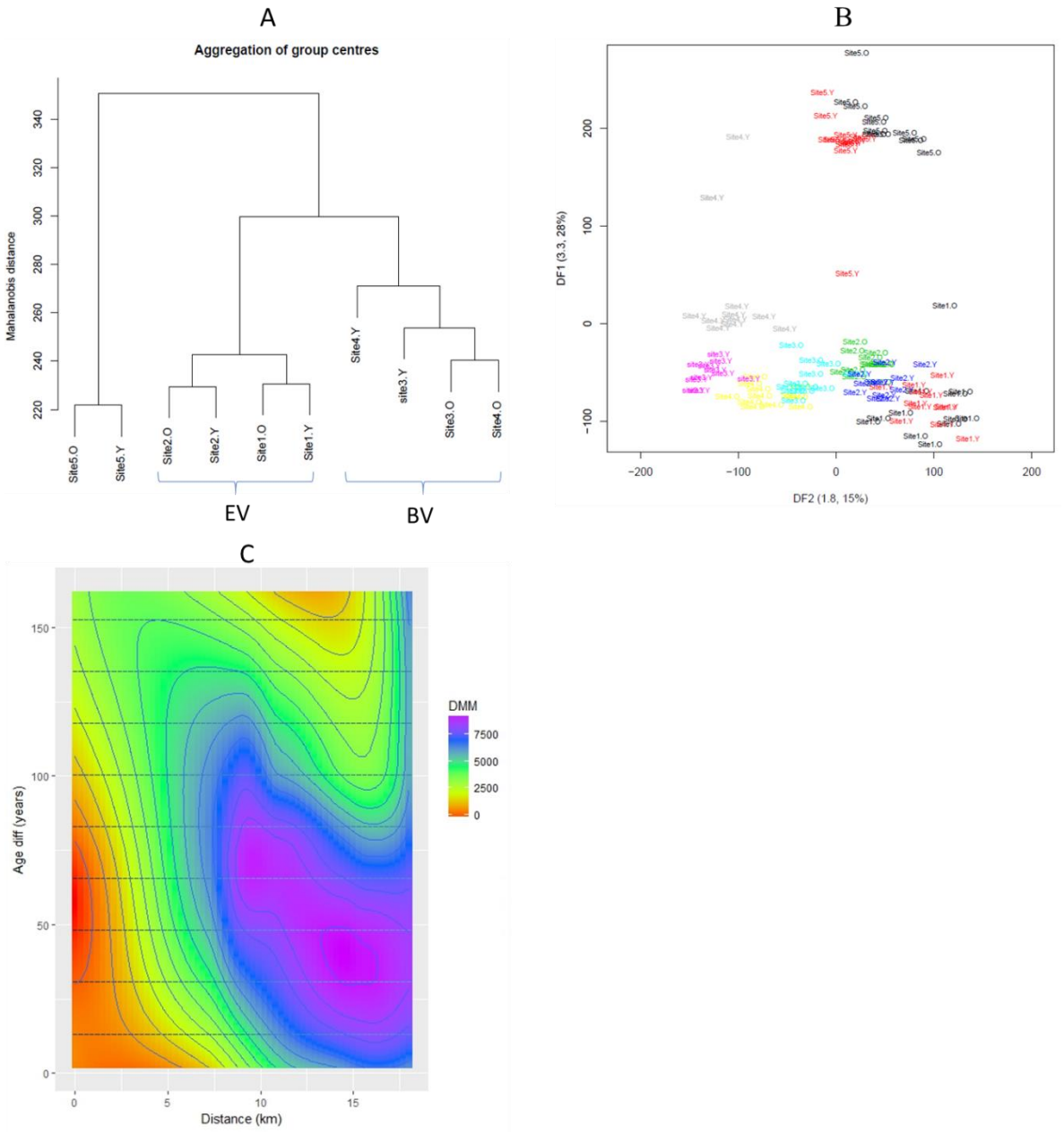


Figure 1. Circos plot of key features which characterise the epigenome of *V.vinifera* vines cv. Shiraz of contrasting planted age, data is based on differential methylation analysis resulting from ms-GBS assay. Track numbers are indicated on the plot and described below.

Track	Description
1	Grapevine chromosome karyotype. Source: *Vitis vinifera* IGGP 12x.
2	Cutsites per million per position across all samples (*Log*CPM scale = 0-15) Grey
3	GC% per chromosome
4	Heatmap of Fold Change comparing old and young at sites 1 to 4. Hypo (-) Hyper (+)
5	Count of DMMs following age comparison of sites 1 through 4. (scale: max=2)
6	Gene density per chromosome (green) genes within 5kb of one of the DMMs on track 5 (red)
7	Fold change comparing samples with age difference of 151 years (site 4)
8	Count of DMMs with an FDR <0.05 with 151 year age difference
9	Fold change comparing samples with age difference of 116 years (site 3)
10	Count of DMMs with an FDR <0.05 with 116 year age difference



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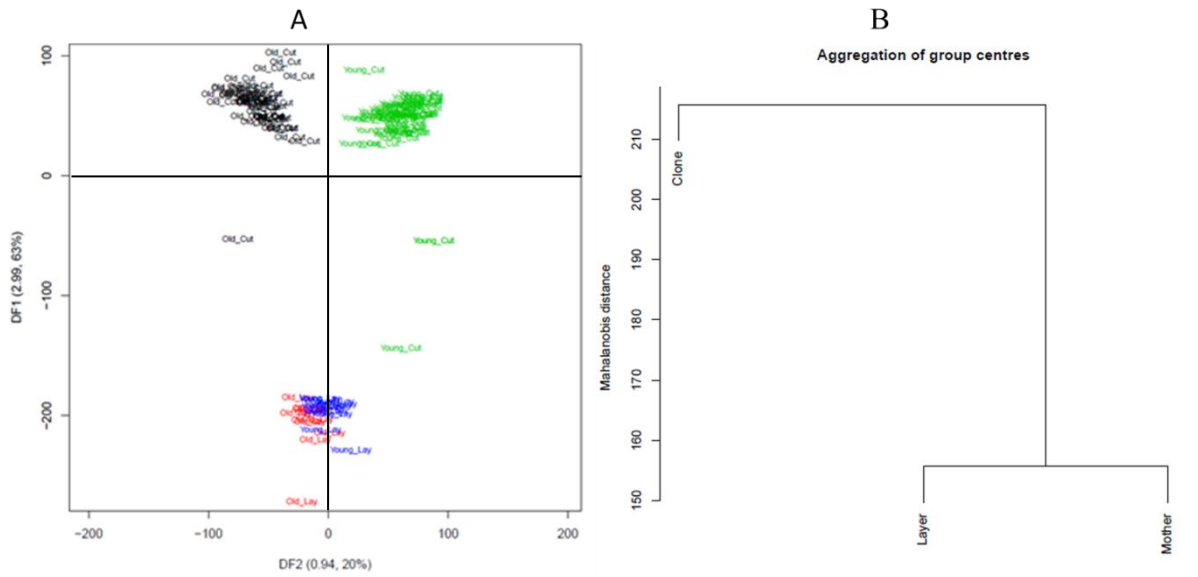

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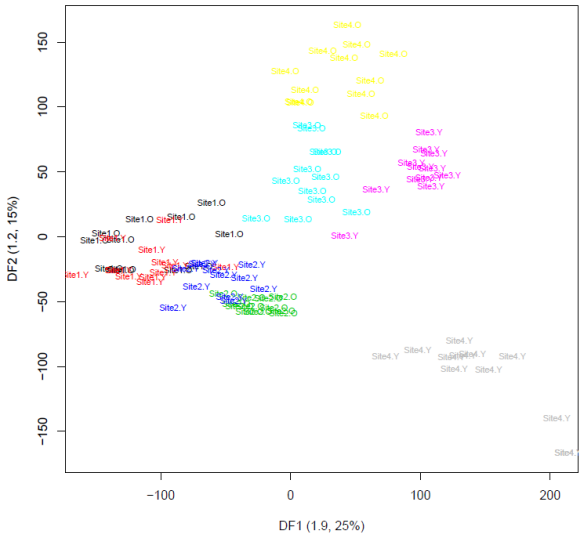

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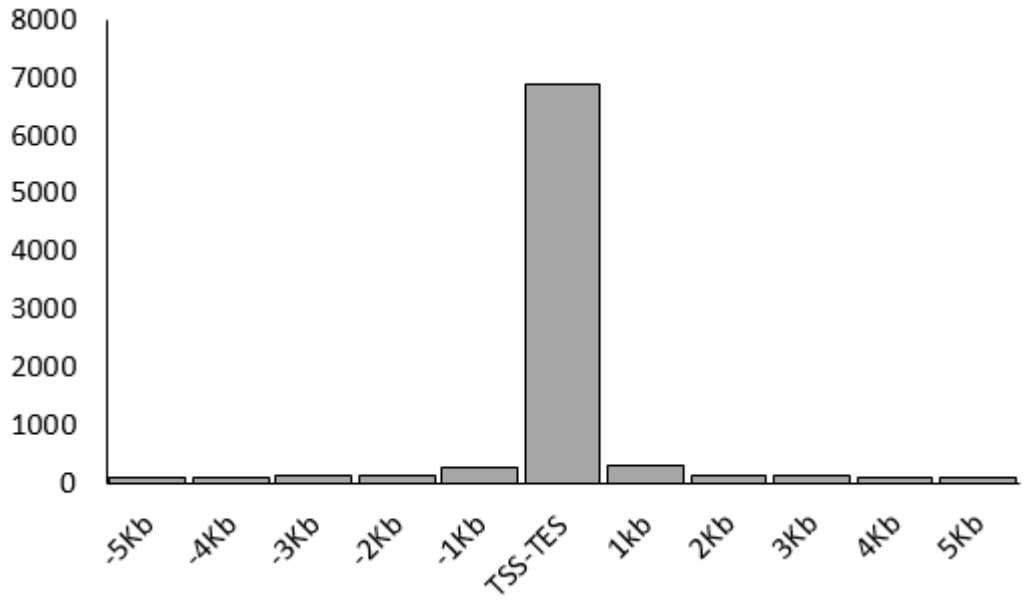

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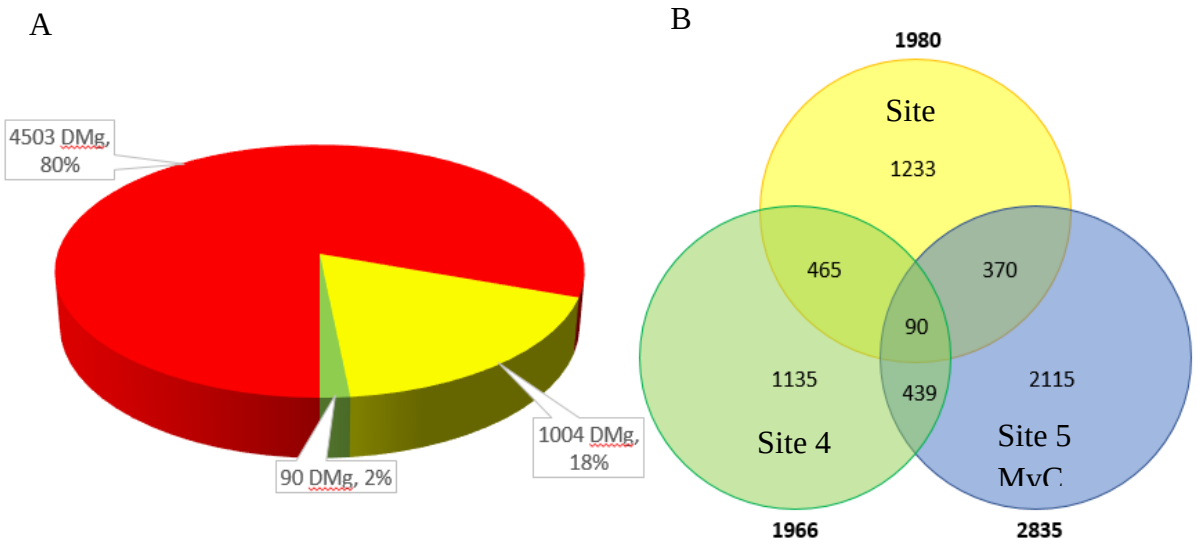

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Supplementary material

Table S1. Retail price of commercial wines produced from study vineyards, prices were publicly available and current at the time of publication.

Current vintage	Site	Age	Retail Price
2010	1 Old	105	699
2009	1 Young	28	325
2012	2 Old	107	100
2013	2 Young	49	93
2014	3 Old	128	60
2013	3 Young	12	30
2013	4 Old	168	125
2013	4 Young	17	30
2012	5 Old	93	100
2012	5 Young	6	100

Table S2. Climatic data for Nuriootpa, Barossa Valley, South Australia. For growing seasons 2013 – 2015, October - April.

Growing season	**Mean daily maximum (°C)**	**Mean daily minimum (°C)**	**E°days****	**Rainfall (mm)**	**MJT (°C)**	**MFT (°C)**
2014-2015	26.0	11.3	1520	145	20.8	23.6
*Long term average**	*25.2*	*11.3*	*1545*	*203*	*21.5*	*21.4*

*Long term average from 1952- 2015, Nuriootpa, Barossa Valley approx. 34°48'S, 139°00'E, Altitude 275m [64]. **Calculated as per [65]

MJT = mean January temperature. MFT = mean February temperature.

Table S3. Location and identification of genes within 5kb either side DMM loci which were consistent between sites 1-4 in leaf samples of Shiraz.

Lower 5kb

Chr	DMM coordinate	Cutsite distance	IGGP_ID	Gene start	Gene end	Transcription start site (TSS)	Gene type	Gene name	Strand	Gene description
chr3	13831970	1591	VIT_03s0110g00570	13833561	13841808	13833561	protein coding		1	Putative uncharacterized protein Acc:F6HRB2
chr4	10807450	3355	VIT_04s0079g00370	10810805	10812671	10812671	protein coding	PERK4	-1	Proline-rich receptor-like protein kinase PERK4
chr4	18101856	538	VIT_04s0023g01570	18102394	18105221	18102394	protein coding		1	Putative uncharacterized protein Acc:F6GWX5
chr4	19723504	610	VIT_04s0023g03170	19724114	19725462	19725462	protein coding		-1	Putative uncharacterized protein Acc:F6GWP0
chr5	7229152	959	VIT_05s0049g00170	7230111	7230818	7230111	protein coding		1	Putative uncharacterized protein Acc:F6H8I0
chr6	7783483	4475	VIT_06s0004g07040	7787958	7788712	7788712	protein coding		-1	Putative uncharacterized protein Acc:D7SJT4
chr7	3229342	1487	VIT_07s0005g00530	3230829	3231377	3231377	protein coding		-1	Putative uncharacterized protein Acc:D7U299
chr7	7533887	1147	VIT_07s0005g04460	7535034	7546064	7546064	protein coding		-1	Putative uncharacterized protein Acc:F6HZR7
chr7	8106019	4122	VIT_07s0005g04850	8110141	8111055	8111055	protein coding		-1	Putative uncharacterized protein Acc:D7U3B1
chr8	8552135	3798	VIT_08s0058g00070	8555933	8569894	8555933	protein coding		1	Putative uncharacterized protein Acc:F6GXV6
chr8	16953551	126	VIT_08s0007g02890	16953677	16959250	16959250	protein coding		-1	Putative uncharacterized protein Acc:D7TJB0
chr8	19340973	1988	VIT_08s0007g05400	19342961	19353562	19342961	protein coding		1	Putative uncharacterized protein Acc:D7TIM9
chr14	25263464	2350	VIT_14s0068g01560	25265814	25273020	25265814	protein coding		1	Putative uncharacterized protein Acc:F6H457
chr15	4708755	485	VIT_15s0045g00150	4709240	4711891	4711891	protein coding		-1	Putative uncharacterized protein Acc:F6I1C2
chr16	20479051	935	VIT_16s0098g00020	20479986	20483310	20479986	protein coding		1	Putative uncharacterized protein Acc:F6H7F8
chr16	20586541	662	VIT_16s0098g00160	20587203	20590853	20587203	protein coding		1	Putative uncharacterized protein Acc:F6H7E9
chr18	4309961	372	VIT_18s0001g05440	4310333	4319306	4319306	protein coding		-1	Putative uncharacterized protein Acc:E0CRJ5
chr18	12898904	1919	VIT_18s0001g14860	12900823	12904890	12900823	protein coding		1	Putative uncharacterized protein Acc:F6GZT1
chr18	21332369	2810	VIT_18s0075g00170	21335179	21344049	21335179	protein coding		1	na

Upper 5kb

Chr	DMM coordinate	Cutsite distance	IGGP_ID	Gene End (bp)	Gene Start (bp)	Transcription start site (TSS)	Gene type	Gene name	Strand	Gene description
chr1	10492715	-191	VIT_01s0026g01480	10492524	10471796	10492524	protein_coding		-1	Putative uncharacterized protein Acc:F6HPG0
chr4	18101856	-1801	VIT_04s0023g01560	18100055	18095521	18095521	protein_coding		1	Putative uncharacterized protein Acc:D7SPF8
chr4	19723504	-46	VIT_04s0023g03160	19723458	19719705	19719705	protein_coding		1	Putative uncharacterized protein Acc:D7SP16
chr5	4921560	-1165	VIT_05s0020g03160	4920395	4912457	4920395	protein_coding		-1	Putative uncharacterized protein Acc:D7T6V7
chr5	7229152	-2084	VIT_05s0049g00160	7227068	7226153	7226153	protein_coding		1	Putative uncharacterized protein Acc:F6H8H9
chr7	7533887	-1273	VIT_07s0005g04450	7532614	7530675	7530675	protein_coding		1	Putative uncharacterized protein Acc:F6HZR6
chr8	8873063	-4514	VIT_08s0058g00180	8868549	8867831	8867831	protein_coding		1	Putative uncharacterized protein Acc:D7SQV8
chr8	19340973	-870	VIT_08s0007g05390	19340103	19337997	19337997	protein_coding		1	Putative uncharacterized protein Acc:F6HK97
chr15	4708755	-3663	VIT_15s0045g00140	4705092	4704955	4704955	protein_coding		1	Putative uncharacterized protein Acc:D7U5M9
chr15	9156889	-351	VIT_15s0021g00030	9156538	9154901	9156538	protein_coding		-1	Putative uncharacterized protein Acc:D7SM85
chr16	16493433	-301	VIT_16s0100g01010	16493132	16491413	16493132	protein_coding		-1	Stilbene synthase 2 Acc:P51070
chr16	17173038	-4272	VIT_16s0050g00350	17168766	17168364	17168364	protein_coding		1	Putative uncharacterized protein Acc:F6H6N4
chr16	17189854	-249	VIT_16s0050g00370	17189605	17187019	17189605	protein_coding		-1	Putative uncharacterized protein Acc:F6H6N3
chr16	19019008	-1857	VIT_16s0050g02050	19017151	19015882	19015882	protein_coding		1	Putative uncharacterized protein Acc:E0CUN8
chr16	20479051	-113	VIT_16s0098g00010	20478938	20476045	20478938	protein_coding		-1	Putative uncharacterized protein Acc:F6H7F9
chr16	20586541	-113	VIT_16s0098g00150	20586428	20583047	20586428	protein_coding		-1	Putative uncharacterized protein Acc:F6H7F0
chr17	65159	-3158	VIT_17s0000g00140	62001	55861	55861	protein_coding		1	Putative uncharacterized protein Acc:D7SH86
chr18	1323900	-1040	VIT_18s0001g00410	1322860	1319712	1322860	protein_coding		-1	Putative uncharacterized protein Acc:E0CQT3
chr18	4309961	-14	VIT_18s0001g05420	4309947	4306846	4306846	protein_coding	UNE2	1	Putative uncharacterized protein Acc:E0CRJ4
chr18	12898904	-557	VIT_18s0001g14850	12898347	12893670	12893670	protein_coding		1	Putative uncharacterized protein Acc:E0CQB1
chr18	27189386	-111	VIT_18s0041g02060	27189275	27186796	27186796	protein_coding		1	Putative uncharacterized protein Acc:F6I449

Table S4. Summary matrix for pairwise comparisons of A. geographic distance and B. DMMs between samples.

A	**Geographic Distance**									
	Site 1 O	Site 1 Y	Site 2 O	Site 2 Y	Site 3 O	Site 3 Y	Site 4 Y	Site 4 O	Site 5 Y	Site 5 O
Site 1 O	0.000									
Site 1 Y	0.250	0.000								
Site 2 O	5.275	5.347	0.000							
Site 2 Y	5.269	5.335	0.105	0.000						
Site 3 O	9.180	8.930	14.336	14.268	0.000					
Site 3 Y	9.249	9.620	14.427	14.437	0.100	0.000				
Site 4 Y	10.620	10.334	14.371	14.303	11.100	11.127	0.000			
Site 4 O	10.578	10.293	14.336	14.268	11.010	11.086	0.046	0.000		
Site 5 Y	16.029	15.716	18.198	18.105	18.100	18.198	7.072	7.113	0.000	
Site 5 O	16.029	15.716	18.198	18.105	18.100	18.198	7.072	7.113	0.000	0.000
	DMM									
	Site 1 O	Site 1 Y	Site 2 O	Site 2 Y	Site 3 O	Site 3 Y	Site 4 Y	Site 4 O	Site 5 Y	Site 5 O
Site 1 O	0									
Site 1 Y	0	0								
Site 2 O	2224	4566	0							
Site 2 Y	15	28	0	0						
Site 3 O	1776	5264	16	82	0					
Site 3 Y	15307	15572	7160	9508	1751	0				
Site 4 Y	21552	16153	9842	10418	4398	1371	0			
Site 4 O	975	3863	26	36	3	73	2186	0		
Site 5 Y	2224	15500	7305	9468	5651	6396	239	5473	0	0
Site 5 O	8949	9544	6420	6146	5733	7504	407	5658	0	0

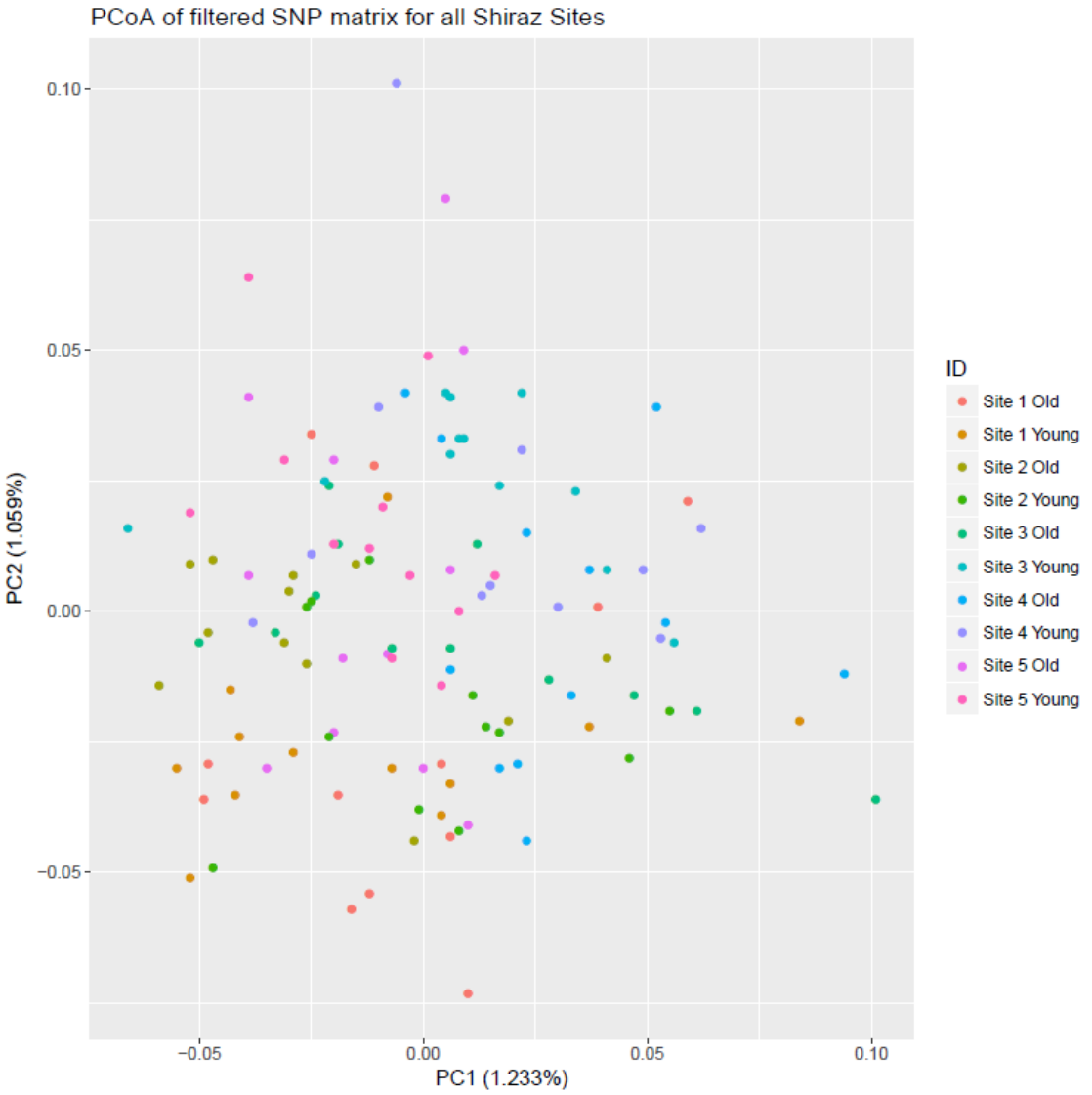
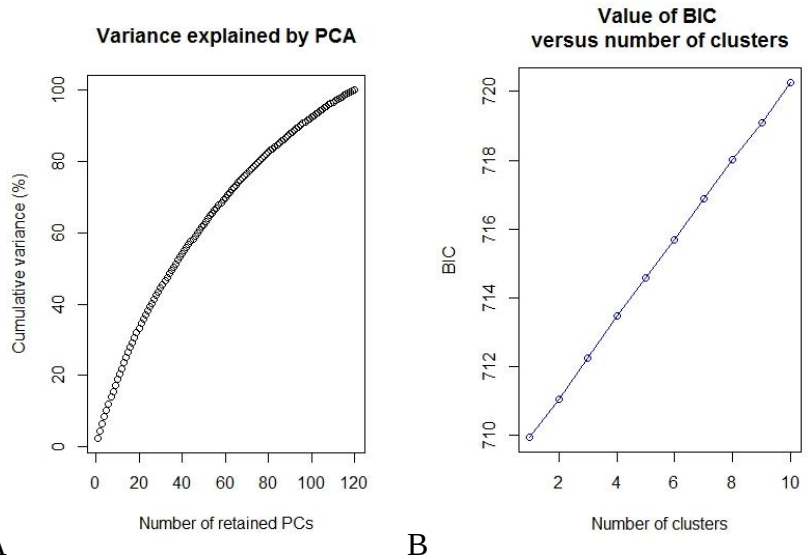


Figure S1. PCoA of all Shiraz sites and all Shiraz regions using filtered SNP distance matrix.



A B

Figure S2. Output from DAPC analysis A. highlighting the maximum number of PC's used for initial clustering and B. showing the BIC number identifying no clustered relationships were present in the genotype data.

Appendix

The digital version of this thesis contains an excel file with the following:

-Differential methylation results of ms-GBS data for all pairwise comparisons.

-List of genes with ms-GBS methylated marker in or within 5kb of coding sequence.

Chapter 7. General Discussion:

This research has provided new knowledge on the current understanding of grapevine age and its influence in several key areas; vine performance, grape and wine chemistry, grape and wine sensory evaluation and via molecular characterisation of genotype and epigenotype. This research will also guide future studies on the influence of grapevine ageing in relation to a range of quality and performance traits and on the effect of propagation systems on the maintenance of age-acquired differences.

Investigation into the vegetative performance of Shiraz vines of significantly different age was performed by comparing vines at both an individual site level, and across multiple sites over a three year period. Despite significant differences in age, measures of vine performance were not found to be consistently influenced by plant age. In isolation, age based comparisons did present significant differences, some of which initially suggested that vine age influenced reproductive performance more than vegetative performance (Chapter 3). Specifically this was observed in relation to bunch architecture, an association which has previously been reported (Ezzili 1992). Older vines did produce higher yields in general; this was observed to be a result of an increase in the yield components berry number and bunch mass. This topic would specifically benefit from a more targeted study to quantify fruit set via specific indices such as the coloure and millerandage indices (Collins and Dry 2009).

If increasing vine age represents a change in reproductive capacity, it was not consistently observed in this study. However, higher yields and differences in reproduction could still be linked to overall capacity or vine reserves. Vines with greater volume of old wood have been previously reported to produce higher yields; specifically due to bunch mass (Koblet and Perret 1980). The result of trunk size correlating with age in this study and also by Tyminski (2013) is an indicator of capacity as ‘old wood’ was in the report of Koblet and Perret (1980). Further work into trunk carbohydrate and nitrogen availability is also suggested, these are known to be important for flower development as well as a range of other vegetative and reproductive variables (Smith and Holzapfel 2003, 2009, Holzapfel et al. 2010, Keller 2015).

Vine size, as measured by permanent wood volume has been shown to influence both vegetative and reproductive measures in several studies (Koblet and Perret 1980,

Reynolds et al. 1994). In this study, vine age was found to have a significant correlation with trunk circumference (R^2=0.88, figure 3, chapter 3). This suggests that as vines age, their buffering capacity for seasonal variation might be greater. Therefore, in more 'challenging' seasons, due to drought or temperature extremes, older vines could be expected to outperform younger vines thereby maintaining long term consistency. However, it was not possible to capture this during this study period.

Variability is an intrinsic property of all biological systems which can be present at many levels. In our case individual seasons presented unique conditions, and with them unique seasonal results. The influence of climate and season on vine growth is well documented (Rankine et al. 1971, Freeman et al. 1979, Gladstones 1992, Keller et al. 2004, Keller 2010). Seasonal variation presents a challenge in any field trial, in this case one limitation on the evaluation of vine performance might be due to the timespan studied. Greater significance in the reported trends could potentially be uncovered if the study was undertaken over a longer time period. This would have the added benefit of increasing the probability of capturing seasonal extremes. This would be of great interest in this study as vine physical size, via the accumulation of perennial wood above and below ground as this is thought to confer resilience and buffer stress (Mullins 1992, Howell 2001). An area that was beyond the scope of this study but should not be overlooked in future is specifically root distribution and its influence in mediating the soil – environment interaction with increasing age.

The analysis of commonly used grape and wine composition found few firm associations with age (Chapter 4). Compositional analysis revealed that both growing season and site significantly affected many of the parameters to a greater extent than vine age. For oenological purposes, a favourable balance of pH and TA with desired sugar level is beneficial (low pH : high TA), and often targeted as a grape quality indicator (Gishen et al. 2001, Iland et al. 2004). Fruit from old vines was frequently found to have lower pH and higher TA at similar °Brix levels than that from younger vines (Table S3 Chapter 4). This observation may again indicate the influence of vine capacity, or more specifically carbohydrate storage capacity. This effect was present in individual seasons but did not hold in seasonally pooled data. In addition, this balance between sugar and acidity may have been an effect related to vine canopy architecture, this was identified via differences in pruning mass in chapter 3. The indication that acid balance or a favourable pH to TA

ratio could be linked with capacity is important to understand in regards to quality outcomes. If vine age resulted in greater natural balance in acid composition it would be beneficial to isolate if this is related to vine age, vine size or canopy architecture, as acid balance is a primary quality determinant in winemaking with potential sensory outcomes. This could be tested if volunteer old vines were cut off and allowed to re-grow, then compared with vines with no change in above-ground storage capacity this would complement previous studies (Koblet and Perret 1980).

The variation in vine performance was somewhat indicative of both grape and wine chemistry. In this study, the majority of compositional measures commonly used to determine harvest time and measure quality did not show a strong relationship with vine age. In addition to seasonal influences, the specific growing conditions at each site had a greater bearing on differentiation of compositional measures each season. Furthermore, consistent similarities were observed between groups of sites; this was revealed to be a regional or 'terroir' effect. Unrelated sites with different management, soil type and vine age still presented similarities in terms of region, particularly in secondary metabolite composition. Traits of regional or even subregional differentiation have been shown to be more discriminatory with time. Analysis of bottle-aged wines from very similar 'terroirs' in Burgundy (France) revealed perfect separation of terroirs after bottle ageing in contrast to analysis immediately after fermentation which did not discriminate (Roullier-Gall et al. 2014). Considering this, further analysis in the future could improve our understanding of wine quality at a regional level and determine if vine age is still secondary to this.

One of the main drivers for undertaking this study was the reputation and claimed quality level (using price as a proxy) of wine produced from vines of different ages (Table 1). The results of sensory descriptive analysis for both grapes and wine revealed unique sensory characteristics and volatile composition associated with vine age. This finding concurs with previous studies that found increased red berry and fruity characters were associated with greater vine age (Heymann and Noble 1987, Zufferey and Maigre 2008). This consistency with these other unrelated studies is compelling for two main reasons; firstly, vine age appears to influence sensory characteristics of wine, and secondly the effect is independent of variety and geographic location; Heymann and Noble (1987) studied Cabernet Sauvignon in California while Zufferey and Maigre (2008) reported age effects for Gamay, Syrah, Humagne Rouge, and Pinot Blanc but not so for white

wines Chasselas or Arvine. If this is an age-related effect, then it is supported by these independent findings. It may indicate an alternate origin causing differences in compounds responsible for traits of quality significance. This could be tested again via manipulation of vine capacity to determine if age-related capacity influences volatile composition of fruit and resultant sensory properties. The experienced panel and sensory protocol in this study was tightly controlled, a greater number of assessors or repeating assessments as wines aged might provide further detail and complement chemical compositional assessments regarding the sensory associations with vine age.

The contribution of secondary metabolites to grape and wine quality and provenance has been widely studied. The link between colour and quality score or bottle price is well established (Somers and Evans 1974, Jackson et al. 1978); however, this is just part of the sensory matrix. The relative impact of phenolic compounds in terms of quality is generally related to mouthfeel properties such as the perception of astringency. These compounds are discriminatory indicators of geographic origin and cultivar (Makris et al. 2006) indicating their sensitivity to environmental cues. Both temperature and UV light are known to influence pathways in the biosynthesis of phenolic compounds, hence its mesoclimatic regional specificity. The microclimate can also be of influence depending on vine vigour, shading or management of the vegetative balance of the vine. The regionally-specific phenolic fingerprints of wines irrespective of age highlights the importance of provenance, terroir and the value of site when discussing wine quality.

Wine regional typicity has been previously linked to genetic differences between cultivars and even between clones cultivated in each region (Schellenbaum et al. 2008). All sites in this study are planted with the same cultivar (Shiraz) and therefore are considered to be similar in genetic composition. To validate this, the analysis of SNPs generated using ms-GBS was undertaken to prove homogeneity amongst the sampled population. No inherent genetic structure or clear relationships were observed. Therefore, the vine plasticity resultant in the varying phenotypes observed throughout the study (specifically Chapter 4 and 5) needs to be explained by some other mechanism(s). Our understanding of phenotypic plasticity, in general, is a rapidly growing field incorporating many species, including grape (Sadras et al. 2007, Dal Santo et al. 2013, Anesi et al. 2015). Epigenetic mechanisms that can alter expressed phenotypes are prevalent in plant genomes, specifically in the form of DNA methylation. This form of epigenetic polymorphism has

been proven to accumulate with age in plants and has been suggested as a form of plant memory. Such environmental memory can help to reduce the severity of a stressful event following previous exposure to it in the form of plant hardening (Boyko and Kovalchuk 2011).

By employing the ms-GBS technique, the level of epigenetic differentiation between the samples was assessed and characterized. Such variability could be used as a potential indicator of differences in the transcriptional level of genes regulating the expression of the observed phenotypes. As observed for the phenotypic traits measured in chapters 3, 4 and 5, the main driver of epigenetic differentiation between the plants included in this study was their geographic origin. This supports previous studies linking epigenetic mechanisms to adaptation to environmental conditions (Raj et al. 2011, Herrera and Bazaga 2013, Gömöry et al. 2014) and opens the door for future research on the importance of such mechanisms affecting fruit quality traits in grapevine.

Not only were vine geographic origin and age found to be associated with a greater level of methylation but also propagation technique was uncovered as an influence on the methylation profile. Hardwood cuttings are known to retain the 'memory' of their paternal environment (Fraga et al. 2002). This has been shown via transcriptome level analysis and suggested to be related to DNA methylation (Raj et al. 2011). What was not expected was the startling similarity in in methylation profile between the young vines propagated via layering at site 5 irrespective of the large age difference. These young vines were classified as such, except during establishment they would be considered 'one plant' still attached to the mother and actually ~87 years old. The *in situ* propagation experiment confirmed the epigenetic differentiation in association to propagation system. The action of severing after some years after establishment appeared not to alter the epigenetic profile or potentially the epigenetic 'age' of the plant. Similarities at this site consistently arose in several chapters, and even age discrimination of sensory analysis found the smallest differences at site 5. This makes it tempting to speculate that layering retains more epigenetic memory from the mother vine than callused cuttings. Could the act of callusing result in a re-setting of epigenetic markers accumulated over time, which can only be re-accumulated once planted in the same environment? This has been demonstrated in the short term via divergence of grapevine tissue following tissue culture

and subsequent convergence over time once grown in the same environment (Baranek et al. 2015).

Although RNA analysis was not undertaken, this initial study will guide further studies in the field of transcriptomic analysis to test if differential methylation is associated with gene expression of quality traits. It would be ideal if transcriptomic analysis was undertaken in a seasons of contrasting environmental stress, providing a comparison for not only the effect of vine capacity and relative plasticity but also transcription-mediated stress tolerance. This last experiment would be made all the more compelling by including vines which had been pre-stressed and established via layering as well as by callused cuttings. The inclusion of alternative propagation techniques would test the hypothesis that 'memory' or environmental conditioning is associated with propagation and plant age.

The novelty of this research makes a very substantial multidisciplinary addition to current knowledge regarding the effect of significant vine age of the cultivar Shiraz, and *V. vinifera* in general. Without the restrictions associated with being confined to just one discipline, this study covered a wide range of fields. This allowed a broad impact and advancement in the collective knowledge of vine age to be made, ranging from characteristics of vine growth to molecular profiles. More in-depth research will still benefit this topic however this study has narrowed the scope for future research to key focussed areas.

The findings as a whole present the power of site and its importance in the unique provenance associated with wine in general. The drivers of geographical or regional influence are important to understand as this facilitates cultivation of long-lived perennial plants such as grapevines. Throughout the process of compiling this thesis, vine age has drifted in, and out of focus numerous times. Irrespective of the findings, vines which are multiple human generations in age are unique, possessing character in both a physical sense being gnarled by time and also in their produce, which inclusive of environment has a unique story to tell.

Discussion Table

Table 1. Retail wine prices for branded bottled products produced from trial sites by commercial wine companies. *prices are recommended retail price in $AU current 01-2017. Due to cellaring and release dates only current vintage is recorded.

Current vintage*	Site	Reported Age	Retail price ($AU)
2010	1 Old	105	699
2009	1 Young	28	325
2012	2 Old	107	100
2013	2 Young	49	93
2014	3 Old	128	60
2013	3 Young	12	30
2013	4 Old	168	125
2013	4 Young	17	30
2012	5 Old	93	100
2012	5 Young	6	100

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