The Influence of climate factors and plant physiological responses on the accumulation of rotundone in *Vitis vinifera* cv. Shiraz grapevines

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Abstract

Rotundone is a sesquiterpene compound that gives grapes and wine a ‘black pepper’ aroma and flavour, which is stylistic important to many Australian Shiraz wines. This PhD project determined the factors important for the concentration of rotundone in Shiraz grapes and wine, including vineyard environmental parameters and grapevine physiological factors. Wine produced from the same Shiraz vineyard over 15 vintages (The Old Block, Mount Langi Ghiran, Victoria, Australia) were analysed for rotundone concentration and compared to historical weather data. Results showed that the highest concentrations of rotundone were consistently found in wines from cool and wet seasons, while the concentration of rotundone in wine was negatively correlated with daily solar exposure and grape bunch zone temperature (veraison to harvest), and positively correlated with vineyard water balance. The influence of vineyard temperature on the concentration of rotundone was further investigated by quantifying rotundone variability within-vineyard, within-vine, and within-bunch. Occurrence of the highest concentration of rotundone was observed in shaded bunch sectors and vines, and from higher vigor vines in the southern-facing areas of the vineyard, which correlated to the lower grape surface and bunch zone air temperature. Modelling further showed that berry temperature exceeding 25 °C negatively affected the rotundone concentration in grape berries. The influence of solar exposure on the concentration of rotundone was investigated by excluding sunlight from grape bunches at different stages of berry development. Significantly lower concentration of rotundone and its precursor \( \alpha \)-guaiene was observed in grape berries with sunlight exclusion at all stages. This indicated that sunlight illumination was important to determine the concentration of rotundone in grapes at harvest, and the biosynthesis pathway from \( \alpha \)-guaiene to rotundone might be light sensitive.
Grapevine phenological stage is the most important physiological factor affecting the concentration of rotundone in grape berries. The evolution of rotundone and other terpenoids in grape berries from 4 weeks post-flowering to maturity was investigated. Results showed that different terpenoids had different production patterns during berry development depending on their biosynthesis pathway, and terpenoids derived from the same biosynthesis pathway shared similar production pattern. The concentration of rotundone and \( \alpha \)-guaiene in grape berries gradually decreased at pre-veraison stages until veraison and increased afterward until maturity. The influence of grapevine non-grape organs on the concentration of rotundone in wine was also investigated. Non-grape organs contained significantly higher amount of rotundone than berries. However, rotundone could not mobilize from these organs into grape berries via the phloem. Despite this, non-grape organs could still be used as a source of rotundone, especially for wineries conducing whole-bunch fermentation and harvest by machine. This project also showed that herbivore activity did not significantly modify the concentration of rotundone in grapevine leaves, and therefore rotundone may not be a member of the herbivore-induced terpenoids. This project investigates numerous factors affecting the production of rotundone in \textit{Vitis vinifera} CV. Shiraz grape and wine, which is very important for wineries and wine regions to establish the ‘terroir’. The knowledge developed from this project could help Australian grape growers and wine industry produce grapes and wine of better quality.
Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Pangzhen Zhang

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Chapter 3

Chapter 4 is in manuscript form, and will be published together with data obtained from other projects.

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Preface

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CHAPTER ONE
Introduction & Project hypothesis

1.1 Introduction

Black peppery aroma has become a favourable Shiraz wine attribute to many wine consumers (Williamson et al. 2012). Rotundone is an oxidized sesquiterpene that has been identified as the primary compound responsible for this peppery characteristic (Siebert et al. 2008, Wood et al. 2008). This compound has been found to be present in Australian wines of different cultivars, including: Shiraz, Merlot, Durif, Pinot Noir and Cabernet Sauvignon. Higher rotundone can be found from grapes produced within many cool climate wine regions, with the highest concentration observed in Shiraz wine produced in the Grampians wine region in west Victoria (Jeffery et al. 2009).

The number of researches and publications on rotundone has been very limited prior to this research project, as rotundone was only first identified from grape and wine in 2008 (Wood et al. 2008). Many other compounds in the terpenes chemical family have been proven to be important for grape/wine aroma and flavour, with most of them belonging to the monoterpenes subgroup. Rotundone represents the first compound in the sesquiterpene subgroup with flavour/aroma importance for grape and wine (Siebert et al. 2008). According to the carbon content within molecules, terpenes can be sub-grouped into hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), homoterpenes (C11 and C16) and diterpenes (C20), derived from the same C5 precursor, isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) (Dudareva et al. 2006). In grapevines, there are two biosynthetic pathways responsible for the synthesis of IPP and DMAPP, which are i) the mevalonic acid (MVA) pathways located in cytosol, and ii) the methyl-erythritol-phosphate (MEP) pathway situated in the plasmids (Dudareva et al. 2006). In both, cytosol and plasmids, IPP and DMAPP can be synthesised into farnesyl diphosphate (FPP), which is the common precursor of all sesquiterpenes. Individual sesquiterpene can be further biosynthesised from FPP, regulating by terpene synthases (TPSs). Up to now, there are more than 89 TPSs genes identified, and a single TPSs gene may be responsible for the production of more than
one compound (Martin et al. 2010). The detailed biosynthetic pathway of rotundone remains unclear. However, recent studies have shown that rotundone can be aerially oxidized or biosynthesized from a precursor compound, namely α-guaiene, which can be biosynthesized by VvPNSeint and VvTPS24 TPSs in grapevines (Drew et al. 2015, Huang et al. 2014, Martin et al. 2010, Takase et al., 2015).

Unlike many other wine aroma compounds formed or released during fermentation, rotundone originates from the grape berries, and is extracted during the winemaking process (Herderich et al. 2012). Rotundone is mainly found in red wine, but also at very low concentration in white varieties (Jeffery et al. 2009). This is mainly due to the wine making process, as rotundone only exists in grape skins but not in grape pulps (Siebert et al. 2008). Human detection threshold of rotundone is 16 ng L⁻¹ in red wine and 8 ng L⁻¹ in water, with around 20 % of the population are not able to detect rotundone, not even at very high concentration (Wood et al. 2008).

The factors affecting rotundone production in grapes were elusive prior to this project. Large variations in rotundone within the same vineyard block were identified by our collaborators during the period of this project (Scarlett et al. 2014). The latter study showed that vineyard vigour, slope and soil conductivity may be related to rotundone production. Since the most ‘peppery’ Shiraz wine observed was produced from cooler climate regions, temperature has been suggested as a possible factor affecting rotundone production in grapes (Scarlett et al. 2014, Jeffery et al. 2009). Irrigation near veraison was also pointed out to be important factor determining the final concentration of rotundone in grape berries (Geffroy et al. 2014).

Rotundone concentration in wine is also affected by the fermentation process, and likely rotundone is not 100 % extracted from the grape skins into wine (Siebert and Solomon 2010). The concentration of rotundone in fermented grape juice mainly increases during the 2nd and 5th day of primary fermentation and reaches a relatively stable level afterward (Siebert and Solomon 2010). From the rotundone quantification methodology developed by Siebert et al. (2008), rotundone in skin could only be fully extracted using 50 % ethanol. This shows that alcohol content in wine could affect the percentage of rotundone.
extraction. Fermentation with addition of grape leaves and stems also significantly increases the concentration of rotundone in the resultant wine, which shows that grape leaves and stems also have high concentration of rotundone (Capone et al. 2012).
Rotundone is a relatively new aroma/flavour compound for grape and wine, and therefore has been less studied. The current project investigates the factors affecting the production of rotundone in grapevine, which could potentially help grape growers to manipulate the concentration of this compound in their products by changing management strategies.
1.2 Project scope and hypothesis

This PhD project focused on studying the environmental and physiological factors that may influence rotundone formation and accumulation in grapevines. The hypothesis of the project includes:

1. The production of rotundone is affected by one or several of the following environmental factors: temperature, solar exposure, vine vigor, vineyard water availability and vineyard aspect;
2. The production of rotundone is affected by one or several of the following physiological factors: grapevine canopy shape, grape bunch location, non-grape material mass (NGM), grapevine phenological stages, vineyard herbivores activity and plant hormones.

![Figure 1.1 Structure of the research project](image)

This PhD project has three stages of experiments: preliminary studies, field trials and glasshouse trials, targeting the environmental and physiological factors affecting rotundone production in grapevine as described in the
hypotheses (Figure 1.1). The preliminary studies include two parts, vineyard variability study and vertical study of wine rotundone. The vineyard variability study aims to characterise the spatial variability of rotundone in grapes across a single vineyard site. This study was initiated by the Rathbone Wine Group collaborating with CSIRO and AWRI. The University of Melbourne (Our research team) joined this project and assisted with grape sampling, rotundone analysis and spatial mapping of rotundone. This work has been published on a peer review journal with acknowledgement for my assistance (Scarlett et al. 2014). Due to copyright, the results of this study is not listed as part of this thesis, nevertheless the data established from this study aided in the design of other experiments. The vertical study of wine investigated the influence of weather parameters on the concentration of rotundone in wine over historical growing seasons, and to predict the possible concentration of rotundone in future wine. This study identifies the most important environmental factors that influence the concentration of rotundone in grapes, and has been described in details in Chapter 2.

The second stage of this project included four field trials studies. Based on the results of Chapter 2, field trials were designed to investigate the impacts of individual environmental factors, temperature (Chapter 3) and solar exposure (Chapter 4) on the concentration of rotundone in grapes. Some grapevine physiological factors, such as grapevine phenological stages (Chapter 5), vine canopy characteristics and non-grape materials (Chapter 6), are related to environmental factors, and their impacts on the concentration of rotundone in grapes were investigated individually.

Potted vines were established in a glasshouse (System Garden, Parkville, The University of Melbourne) to study physiological parameters as the third stage of the project. Potted vines establishment is described in detail in the appendix A. The possibility of rotundone translocation between different grapevine organs was investigated using stable isotope tracer on the potted vine (Chapter 6). The impacts of herbivore on rotundone productions were investigated in three trials by applying insects, herbivore related hormones, mimic herbivorous physical damage to potted grapevine leaves (Chapter 6).
1.3 Objectives

The ultimate goal of this project is to benefit Australian grape growers and wine producers by maximizing rotundone concentration in their products. To achieve this goal, objectives at each stage were specified as below:

Stage one:
1. To investigate if grapevine growing environment could affect the concentration of rotundone in grape berries;
2. To identify the most important environmental factors for rotundone production.

Stage Two:
1. To assess the importance of individual environmental factors to the concentration of rotundone in grape berries;
2. To study the concentration of rotundone and other sesquiterpenes compounds in grape berries at different grapevine phenological stages;
3. To analyse the influence of canopy characteristic and non-grape materials on the concentration of rotundone in different grapevine organs.

Stage Three:
1. To investigate the possible interactions between different grapevine organs in rotundone production;
2. To test whether rotundone production in grapevine leaves can be stimulated by herbivores.

The final objective is to establish a protocol that can be practically used by grape growers to maximize the rotundone concentration in their product based on the scientific research results.
1.4 References


CHAPTER TWO

Environmental factors and seasonality affect the concentration of rotundone in Vitis vinifera L. cv. Shiraz

3.1 Introduction

This chapter is the preliminary research (Stage One) on the environmental factors potentially important to the concentration of rotundone in Shiraz wine. This study compares the concentration of rotundone in Shiraz wine produced from different vintages in the past 20 years with historical weather data, and indentifies the most important weather parameters affecting the concentration of rotundone in Shiraz grape and wine.

The aim of this chapter was to identify the weather parameters important to the concentration of rotundone in Shiraz grapes and wine. The hypothesis of this chapter was that:

1. Variation in the concentration of rotundone in Shiraz wine is related to the variation in historical weather data.
2. Temperature, precipitation and solar exposure may be the important factors affecting the concentration of rotundone in Shiraz grapes and wine.
3. Weather parameters only at post-veraison stage of grape development are important to the concentration of rotundone, as rotundone in grape berries mainly accumulates at this phenological stage.
Environmental Factors and Seasonality Affect the Concentration of Rotundone in Vitis vinifera L. cv. Shiraz Wine

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Abstract
Rotundone is a sesquiterpene that gives grapes and wine a desirable 'peppery' aroma. Previous research has reported that growing grapevines in a cool climate is an important factor that drives rotundone accumulation in grape berries and wine. This study used historical data sets to investigate which weather parameters are most influencing rotundone concentration in grape berries and wine. For this purpose, wines produced from 15 varieties from the same Shiraz vineyard (The Old Block, Mount Langi Ghiran, Victoria, Australia) were analysed for rotundone concentration and compared to comprehensive weather data and minimal temperature information. Degree hours were obtained by interpolating available temperature information from the vineyard site using a simple piecewise cubic hermite interpolating polynomial method (PCHIP). Results showed that the highest concentrations of rotundone were consistently found in wines from cool and wet seasons. The Principal Component Analysis (PCA) showed that the concentration of rotundone in wine was negatively correlated with daily solar exposure and grape bunch zone temperature, and positively correlated with vineyard water balance. Finally, models were constructed based on the Gompertz function to describe the dynamics of rotundone concentration in berries through the ripening process according to phenological and thermal times. This characterisation is an important step forward to potentially predict the final quality of the resultant wines based on the evolution of specific compounds in berries according to critical environmental and micrometeorological variables. The modelling techniques described in this paper were able to describe the behaviour of rotundone concentration based on seasonal weather conditions and grapevine phenological stages, and could be potentially used to predict the final rotundone concentration early in future growing seasons. This could enable the adoption of precision irrigation and canopy management strategies to effectively mitigate adverse impacts related to climate change and microclimatic variability, such as heat waves, within a vineyard on wine quality.
Introduction

Predicting the quality of wine by analysing the vineyard weather parameters has proven to be an attractive, yet elusive goal for scientists and viticulturists. Mean January temperature (MJT) and growing degree days (GDD) are the most commonly used viticultural weather parameters to estimate the quality and potential grape points of the wine for Vitis vinifera L. cultivars [1]. More broadly, the number of days with a specific range of temperatures, solar radiation and relative humidity during grape ripening has been associated with resulting wine quality in several Australian wine regions [2]. Vineyard micrometric data, including visible light radiation and canopy temperature were also shown to be good indicators of the final quality for Sauvignon blanc wine in South Africa [3].

Newly available databases provide more comprehensive environmental information to viticulturists that could be helpful to select agroclimatic regions suitable for viticulture growing. Specifically, the Australian water availability project (AWAP) has established an Australian-wide fine scale climate map database, which can provide precise historical weather data (from 1900 onwards) for any Australian agricultural location [5]. This gives an opportunity to study the influence of climatic and micrometric parameters on grape and wine quality throughout historical seasons. Thus, wine quality estimation models could be established to characterise and estimate quality trait parameters for wine based on the AWAP dataset for a specific viticultural site or region in Australia.

Rotundone is an oxygenated bicyclic sesquiterpene that contributes to the 'black pepper' character of Shiraz grapes and wine, which is favourable to many wine consumers, especially experienced wine drinkers [6,7]. This peppery characteristic is stylistically crucial to high-quality Australian Shiraz wine [8]. Unlike many other wine aroma compounds, rotundone originates from the grapes berry encarp (skin) [9], and is extracted during the wine primary fermentation process [8]. Rotundone is a very stable chemical compound in the wine matrix, with little loss under different bottling and accelerated aging conditions [10]. Therefore, grape rotundone concentration is crucial to the 'peppery' character in the finished wine, and the rotundone found in wine can be used as an accurate proxy for the amount found in wine grapes from the same vintage. And as rotundone concentration is directly related to the sensory quality of the Shiraz wine, measuring this compound and its relation to environmental/weather measures provides an opportunity to predict and model wine quality.

The influence of vineyard microclimate on the production of rotundone is currently elusive in the literature. In general terms, Shiraz grapes grown in cooler climates have been associated with higher rotundone concentration in grape berries and therefore in wines produced. The latter, based on the organoleptic observation that the most 'peppery' Shiraz wines originates from 'cooler' geographic production areas [8]. More recently, it has been demonstrated that rotundone in berries varies considerably within the same vineyard [11], within the same vine and even within the same bunch [12], which may be related to the differences in bunch zone microclimate. This same research showed that temperatures at the grape surface bunch zone and ambient air, were important for rotundone production, with temperatures exceeding 25°C being associated to negative impacts on berry rotundone concentration.

Seasonal water balance in a vineyard is critical to the development of many flavour and quality traits in grapes and wine [13,14]. Vine water deficit has been linked to increments of monoterpenoids and isoprenoids concentration in reached Merlot wine [14]; ii) affecting the pheophytin, abscisic acid, isoprenoid, carotenoid, amino acid and fatty acid metabolic pathway in Cabernet Sauvignon and Chardonnay [13]; iii) significantly increasing the transcription of one sesquiterpene related terpene synthetase at maturity in Chardonnay and at early ripening stages in Cabernet Sauvignon [15]. Even though, the metabolic pathway of...
rotundone has not been fully unravelled, one possible precursor namely o-guaiene has been reported [19], and there is the possibility that water deficit may affect the rotundone biosynthetic pathway. For example, a recent study showed that increased irrigation over the veraison-harvest period resulted in higher rotundone concentration in grapes and resulted wine [16].

Solar exposure of grape berries is also important for the accumulation of berry aroma and flavour compounds. It has been shown that moderate solar exposure increased grape berry monoterpenes concentration at harvest in Traminette grapes [17]. On the contrary, complete sunlight exclusion from berries, especially from veraison to harvest, significantly inhibits the synthesis and accumulation of monoterpenes in Muscat grapes [18]. Sunlight exposure influences grape metabolites synthesis either by increasing grape surface temperature or via higher UV-B radiation [12, 17, 18]. The latter also influences the concentration of abscisic acid, salicylic acid, jasmonic acid and ethylene in plants, which play important roles in the up-regulation and down-regulation of gene expression for plant immune responses, especially terpenoids synthesis [19–21]. The influence of sunlight on the concentration of rotundone has not yet been studied. Nevertheless, previous studies suggest that sunlight could be a factor important to the final concentration of rotundone in grapes at harvest.

The influence of weather parameters on grape quality varies at different grapevine physiological stages, especially when some compounds such as sesquiterpenes mainly develop at certain time of the ripening stage in Vitis vinifera cultivars [22]. Bicyclic sesquiterpenes were mainly detected in the post-veraison period of berry development in Shiraz [23], and therefore the weather parameters during this period may be more important to the concentration of rotundone in grapes at harvest. In western Victoria (Australia), grapevines start the annual cycle around late September to October, where the dormant vines enter the bud burst stage and start to grow. In this region, veraison (start of berry softening and colour change) occurs typically from January to early February for the Shiraz cultivar [24], but usually occurs in middle February for the studied vineyard. Commercial harvest typically happens from middle March to early April in this region, but usually middle to late April for the studied vineyard. The critical stage for quality grape production is from the post-veraison to harvest period, where grape berries rapidly accumulate sugar, change colour, shift metabolism and accumulate flavour compounds [24]. It is likely that meso- and micro-climatic parameters at the post-veraison period play a critical role in the final rotundone concentration in wine grapes, as rapid rotundone accumulation has been only observed to occur around two weeks before harvest [24].

This paper describes a detailed study on the interaction between weather parameters and rotundone concentration in Shiraz grapes and wine over 15 growing seasons (from 1996–97 until 2013–14). This study implements interpolation techniques to obtain hourly air temperature in the bunch zone from historical growing seasons, and establishes descriptive models to characterise grape and wine rotundone concentrations in berries within a season. Even though the models described in this paper are site specific, the modelling techniques are applicable to any vineyard with adequate environmental and berry quality records and information. These techniques can be used to link historical weather data with rotundone production considering the availability of critical parameters, and therefore potentially be applied to predict rotundone concentration at harvest from real-time weather information in the growing season.

Materials and Methods

Site and plant material description

All grapes and wines used for this study were obtained from a commercial vineyard planted with Vitis vinifera L cv Shiraz (The Old Block, Mount Langi Ghiran 37°31′S, 143°15′E) located in the Grampians wine region of Victoria, Australia. The vineyard was planted in 1968 on its...
own roots at 3.0 m between rows and 1.8 m between vines, with rows oriented northeast to southwest. Grapevines are trained to a vertical shoot positioning trellis (VSP). Regional climatic data is described in the following sections.

Ethics statement
All the samples in this study were collected from private land (Mount Langi Ghiran), and the owners of the vineyards gave permission to conduct the study on these sites. No specific permissions were required for these locations, because there were no endangered or protected species in these areas, and this study did not involve endangered or protected species.

Wine and grape sampling for rotundone concentration assessment
Shiraz wines from 15 different vintages were studied, with the earliest growing season from 1995–1996 (vintage 1996) and the most recent growing season of 2013–2014 (vintage 2014). Due to the limitation of wine stock from historical seasons, only selected seasons were studied. Two bottles of Shiraz wine produced in each selected vintage were sampled using 100 ml sealed bottles, and transferred to the laboratory for chemical analysis. It has been shown that winemaking techniques potentially affect the concentration of rotundone in wines as rotundone accumulates during the first few days of primary fermentation and remains constant afterward [9]. However, the winemaking protocol at the studied commercial winery was consistent throughout the studied vintages. Therefore, the winemaking process was not considered as a major factor contributing to the differences in wine rotundone concentration in this study.

Grape branches were randomly sampled across the vineyard in triplicate (2kg per field replicate) at fortnightly intervals from 80% veraison to commercial harvest in three continuous seasons (2011–12, 2012–13 and 2013–14). Grape samples were collected in zip-lock plastic bags, frozen at -20°C and transferred to the laboratory in styrofoam boxes on dry ice, and stored at -20°C before chemical analysis. The rotundone concentration in grapes and wine has previously been reported to be stable under proper storage conditions, and unlikely to change drastically during wine aging [10].

Preparation of samples and SPME-GC-MS analysis of rotundone
Grape and wine samples were prepared for rotundone analysis based on the protocol described by Siebert et al. [11]. The 100 ml of wine samples described before were sub-sampled before analysis. 100 ml of 10% ethanol (5.36 ng/ml in ethanol) was added as internal standard to each sample, and then subjected to solid phase extraction (SPE) performed as reported previously [9]. For each of the grape samples, 300 g of destemmed grapes were sub-sampled before being homogenised using a hand-held blender. Sub-samples were centrifuged to separate the juice and solid parts. The solid parts were mixed with 30 ml of ethanol, 30 ml of water and 106 μl of d5-rotundone (515ng/ml in ethanol) as internal standard, then shaken for 24 hrs at 22°C and sonicated before adding the juice back. Sub-samples were then centrifuged and filtrated (1.6 μm glass fibre) to obtain berry extract filtrate, which was topped up to 200ml with Milli-Q deionised water before subjected to solid phase extraction (SPE) following the same method used for wine samples. For both grape and wine samples, the SPE residue supernatant collected was air dried with nitrogen, and reconstituted in 0.3 ml of ethanol and 9ml of Milli-Q deionised water. The samples were then analysed and quantified with SPME-GC-MS using the parameters described by Geoffrey et al. [24].
Regional historical climatic data and irrigation scheduling

The weather data of each selected season was obtained from the nearest Bureau of Meteorology (BOM) weather station located at Ararat Prison (Australian BOM Station No. 0959083), which is approximately 13.5 km west from the vineyard. The long-term mean January temperature (MIT) recorded at this weather station was 18.9°C with annual average rainfall of 596 mm, which classify it as a cool climate wine region [23]. The seasonal MIT and mean daily solar exposure (E_d) from October to harvest were also calculated for each studied season (SI Table). The vineyard is under drip irrigation system with dripper spacing of 0.5 m, and discharge of 1.5 l h⁻¹ since 1998. Before 1998, a different drip irrigation layout was in place with dripper spacing of 1.8 m, and dripper output of 4 l h⁻¹. Irrigation data from each vintage was recorded by the winery. Crop evapotranspiration (ET_c) from each season has been calculated as reference evapotranspiration (ET_o) × crop coefficient (K_c), where ET_o was calculated from the temperature and dew point data (BOM station) based on the simplified Penman formula [29], while K_c used were 0.28, 0.35, 0.42, 0.49, 0.55 and 0 for October, November, December-January, February, March, April and May, respectively, based on Pochey et al. [28]. Cumulative growing degree-days (DD) from each season from Oct to harvest were calculated from the BOM station temperature data with a base temperature of 10°C following the method described by Gething et al. [22] and a proposed interpolation method to obtain half-hourly temperature data from daily maximum and minimum temperatures, which will be described in detail later in this paper. The MIT, E_d, irrigation volume, ET_c and DD, for studied seasons are described in SI Table.

Interpolated vineyard weather data to obtain thermal time

A simulated temperature model (STM) obtained with minimal data, and specific for the experimental vineyard, was developed based on the AWAP database. The Australian Bureau of Meteorology have generated high-resolution spatial climate maps for AWAP based on the Australian Data Archive for Meteorology [14], which includes daily/monthly rainfall, temperature and solar exposure data from 1980 to 2014 (continuously updated). Weather data of the studied vineyard was extracted using the vineyard GPS location from the AWAP high-resolution spatial climate maps using ArcMap software (ver.10, Esri, Redlands, CA, USA). Mean daily maximum temperature (T_max), mean daily minimum temperature (T_min) and mean daily solar exposure (E_p) from version to version were extracted for each studied season. Since historical version time from the studied vineyard was not available, the estimated version time was the 15th February for most of the growing seasons (Mr Damien Sheehan, vineyard manager, personal communication; dibsheehan@iangi.com.au). However, for the growing seasons where harvest was conducted earlier than the 14th April, the estimated version time was approximately 60 days before harvest (Mr Damien Sheehan, dibsheehan@iangi.com.au). Water balance (P_n) for each growing season (October to harvest) was calculated using a simplified water balance method as total rainfall + total irrigation - ET_c, where total rainfall was calculated from AWAP maps. Furthermore, T_max, T_min, E_p, total rainfall, P_n, and wine rotundone concentration for studied seasons are described in SI Table.

The STM estimates the half-hourly temperature of the vineyard from version to harvest based on the daily maximum and minimum temperature obtained from AWAP high-resolution spatial climate maps using the piecewise cubic hermite interpolating polynomial (PCHIP) function and a customised code written in MatLab ver. 2014a (The MathWorks, Inc. Matick, MA, USA). The PCHIP function can be expressed as:

\[ T_i = \text{PCHIP}(T_{in}, T_{out}, t_i). \]
where \( T_p \) is the predicted temperature at the time point \( t_p \), \( T_{\text{prev}} \) is the daily maximum/minimum temperature at their corresponding time \( t_{\text{prev}} \). PCHIP finds temperature values of an underlying interpolating function \( T(t) \) at each time point between the time points of daily maximum and minimum temperatures, such that on each time subinterval, \( 0 \leq t \leq t_{\text{prev}} \), \( T(t) \) is the cubic Hermite interpolant at the given temperature values and certain slopes at two endpoints; \( T(t) \) interpolates \( T_{\text{prev}} \), for example, \( T(t_{\text{prev}}) = T_{\text{prev}} \) and the slopes at the \( t_{\text{prev}} \) are chosen in a way that \( T(t) \) preserves the shape of the \( (t_{\text{prev}}, T_{\text{prev}}) \) data and respects monotonicity. This means that, on intervals where the data are monotonic, so is \( T(t) \), at points where the data has a local extreme, so does \( T(t) \).

Temperatures from veraison to harvest were estimated at half-hourly intervals using the STM proposed for all growing seasons studied. The AWAP temperature data and predicted half-hourly temperatures from STM were plotted against time (Fig 1). Vineyard thermal time was calculated as degree hours using the data obtained from STM following the established protocol [27], which represents the heat-hours accumulated in the vineyard (S3 Table). Percentage of degree hours above 35°C (DH35), above 30°C (DH30), and above 25°C (DH25) from the total degree hours were calculated using a customised code written in Matlab ver. 2014a (The MathWorks, Inc. Natick, MA, USA) (S3 Table). Cumulative growing degree days from veraison to harvest (\( \Delta GD \)) were calculated by dividing degree hours higher than 10°C by 24 (S3 Table) [23].

**Vineyard micrometeorological data**

Fruit zone temperature of the studied vineyard was measured by temperature loggers (Tinytag transit 2, Gemini Data Logger Ltd, Chichester, UK) in the 2012–13 and 2013–14 growing seasons to validate the estimated temperature data from the STM. In the 2012–13 growing season, one logger (L2013A) was placed in the canopy of a representative vine, while the second logger (L2013B) was placed in another representative vine canopy covered by a commercial UV-stabilised high density polyethylene (HDPE) shade cloth, which blocks 57% of light and 69% of UV (Coolaroo, Gale Pacific Ltd, Australia). In the 2013–14 growing season, one logger (L2014A) was placed in a representative vine canopy, while the second logger (L2014B) was placed in a shading box, and installed next to the latter logger. The shading boxes used in this experiment were similar to those described in Downten et al. [28], which are made from white polypropylene sheeting painted black on the inside. Artificial shadings were used to minimise direct solar radiation to temperature loggers, and therefore shaded loggers could better reflect the air temperature at the bunch zone. The logger has an operational temperature range of -40°C to 70°C with a 0.01°C resolution. Temperature was measured and logged every 30 mins from veraison to harvest (14th Feb—10th Apr 2013 and 8th Feb—8th Apr 2014). The recorded data was downloaded and analysed using the Tinytag Explorer software (version 4.7, Gemini Data Logger Ltd, Chichester, UK) following the method described previously [27].

**Rotundone accumulation model**

A rotundone accumulation model was proposed to characterise the accumulation dynamics of rotundone in grape berries after veraison based on the Gompertz function, which was parameterised using Matlab ver. 2014a (The MathWorks, Inc. Natick, MA, USA) as follows:

\[
R_{\text{tot}}(t) = \text{Gompertz}(a, b, c, T_p) = ae^{-be^{-ct}},
\]

where \( R_{\text{tot}} \) is the rotundone concentration of grape berries at the time point \( t_p \) during grape ripening process (Fig 2). The parameter \( a \) is the upper asymptote, which represents the maximum concentration of rotundone the grape may achieve in a specific season. The parameter \( b \)
sets the displacement of the Gompertz curve along the time (T) axis, and therefore determines when rotundone in grape starts to increase rapidly. The parameter \( c \) sets the growth rate of the Gompertz curve, which reflects the speed of rotundone accumulation in grape berries.

**Statistical analysis**

Estimated temperatures using the STM and observed vineyard temperature data were compared using the CoStat software (version 6.4. CoStat Software, Monterey, USA). Weather data from BOM stations, AWAP-based weather data, calculated weather data from STM and wine...
rotundone concentration (RotC) were analysed using principal component analysis (PCA) with a customised code written in the Matlab ver. 2014a (The MathWorks, Inc., Math, MA, USA). The statistical parameters and significance of relationships between weather parameters and RotC were calculated using the CoStat software (version 6.4, CoHort Software, Monterey, USA). Weather data and RotC were also analysed with the k-mean clustering and stepwise linear discriminant analysis (SLDA) using SPSS 21 (SPSS Inc., Chicago, IL, USA).

Results and Discussions
Regional weather condition of the studied vineyard
In the past 20 years, the studied wine region experienced a relatively warm period, as the average 15-station daily average temperature has been lower than the long-term average (18.8°C) described as climatic condition for the area. This is consistent with a climate variation study showing that annual temperature of major Australian wine-growing regions has been increasing in the past 20 years and will continue to increase in the next 40 years [29, 30]. Despite the observed increases in temperature, the average daily average temperature at all studied stations (19.8°C) is still within the range of a cool climate wine region classification (MT: <20.5°C) [31]. However, four of the selected seasons (1998–99, 2005–06, 2011–12 and 2013–14) had MT above this range, and could be considered as warm seasons. Seasons with both high and low ET were selected for this study to investigate the influence of solar exposure on the quality trait analyses of rotundone. The 1995–96, 2005–06 and 2010–11 growing seasons had low level of ET, which was high for the 2006–07 and 2007–08 growing seasons (SI Table). The studied seasons also had a wide variation of DD, ranging from 1,090 to 1,424 degree days. Large variation in precipitation was also observed among the studied seasons from a minimum of 124 mm up to a maximum of 605 mm. Vineyard irrigation volume was relatively small compared to the precipitation, which ranged from 0 to 112 mm of water applied per season (Oct–Harvest). ET did not vary as much as precipitation among the studied seasons, from 328 to 420 millimetres.

Validation of the Interpolated weather data
A linear regression analysis was performed between observed (temperature loggers) and estimated data (from AWAP and STM) for both 2012–13 and 2013–14 growing seasons using the curve fitting tool in Matlab (Fig 3). A strong positive linear relationship was established for both 2012–13 and 2013–14 (Fig 3a, y = 0.842x + 3.393, R² = 0.84, RMSE = 2.255, p < 0.0001) and 2013–14 (Fig 3b, y = 0.8135x + 4.228, R² = 0.86, RMSE = 2.478, p < 0.0001) growing seasons. The STM consistently underestimated daily maximum temperature and overestimated daily minimum temperature (Fig 3). Compared to the loggers (L2013A and L2014A) positioned in representative canopies of vines, the predicted percentage of degree hours from artificially shaded loggers (L2013B and L2014B) were closer to the observed value. Artificially shaded loggers (L2013B and L2014B) had minimum influence from direct solar exposure, and therefore their data better reflected the bunch zone air temperature. Thus, the estimated temperature and degree hours from the STM model were suitable to represent the bunch zone air temperature of the studied vineyard.

Limitations of AWAP dataset and STM estimation
The IOM high-resolution spatial climate maps provides data to accurately estimate vineyard temperatures over time. However, the accuracy of the AWAP weather data can be hampered by two factors: 1) the AWAP spatial climatic maps have a resolution of 0.05° (longitude) X 0.05° (latitude) (approximately 5 km²), and therefore the diurnal temperature data obtained from
AWAP maps were averaged values from an area with a wide variation of plant species and vigour [1]. The measured area on the AWAP maps for the studied vineyard includes high vigour eucalyptus forest surrounding vineyards, as a result the AWAP temperature data could underestimate the maximum temperature from the vineyard (Fig 3a, b). Secondly, large spatial variations in topography, slope and soil properties exists in the studied vineyard [11], which is associated to the variation in vineyard vigour [11, 12]. These variations could explain inconsistency in the interpolation of microclimate data within the studied vineyard, resulting in relatively higher vineyard surface temperature in low vigour area during the day [13]. Therefore, the actual vineyard temperature, especially in lower vigour areas may be higher than the calculated value from AWAP. Despite these differences, the eucalyptus forest and vineyard land area of the studied vineyard has limited variation among studied seasons, and the spatial variation of vigour in the vineyard is relatively stable across different seasons [13]. Therefore, differences between AWAP and actual temperature variations are consistent over years, which validate the use of interpolated temperature from AWAP as an alternative to actual time resolved temperature readings for the comparison of vineyard temperature profiles among different growing seasons.

The STM was established to estimate vineyard bunch zone air temperature over time whenever actual measurements were not available. Even though, the STM described in this paper is site specific, the STM modelling techniques could be applied to most wine growing regions in Australia wherever the high-resolution spatial climate maps data are available. The accuracy of STM largely depends on the temperature data obtained from AWAP, which may underestimate vineyard diurnal temperature (Fig 3). As a result, the predicted percentage of degree hours from STM (STM Table) is relatively smaller than the observed value (S4 Table). Similarly as the differences between AWAP and actual temperature, the differences between estimated
and actual degree hours are also consistent over the years. The accuracy of STM is also affected by the cloud fraction during daytime since the ground surface temperature with vegetation layer is affected by this factor [34]. Therefore, temperature changes rapidly within a day with cloud coverage variability. On clear and totally cloudy days, ground surface temperature usually increase/decrease smoothly (Fig. 4a), which can be well predicted by the STM. However, on partially cloudy days, where ground surface temperature changes irregularly due to cloud cover variability, the predicted temperature from STM may not accurately reflect the real temperature changes (Fig. 4b).

**Outputs from AWAP maps, STM and rotundone concentration in wine**

The studied seasons had clear differences in the majority of weather parameters obtained from AWAP maps. From all of the seasons studied, 2006–07 and 2007–08 were typically hot with associated water deficits and strong solar exposure from vernal to harvest \( T_{\text{max}} \), 26.7 and 27.5°C, \( T_{\text{mean}} \), 12.7 and 12.8°C, \( P_{\text{tot}} \), 234 and 1.32 mm, \( E_{\text{tot}} \), 207 and 23.8 Mm \(^3\) [32 Table]. While the 1995–96, 1998–99 and 2010–11 seasons were typically cooler and wet seasons, with relatively lower solar radiation from vernal to harvest \( T_{\text{max}} \), 21.4, 21.6 and 21.0°C, \( T_{\text{mean}} \), 9.0, 7.9 and 10.2°C, \( P_{\text{tot}} \), 36, 28.2 and 254.8 mm, \( E_{\text{tot}} \), 14.5, 17.0 and 14.2 Mm \(^3\) [32 Table]. The 2003–04 growing season was a typically cool season, but with water deficit and moderate solar exposure from vernal to harvest \( T_{\text{max}} \), 22.6°C, \( T_{\text{mean}} \), 9.9°C, \( P_{\text{tot}} \), 194.9 mm, \( E_{\text{tot}} \), 47.8 Mm \(^3\) [32 Table]. The remaining seasons had relatively moderate weather parameters [32 Table] representative of the climate classification for the region. Initial data analysis showed that degree hours rather than mean temperatures were more suitable for modelling the relationship between vineyard temperature and Rot\(_{0}\). For example, the 2007–08 growing season only had slightly higher \( T_{\text{max}} \) (22.4°C), compared to the 2008–09 growing season (26.7°C), while it had much higher DHI\(_{0}\) (2005–08: 8.47%, 2006–07: 2.6%) [32 Table]. This is indicative of extreme hot weather in the 2007–08 growing season. Cool seasons did not necessarily have consistent low temperature from the vernal to harvest period. Even though the 2003–04 growing season had relatively low \( T_{\text{max}} \) (21.4°C) and \( T_{\text{mean}} \) (9.0°C) compared to the other seasons, it also had relatively high percentage of hot weather condition with almost 3% of DHI\(_{0}\) throughout the season. The highest concentration of rotundone was found in wines from the 1998–99 growing season (116 ng/L), while the 2005–06, 2006–07, 2007–08 growing seasons had similarly low concentration of rotundone (3.6, 4.6 and 2.5 ng/L) [32 Table]. All five growing seasons (1995–96, 1998–99, 2001–02, 2010–11 and 2011–12) with moderate to high concentration of rotundone (68.3, 115.8, 60.5, 52.5 and 67.1 ng/L, respectively) had none or very low DHI\(_{0}\), and relatively low DHI\(_{1}\) and DHI\(_{2}\) [32 Table].

**Impacts of climatic parameters on wine rotundone concentration (Rot\(_{0}\))**

From the PCA, it can be seen that the first two principal components combined explained almost 76% of the total variability in the data. The 15 studied seasons were widely spread in the PCA biplots, showing that there were high variation among seasons in weather parameters and Rot\(_{0}\). Seasons were separated along PC1 (59%) mainly on the basis of \( E_{\text{tot}} \), Rot\(_{0}\), \( T_{\text{mean}} \) and DHI\(_{0}\). PC1 shows a positive correlation between Rot\(_{0}\) and DHI\(_{0}\). An inverse correlation was found for the previous two variables with \( E_{\text{tot}} \), \( T_{\text{mean}} \) and DHI\(_{0}\). PC2 (16%) separated the seasons mainly according to the MAT, DD\(_{1}\) and DD\(_{2}\).

The relationships between Rot\(_{0}\) and individual weather parameters were further analysed (Table 1). A positive exponential curve was established between Rot\(_{0}\) and \( P_{\text{tot}} \) (\( r = 0.78, R^{2} = 0.59 \), \( F = 7.3, p = 0.0005 \)) (Table 1). Wine rotundone was also found to have a significant exponential relationship with \( T_{\text{mean}} \) (\( r = 1.79, R^{2} = 0.58 \)).
Fig. 4. Limitations of the simulated temperature model (STM). (a) Best estimation of half hourly temperature on a clear day (8th Feb 2014, Day of the year 40). (b) Worst estimation of half hourly temperature on a partially cloudy day (21st Mar 2016, Day of the year 80).

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$$R^2 = 0.51, \text{RMSE} = 23.78, p = 0.0003$$, $T_{\text{min}}(y = 959.4e^{-0.3770x}, R^2 = 0.58, \text{RMSE} = 22.17, p = 0.0385$ and $T_{\text{max}}(y = 279.9e^{-0.2630x}, R^2 = 0.32, \text{RMSE} = 26.66, p = 0.0003$ (Table 1). The model was further analyzed against calculated percentage of degree hours obtained from the STM, and it was found to have an exponential relationship with $DDH_3(y = 1.141000e^{0.0356x}, R^2 = 0.53, \text{RMSE} = 23.35, p = 0.0366$), $DH_{15}(y = 35.56e^{0.0334x}, R^2 = 0.46, \text{RMSE} = 27.15, p = 0.0336$), $DH_{30}(y = 85.56e^{-0.2740x}, R^2 = 0.53, \text{RMSE} = 23.43, p = 0.0041$) and $DH_{90}(y = 157.70e^{-0.4038x}, R^2 = 0.61, \text{RMSE} = 21.32, p = 0.0001$ (Table 1). No significant

Table 1. Exponential relationships (y = a exp(bx)) between rothundone concentration in wine (RothL) and climatic parameters.

<table>
<thead>
<tr>
<th>Climate factors</th>
<th>Coefficients</th>
<th>$\text{SSE}^a$</th>
<th>$R^2$-square</th>
<th>$\text{RMSE}^b$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DOM</strong> data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean January temperature °C (MJT)</td>
<td>36.61</td>
<td>-0.0988</td>
<td>10570</td>
<td>0.0001</td>
<td>34.95</td>
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<td>Mean daily solar exposure Mm³ (EJB)</td>
<td>272.2</td>
<td>-0.1959</td>
<td>12690</td>
<td>0.0774</td>
<td>32.76</td>
</tr>
<tr>
<td>Cumulative growing degree days (DDH)</td>
<td>6319</td>
<td>-0.0043</td>
<td>11510</td>
<td>0.2364</td>
<td>29.76</td>
</tr>
<tr>
<td><strong>AWAP</strong> data</td>
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</tr>
<tr>
<td>Water Balance ($P_{aw}$)</td>
<td>37.84</td>
<td>0.0026</td>
<td>11110</td>
<td>0.2632</td>
<td>29.23</td>
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<tr>
<td>Mean maximum temperature °C ($T_{\text{max}}$)</td>
<td>11700</td>
<td>-0.3489</td>
<td>7256</td>
<td>0.1210</td>
<td>23.76</td>
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<tr>
<td>Mean minimum temperature °C ($T_{\text{min}}$)</td>
<td>9580</td>
<td>-0.5710</td>
<td>6389</td>
<td>0.5701</td>
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<tr>
<td>Mean daily solar exposure Mm³ (EJB)</td>
<td>2799</td>
<td>-0.2610</td>
<td>10240</td>
<td>0.3208</td>
<td>29.06</td>
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<tr>
<td><strong>STM</strong> data</td>
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</tr>
<tr>
<td>Cumulative growing degree days (DDH)</td>
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<td>-0.0386</td>
<td>7091</td>
<td>0.5296</td>
<td>23.38</td>
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<tr>
<td>% of Degree days above 35°C ($DH_{35}$)</td>
<td>55.58</td>
<td>-14.39</td>
<td>9981</td>
<td>0.3843</td>
<td>27.18</td>
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<tr>
<td>% of Degree days above 40°C ($DH_{40}$)</td>
<td>85.66</td>
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<td>7138</td>
<td>0.5264</td>
<td>23.43</td>
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<tr>
<td>% of Degree days above 25°C ($DH_{25}$)</td>
<td>197.7</td>
<td>-0.4031</td>
<td>5968</td>
<td>0.5680</td>
<td>21.32</td>
</tr>
</tbody>
</table>

*The climate data is for the period from October to harvest.

*The climate data is for the period from verasion to harvest of each season. Verasion is approximately 15th February for most seasons. For seasons harvested early than 15th Apr, the approximate verasion time is 60 days before harvest.

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correlations could be established between Rot_{w} and other climatic parameters including MTT, P_{w} and DD_{w} (Table 1).

Differences in Rot_{w} between seasons appeared to reflect the combined influence of weather parameters along PCL. Temp_{max}, Temp_{min}, DH_{max} and DD_{w} represent the temperature condition of vineyard microclimate, while P_{w} is also associated with grape surface temperature. Negative relationships were observed between wine rotundone and Temp_{max}, DH_{max}, DD_{w} and E_{w} (Table 1), thus a higher Temp_{max}, DH_{max}, DD_{w} and E_{w} are likely to result in lower Rot_{w} (Fig 3). This was consistent with a previous study that demonstrated the negative impacts of high surface and bunch zone temperature on grape rotundone concentration [12]. Furthermore, sunlight exposure was found to enhance grape monoterpene concentration in Traminer [17], while elevated UV-B radiation would increase monoterpens and a sesquiterpene, namely E-menthol in Malbec leaves [21]. On the contrary, complete exclusion of sunlight was found to inhibit the synthesis and accumulation of monoterpenes and a sesquiterpene, caryophyllene in Muscat [18]. At this stage, no information, as far as the authors’ knowledge, is available about the regulation of sesquiterpene synthesis in V. vinifera cv. Shiraz by sunlight exposure and ambient temperature. Therefore, further studies are required to separately investigate the influence of direct solar illumination and indirect solar radiation induced temperature increase on grape rotundone concentration.

Water balance was also associated with rotundone concentration as shown by the relationship between P_{w} and Rot_{w} (Table 1). Thus, a higher overall water balance would lead to increased rotundone concentration in wine (Fig 5), which was consistent with a previous study in V. vinifera cv. Duras [24]. This effect may have two main reasons: i) increased water availability can lead to higher vine vigour [16], resulting in increased bunch zone shading and lower bunch zone air temperature [19], which tends to promote rotundone production and accumulation in grape berries [12]; ii) higher vigour vines with increased mass of leaves and stems organs may result in increased rotundone from non-grape sources. Higher concentration of rotundone was reported in grape leaves and stems compared to berries [30]. The same study also reported that fermentation with these non-grape materials amongst harvested grapes could lead to elevated rotundone concentration in the resulted wine. In addition, a potential source-sink relationship could exist between leaves/stems and grape berries, as it has been reported that some monoterpene derivatives could be translocated via phloem transportation in other plants [32, 33]. In V. vinifera, an active transport mechanism may be necessary for translocation of terpene related compounds into grape berries via phloem [34], and this warrants further investigation.

The weather parameters MTT, DD_{w} and E_{w} are commonly used as indices to describe wine regional seasonal temperature and solar radiation conditions [24]. However, no significant correlations were observed between Rot_{w} and these parameters (Table 1). This may be due to the development time of rotundone, which mainly accumulates in berries from veraison to harvest in V. vinifera cv. Duras [24] and Shiraz as described in this paper. This may explain findings that showed rotundone production been sensitive to ambient temperature Temp_{max}, Temp_{min}, DD_{w} and DH_{max} and solar exposure (E_{w}) only from veraison to harvest (Table 1). Furthermore, DD_{w} and MTT were found on the opposite side from DD_{w} along PC2 (Fig 5), which indicated that a warmer overall season might not necessarily indicate a warm post-veraison ripening period, and vice versa. Therefore, traditional seasonal weather indicators used in the viticultural industry (MTT, DD_{w} and P_{w}) are not suitable for wine quality studies focused on certain quality traits as rotundone concentration. Further research is required to characterise the influences of environmental factors on the sesquiterpene/biosynthesis in Vitis vinifera tissues, including rotundone and its precursor α-guaiene.
Fig 6. Principal component analysis biplot of the mean PC scores of each weather parameters as vectors, together with the PC scores of each season. PC1 and PC2 account for 59.3% and 16% of total variance, respectively. The abbreviations used in the figure are: wind rotation in concentration (Rot), mean maximum temperature (Tmax), mean minimum temperature (Tmin), mean daily solar exposure (E), percentage of growing degree days (≥10°C) (DD10), mean January temperature (MIT), vineyard water balance (Pw), cumulative growing degree days from October to harvest (DDA) and cumulative growing degree days from vernalization to harvest (DDA).

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Wine rottenone projection based on DH25

For practical applications in the wine industry, it is necessary to use the last number of weather parameters to characterize Rot. From this study and analysing all the weather parameters and their relationships with Rot, the DH25 was the most significant vector in separating seasons (Fig 5), and it also had the most significant relationship with Rot (p < 0.0001) (Table 1). Using this criterion, the studied seasons were separated into four groups based on K-mean clustering analysis using DH25 in SPSS 23 (SPSS Inc., Chicago, IL, USA) (85 Table). The separated seasonal groups also discriminated according to Rot, Tmax, E, DD10, DDA and Pw (85 Table). Therefore, DH25 could be potentially used as a potential predictor of Rot. This could provide a convenient way for wineries and viticulturists to estimate Rot from the weather experienced in a specific growing season. The DH25 and Rot range of each k-mean clusters (85 Table) were plotted with areas demarked between groups by dash lines (Fig 6).
projection of wine rotundone concentration range ($\text{Rot}_w$) could be generated based on the $\text{DH}_{13}$.

$$\text{Rot}_w = f(\text{DH}_{13}),$$

where $\text{Rot}_w$ is the rotundone concentration range of grape berries at the $\text{DH}_{13}$ level of a specific growing season (Fig. 6). The dash lines connecting each seasonal group shows the $\text{Rot}_w$ range at a specific $\text{DH}_{13}$, with two exceptions. If $\text{DH}_{13}$ is lower than the range of group 3 (Fig. 6 region a) or higher than the range of group 4 (Fig. 6 region b), where no actual wine data is available, $\text{Rot}_w$ is likely be within the range indicated by the dash lines. The detailed $\text{Rot}_w$ projection at each $\text{DH}_{13}$ range is specified in Table 5. Since the averaged human detection threshold for rotundone is 18 ng/L in red wine [7], it is highly unlikely to have detectable rotundone in wine if $\text{DH}_{13}$ is higher than 6%, and most likely to have detectable rotundone in wine if $\text{DH}_{13}$ is lower than 3% (Fig. 6). Based on this results and the STM modelling technique proposed in this paper, an Australia wide potential peppery aroma production map could be generated to estimate wine regions capable of producing detectable concentrations of rotundone in Shiraz wine. This may also be related with future climate projections of Australian wine producing regions to estimate the potential peppy wine production regions in the future.

**Seasonal pattern differentiation**

By using SLDA biplots (Fig. 7), it is possible to recognize the seasonal patterns of weather parameters, which explained most of the variance (99.9%) with the first two discriminant functions. The SLDA analysis considered $\text{P}_{60}$, $\text{DD}_{0}$, and $\text{MJT}$ as the most significant variables in the
stepwise analysis. Seasons were separated into four groups mainly along the first discriminant function (97.8% of explained variance) by DJF, on the negative side, and by MJJ and DD, on the positive side of the discriminant function 1. Seasons before 2005 (Group 1, 3) were clearly separated from seasons after 2005 (Group 4) considering DJF, DD, and MJT as the major determinants (Fig. 7) with 2010–11 growing season as an exception, which corresponded to a wet season (Table 1). These results showed that the seasonal weather pattern from the studied vineyard is shifting from a wet and cool type to a drier and warmer type in the past two decades. This is consistent with previous studies showing that south-eastern Australian had and would continue having a gradually decrease in precipitations and an increase annual mean temperature [29, 40, 41]. However, no significant differences in vs, were observed between seasons before (Group 1, 3) and after 2005 (Group 4). The seasons before 2005 may have overall cooler and wet weather throughout the vintage (October–Harvest), but not necessarily for the period between vintage to harvest. The later period is more critical to grape rotundone production in V. vinifera cv. Durif and Shiraz [24]. Australian temperatures have warmed by 0.9°C from 1910 to 2014, and will continue to increase up to 5.1°C by 2090 [19]. The annual temperature of the Grampians wine growing region is estimated to increase for 2 to 2.4°C by 2090 [29, 30]. Despite of the projected annual temperature increase, seasonal temperature may continue fluctuating, resulting in relatively cooler seasons [40]. In addition, south-eastern Australia is affected by El Niño Southern Oscillation, which is associated with the periodical fluctuations in temperature and precipitation [42, 43]. Therefore, relatively wetter and cooler season
is expected with the occurrence of this phenomenon, and it is still possible to produce significant rotundone concentrations in those seasons.

**Rotundone accumulation model**

The rotundone accumulation model was established to describe the rotundone accumulation dynamics in grape berries within seasons, and to identify the importance of vine phenological stages in the final grape rotundone concentration. By understanding the behaviour of rotundone concentration according to seasonality, it would help grape growers to maximize their grape rotundone concentration using management strategies, such as irrigation scheduling, canopy management or by selecting the optimum harvest time. Rotundone in grape berries mainly accumulates at late stages of ripening and reaches a relatively stable concentration 41 days after mid-veraison in **V. vinifera** cv. Duras [24]. The Gompertz function (Rot = Gompertz (a, b, c, Tt)) was proposed here to mathematically describe the accumulation curve of rotundone in grape berries from veraison to harvest. This modeling tool was able to reflect the start time and accumulation rate at the maximum rotundone concentration (or plateau) (Fig 3). In cooler and wetter growing seasons, a higher rotundone concentration in grape is expected (Figs 5 and 6), and therefore a higher parameter ‘a’. Cooler and wetter growing seasons may have earlier accumulation starting point and faster rotundone accumulation rate, and therefore a lower parameter ‘b’ and higher parameter ‘c’ is expected (Fig 2).

The Gompertz function accurately described the accumulation trend in rotundone calculated against other calendar days (Fig 6) (2011–12: Rot = Gompertz (140.2, 822796, 0.1464, Tt), R² = 0.99, RMSE = 3.73; 2012–13: Rot = Gompertz (64.06, 99.3, 0.0518, Tt), R² = 0.95, RMSE = 10.3), or cumulative degree hours (thermal time) above 25°C (DH<sub>25°C</sub>) (Fig 8) (2011–12: Rot = Gompertz (162.8, 95280, 0.0144, Tt), R² = 0.99, RMSE = 3.73; 2012–13: Rot = Gompertz (154.09, 124400000, 0.0032, Tt), R² = 0.99, RMSE = 10.27). However, in the 2013–14 growing season, grape berries samples were harvested before rotundone concentration reached the plateau. As a result, for this season, the Gompertz function only described parameters ‘b’ and ‘c’, but could not predict the plateau parameter ‘a’. (Calendar day: Rot = Gompertz (could not be determined), Tc = 17.2, 0.0047, Tt), R² = 0.73, RMSE = 6.812; DH<sub>25°C</sub>; Rot = Gompertz (could not be determined), 20.52, 0.00007056, Tt), R² = 0.67, RMSE = 7.50). Therefore, at least 41 days from mid-veraison is required before rotundone concentration in berries can reach the plateau for **V. vinifera** cv. Duras variety in the Gallic region (France) (MIT 21.2°C) [24], and it may even take longer for the Shiraz cultivar in a cooler climate region (Geaipans, Australia; MIT 18.9°C). In the 2011–12 and 2012–13 growing seasons, the time from 80% veraison to harvest were both longer than 51 days (Fig 8b). Veraison in the 2013–14 growing season occurred around 10 days later than 2011–12 and 2012–13 growing seasons, but was harvested earlier than the other two seasons (Fig 8a). Therefore, a shorter ripening time has resulted in lower rotundone concentration in the 2013–14 growing season. The 2012–13 growing season had clearly higher DH<sub>25°C</sub> compared to the 2011–12 growing season, which explained it lower maximum rotundone concentration or plateau (Fig 8b). Despite that the 2013–14 growing season had similar DH<sub>25°C</sub> to the 2011–12 at harvest, the former had much lower rotundone concentration in grape berries. This may be explained by the later veraison time achieved in the 2013–14 growing seasons (Fig 8b). Since the 2013–14 growing season reached a higher concentration of rotundone at a relatively low DH<sub>25°C</sub> compared to the 2012–13 growing season, it might have reached an even higher maximum rotundone concentration plateau if later harvest date was allowed (Fig 8b). Therefore, vine phenological stage and harvest time selection, especially before rotundone reaches the critical plateau, will impact upon the final concentration of rotundone found in grapes and thus wine.
Conclusions

In this paper, accurate historical weather data was generated from high resolution climate maps and modelled vineyard fruit zone air temperature using the PCHIP modelling approach. Extrapolated data provided a reliable estimation of vineyard temperature in any historical season. The basis of the PCHIP interpolation technique allowed its application to any vineyard located in Australia with similar limited weather information. Fruit zone air temperature, daily solar exposure and vineyard water balance were correlated with the concentration of rotundone in Shiraz wine produced. While the studied vineyard experienced a change in weather conditions after 2005, with an increase in seasonal cumulative GDD and MJT, the rotundone concentration in wine was not affected. MJT, daily solar exposure and GDD over an entire
growing season does not relate well to weather indices between veraison to harvest. Temperature from veraison to harvest, in particular the bunch zone air temperature, appeared to represent better the final wine rotundone concentration. Identification of the major environmental factors affecting rotundone concentrations in grape and wine could allow the implementation of precision irrigation techniques and management strategies to manipulate rotundone concentration in grapes at harvest. This study further developed projection models to characterise the possible rotundone concentration range in finished wine, and berry rotundone accumulation trend during the grape ripening process. The use of these two models could allow wine-makers to estimate rotundone concentrations in final Shiraz wines based on seasonal climatic conditions, and to adjust winemaking techniques to achieve high quality 'peppery' Shiraz wine. This research also provides a guideline to help viticulturists adjust vineyard management practices and identify potentially 'peppery' grape growing regions.

Supporting Information
S1 Table. Weather record of the studied wine region in selected growing seasons (Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085).

S2 Table. Summary of weather data for the vineyard in selected growing seasons (Data from Australian Water Availability Project, Australian Bureau of Meteorology) and rotundone concentration in wine (Rot$_{w}$).

S3 Table. Summary of thermal data from simulated temperature model from each studied season.7

S4 Table. Validation of the simulated temperature model. Regression analysis between predicted temperature data and observed temperature data in growing seasons 2012-13 and 2013-14.

S5 Table. Comparison of the groups separated by k-mean clustering using DH$_{25}$ in wine rotundone concentration and climate parameters.

S6 Table. Specification of estimated wine rotundone concentration (Rot$_{w}$) range at different percentage of degree hours above 25°C (DH$_{25}$) from veraison to harvest (Rot$_{w} = f$ (DH$_{25}$)).

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Author Contributions
Conceived and designed the experiments: PZ KH MK MH EWRB SF. Performed the experiments: PZ SF. Analyzed the data: PZ SF. Contributed reagents/materials/analysis tools: PZ KH MK MH EWRB SF. Wrote the paper: PZ KH MK MH EWRB SF.

References
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PMID: 18491180
PMID: 19424446


2.3 Addendum

Note: Page 25 Session preparation of samples and SPME-GC-MS analysis of rotundone, line 9, ‘filtrated’ should be corrected as ‘filtered’.

Note: Page 30 Fig 3. Validation of simulated temperature model

The relationship between estimated temperature and observed temperature could be well explained by the regression equation. However, differences still exist between estimated and observed values, which could be large around 20 to 30 °C. This could be explained by the discussion based on Fig 4., where cloud could influence ground ambient temperature.
2.4 Supplementary data

Supporting information to the publication in Chapter 2 is attached below:

Table S2.4.1. Weather record of the studied wine region in selected growing seasons (Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085)

<table>
<thead>
<tr>
<th>Growing Seasons</th>
<th>Mean January Temperature °C (MJT)</th>
<th>Mean January Maximum Temperature °C</th>
<th>Mean January Minimum Temperature °C</th>
<th>Mean Daily Solar exposure MJm(^2) ((E_s)) (^a)</th>
<th>Cumulative Growing Degree days (DD(_s)) (^a)</th>
<th>Total Rainfall millimetres (^a)</th>
<th>Total Irrigation millimetres (^a)</th>
<th>Total Evapotranspiration millimetres (ET(_c)) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995-1996</td>
<td>18.4</td>
<td>25.9</td>
<td>10.8</td>
<td>19.6</td>
<td>1059.9</td>
<td>246.2</td>
<td>93.3</td>
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</tr>
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<td>1998-1999</td>
<td>21.5</td>
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<td>13.0</td>
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<td>1188.3</td>
<td>263.8</td>
<td>112.0</td>
<td>362.6</td>
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<td>25.9</td>
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<td>1277.8</td>
<td>242.4</td>
<td>78.9</td>
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<td>248.2</td>
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\(^a\)Climate data is for the period from October to harvest of each growing season.
Table S2.4.2. Summary of weather data for the vineyard in selected growing seasons (Data from Australian Water Availability Project, Australian Bureau of Meteorology) and rotundone concentration in wine (Rotw)

<table>
<thead>
<tr>
<th>Growing Seasons</th>
<th>Harvest date</th>
<th>Mean Maximum Temperature °C (T&lt;sub&gt;max&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Minimum Temperature °C (T&lt;sub&gt;min&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Daily Solar exposure MJm&lt;sup&gt;-2&lt;/sup&gt; (E&lt;sub&gt;vh&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total Rainfall millimetres&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Water Balance millimetres (P&lt;sub&gt;wb&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rotundone concentration in wine (ng/L) (Rotw)</th>
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</thead>
<tbody>
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</tbody>
</table>

<sup>a</sup>The weather data is for the period from veraison to harvest of each season. Veraison is approximately 15<sup>th</sup> February for most seasons. For seasons harvested early than 15<sup>th</sup> Apr, the approximate veraison time is 60 days before harvest.

<sup>b</sup>Total rainfall and water balance data is for the period from Oct to harvest.

<sup>c</sup>na, not applicable.
<table>
<thead>
<tr>
<th>Growing Seasons</th>
<th>Total degree hours</th>
<th>Cumulative growing degree days (DD&lt;sub&gt;m&lt;/sub&gt;)</th>
<th>% of Degree hours above 35°C (DH&lt;sub&gt;35&lt;/sub&gt;)</th>
<th>% of Degree hours above 30°C (DH&lt;sub&gt;30&lt;/sub&gt;)</th>
<th>% of Degree hours above 25°C (DH&lt;sub&gt;25&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995-1996</td>
<td>20732</td>
<td>327.4</td>
<td>0.00</td>
<td>0.75</td>
<td>3.60</td>
</tr>
<tr>
<td>1998-1999</td>
<td>18620</td>
<td>300.1</td>
<td>0.00</td>
<td>0.33</td>
<td>1.72</td>
</tr>
<tr>
<td>1999-2000</td>
<td>17993</td>
<td>359.4</td>
<td>0.49</td>
<td>1.46</td>
<td>4.82</td>
</tr>
<tr>
<td>2001-2002</td>
<td>22995</td>
<td>390.6</td>
<td>0.00</td>
<td>0.91</td>
<td>3.44</td>
</tr>
<tr>
<td>2003-2004</td>
<td>20500</td>
<td>342.6</td>
<td>0.45</td>
<td>2.59</td>
<td>4.11</td>
</tr>
<tr>
<td>2004-2005</td>
<td>20223</td>
<td>352.0</td>
<td>0.10</td>
<td>1.87</td>
<td>4.17</td>
</tr>
<tr>
<td>2005-2006</td>
<td>17668</td>
<td>324.0</td>
<td>0.02</td>
<td>2.10</td>
<td>5.11</td>
</tr>
<tr>
<td>2006-2007</td>
<td>18715</td>
<td>388.5</td>
<td>0.68</td>
<td>2.46</td>
<td>6.22</td>
</tr>
<tr>
<td>2007-2008</td>
<td>19180</td>
<td>408.7</td>
<td>0.44</td>
<td>3.47</td>
<td>7.63</td>
</tr>
<tr>
<td>2008-2009</td>
<td>18168</td>
<td>327.9</td>
<td>0.01</td>
<td>1.11</td>
<td>4.47</td>
</tr>
<tr>
<td>2009-2010</td>
<td>17317</td>
<td>329.7</td>
<td>0.00</td>
<td>0.88</td>
<td>3.16</td>
</tr>
<tr>
<td>2010-2011</td>
<td>19814</td>
<td>313.3</td>
<td>0.00</td>
<td>0.00</td>
<td>1.41</td>
</tr>
<tr>
<td>2011-2012</td>
<td>17691</td>
<td>327.0</td>
<td>0.04</td>
<td>1.33</td>
<td>3.65</td>
</tr>
<tr>
<td>2012-2013</td>
<td>18983</td>
<td>400.5</td>
<td>0.18</td>
<td>2.40</td>
<td>7.32</td>
</tr>
<tr>
<td>2013-2014</td>
<td>17958</td>
<td>357.8</td>
<td>0.31</td>
<td>2.39</td>
<td>5.75</td>
</tr>
<tr>
<td>Mean</td>
<td>19201.23</td>
<td>350.0</td>
<td>0.18</td>
<td>1.60</td>
<td>4.44</td>
</tr>
</tbody>
</table>

*All weather data is for the period from veraison to harvest of each season. Veraison is approximately 15th February for most seasons. For seasons harvested early than 15th Apr, the approximate veraison time is 60 days before harvest.*
Table S2.4.4. Validation of the simulated temperature model. Regression analysis between predicted temperature data and observed temperature data in growing seasons 2012-13 and 2013-14.

<table>
<thead>
<tr>
<th>Growing Seasons</th>
<th>Temperature Loggers</th>
<th>Equation</th>
<th>R-square</th>
<th>SSE&lt;sup&gt;c&lt;/sup&gt;</th>
<th>RMSE&lt;sup&gt;d&lt;/sup&gt;</th>
<th>% of Outliers</th>
<th>P value</th>
<th>Data Type</th>
<th>Total degree hours</th>
<th>% of Degree hours above 35°C (DH&lt;sub&gt;35&lt;/sub&gt;)</th>
<th>% of Degree hours above 30°C (DH&lt;sub&gt;30&lt;/sub&gt;)</th>
<th>% of Degree hours above 25°C (DH&lt;sub&gt;25&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>L2013A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>y=0.7531x+4.631</td>
<td>0.7789</td>
<td>29730</td>
<td>3.362</td>
<td>0.38</td>
<td>&lt;0.0001</td>
<td>Ob. Val.</td>
<td>17949</td>
<td>5.04</td>
<td>6.94</td>
<td>12.88</td>
</tr>
<tr>
<td></td>
<td>L2013B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>y=0.8423x+3.383</td>
<td>0.8403</td>
<td>21440</td>
<td>2.855</td>
<td>0.34</td>
<td>&lt;0.0001</td>
<td>Ob. Val.</td>
<td>17328</td>
<td>1.39</td>
<td>4.93</td>
<td>9.90</td>
</tr>
<tr>
<td>2014</td>
<td>L2014A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>y=0.7814+4.485</td>
<td>0.8528</td>
<td>17970</td>
<td>2.52</td>
<td>0.25</td>
<td>&lt;0.0001</td>
<td>Ob. Val.</td>
<td>17494</td>
<td>3.13</td>
<td>5.06</td>
<td>9.00</td>
</tr>
<tr>
<td></td>
<td>L2014B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>y=0.8139+4.228</td>
<td>0.8576</td>
<td>17360</td>
<td>2.478</td>
<td>0.28</td>
<td>&lt;0.0001</td>
<td>Ob. Val.</td>
<td>17066</td>
<td>0.38</td>
<td>3.58</td>
<td>8.73</td>
</tr>
</tbody>
</table>

*Temperature logger L2013A and L2013B measure vineyard temperature in 2013, with L2013A placed inside vine canopy and L2013B placed inside canopy of vine covered with UV-stabilized HDPE shade cloth;


<sup>a</sup>error sum of square.

<sup>b</sup>root mean square deviation.

<sup>e</sup>observed value.
Table S2.4.5. Comparison of the groups separated by k-mean clustering using DH$_{25}$ in wine rotundone concentration and climate parameters.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean January temperature °C (MJT)</td>
<td>20.9±0.8</td>
<td>18.9±1.6</td>
<td>20.6±1.3</td>
<td>20.3±0.4</td>
</tr>
<tr>
<td>Cumulative growing degree days (DD$_{a}$)</td>
<td>1216.9±40.4</td>
<td>1243.5±137.6</td>
<td>1283.8±36.8</td>
<td>1347.6±63.0</td>
</tr>
<tr>
<td>Mean maximum temperature °C (T$_{max}$)</td>
<td>21.3±0.5</td>
<td>23.2±0.9</td>
<td>25.7±1.1</td>
<td>27.3±0.3</td>
</tr>
<tr>
<td>Mean minimum temperature °C (T$_{min}$)</td>
<td>9.0±1.7</td>
<td>10.0±1.2</td>
<td>13.0±3.0</td>
<td>15.6±3.9</td>
</tr>
<tr>
<td>Water Balance ($P_{wb}$)</td>
<td>140.0±158.1</td>
<td>66.2±91.9</td>
<td>139.9±84.8</td>
<td>179.1±38.8</td>
</tr>
<tr>
<td>Mean daily solar exposure MJm$^{-2}$ (E$_{vh}$)</td>
<td>15.6±2.0</td>
<td>16.6±1.2</td>
<td>18.7±1.5</td>
<td>20.3±3.2</td>
</tr>
<tr>
<td>Rotundone concentration in wine (ng/L) ($Rot_{w}$)</td>
<td>84.2±44.8</td>
<td>38.5±23.6</td>
<td>7.8±4.5</td>
<td>3.9±1.9</td>
</tr>
<tr>
<td>Cumulative growing degree days (DD$_{vh}$)</td>
<td>306.7±9.3</td>
<td>342.5±23.3</td>
<td>357.4±26.4</td>
<td>404.6±5.8</td>
</tr>
<tr>
<td>% of Degree days above 25°C (DH$_{25}$)</td>
<td>1.6±0.2</td>
<td>3.8±0.5</td>
<td>5.5±0.6</td>
<td>7.5±0.2</td>
</tr>
</tbody>
</table>

*The climate data is for the period from October to harvest.

*The climate data is for the period from veraison to harvest of each season. Verasion is approximately 15th February for most seasons. For seasons harvested early than 15th Apr, the approximate veraison time is 60 days before harvest.
Table S2.4.6. Specification of estimated wine rotundone concentration (Rot<sub>e</sub>) range at different percentage of degree hours above 25°C (DH<sub>25i</sub>) from veraison to harvest (Rot<sub>e</sub> = f (DH<sub>25i</sub>)).

<table>
<thead>
<tr>
<th>DH&lt;sub&gt;25i&lt;/sub&gt; range</th>
<th>Rot&lt;sub&gt;e&lt;/sub&gt; range</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 1.3%</td>
<td>(-12.16 x DH&lt;sub&gt;25i&lt;/sub&gt; + 55.64) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-26.58 x DH&lt;sub&gt;25i&lt;/sub&gt; + 175.87) ng/L</td>
</tr>
<tr>
<td>(b) 1.3% ≤ DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 3.3%</td>
<td>(-12.16 x DH&lt;sub&gt;25i&lt;/sub&gt; + 55.64) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-26.58 x DH&lt;sub&gt;25i&lt;/sub&gt; + 175.87) ng/L</td>
</tr>
<tr>
<td>(c) 3.3% ≤ DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 4.3%</td>
<td>(-7.77 x DH&lt;sub&gt;25i&lt;/sub&gt; + 40.96) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-26.58 x DH&lt;sub&gt;25i&lt;/sub&gt; + 175.87) ng/L</td>
</tr>
<tr>
<td>(d) 4.3% ≤ DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 4.8%</td>
<td>(-7.77 x DH&lt;sub&gt;25i&lt;/sub&gt; + 40.96) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-27.15 x DH&lt;sub&gt;25i&lt;/sub&gt; + 178.31) ng/L</td>
</tr>
<tr>
<td>(e) 4.8% ≤ DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 6.1%</td>
<td>(-0.59 x DH&lt;sub&gt;25i&lt;/sub&gt; + 6.23) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-27.15 x DH&lt;sub&gt;25i&lt;/sub&gt; + 178.31) ng/L</td>
</tr>
<tr>
<td>(f) 6.1% ≤ DH&lt;sub&gt;25i&lt;/sub&gt; &lt; 7.7%</td>
<td>(-0.59 x DH&lt;sub&gt;25i&lt;/sub&gt; + 6.23) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-4.19 x DH&lt;sub&gt;25i&lt;/sub&gt; + 37.91) ng/L</td>
</tr>
<tr>
<td>(g) DH&lt;sub&gt;25i&lt;/sub&gt; ≥ 7.7%</td>
<td>(-0.59 x DH&lt;sub&gt;25i&lt;/sub&gt; + 6.23) ng/L ≤ Rot&lt;sub&gt;e&lt;/sub&gt; ≤ (-4.19 x DH&lt;sub&gt;25i&lt;/sub&gt; + 37.91) ng/L</td>
</tr>
</tbody>
</table>
CHAPTER THREE
Within-vineyard, Within-vine and Within-bunch Variability of Rotundone Concentration in Berries of Vitis vinifera L. cv. Shiraz

3.1 Introduction
This chapter is the first experiment of the Stage Two Field Trials, and investigates the impacts of temperature on rotundone concentration in grape berries by studying the variability of rotundone within-vineyard, within-vine and within-bunch. The aim of this chapter was to investigate how temperature affects the concentration of rotundone in grape berries. The hypothesis of this chapter was:
1. Vineyard zones with high vigor and southern aspect, shaded side of individual vine and shaded sector of individual bunch has less direct sunlight exposure, and therefore less berry surface temperature and bunch zone air temperature.
2. Lower berry surface and bunch zone air temperatures are associated with higher concentration of rotundone in grape berries.
3. Bunch zone temperature higher than a certain level has negative impacts on the rotundone concentration of grape berries at harvest.
3.2 Published Paper

Within-Vineyard, Within-Vine, and Within-Bunch Variability of the Rotundone Concentration in Berries of *Vitis vinifera* L. cv. Shiraz

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‡Australian Wine Research Institute, Port Melbourne, Victoria 3207, Australia
§Australian Wine Research Institute, Urrbrae, South Australia 5064, Australia

Supporting Information

**ABSTRACT:** This study characterizes the environmental factors driving rotundone concentrations in grape berries by quantifying rotundone variability and correlating it with viticultural parameters. Dissection of the vineyard into distinct zones (on the basis of vigor, electrical soil conductivity, and slope), vine into orientations to sun (shaded/unshaded), and grape bunches into sectors (upper and lower and front and back) shows the influence of vine vigor, sunlight, and temperature. Occurrence of the highest rotundone concentration was observed in shaded bunch sectors and vines and from higher vigor vines in the southern-facing areas of the vineyard. The highest concentration of rotundone is consistently found at the top and in shaded sectors of bunches, and this correlates to lower grape surface temperatures. Modeling showed that berry temperature exceeding 25 °C negatively affects the rotundone concentration in Shiraz. Both natural and artificial shading modulated the grape surface and air temperature at the bunch zone and increased the rotundone concentration, without affecting other grape berry quality parameters. Thus, temperature and possibly sunlight interception are the main determinants of rotundone in grape berries. Vineyard topography, vine vigor, vine row, and grape bunch orientation influence the level of berry shading and can, therefore, adjust bunch surface and zone temperatures and influence the berry rotundone concentration.

**KEYWORDS:** rotundone, *Vitis vinifera* (cv. Shiraz), degree hour, vigor, artificial shading

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**INTRODUCTION**

Terpenoids and their derivatives in grapes and wine are important for wine flavor and aroma. The majority of these compounds are monoterpenes, but a member of the sesquiterpene subgroup has been reported as an important aroma or flavor compound of grapes and wine. This compound, named rotundone, is an oxygenated bicyclic sesquiterpene and gives a "peppery" character to grapes and wine. A survey showed that 19% of Australian red wines of different varieties and vintages contained detectable levels of rotundone and rotundone is predominately found in Shiraz wines. This "peppery" characteristic is considered desirable by red wine consumers and is stylistically important in some high-quality Australian Shiraz wine. Unlike many other wine aroma compounds, which are either formed or released during fermentation, rotundone originates from the grapes and is extracted from grapes during the winemaking process. Vine material analysis shows the presence of rotundone in grape berries, grape leaves, and grape stems. Recent research reported that sesquiterpene biosynthesis is primarily located in the grape berry epidermis (skin), with no detectable activity in the berry mesocarp (flesh). This latter study is consistent with previous findings that showed that rotundone only exists in grape berry skin but not in the pulp or seeds. The biochemical pathway of rotundone in Shiraz grapes has not yet been elucidated. A recent publication demonstrates that the formation of rotundone can occur through aerobic oxidation of the precursor α-guaiene. The factors affecting the presence and concentration of rotundone in grapes are elusive. Because the most "peppery" Shiraz wines appear to originate from "cooler" geographic production areas, growth of vines in cooler climates has been suggested as a contributing factor to increased rotundone concentration in grape berries. In addition, significant temporal differences in berry and wine rotundone concentrations have been observed. Most recently, it was demonstrated that rotundone varies considerably between grapes within a vineyard site, and this may be due to differences in vineyard soil properties and topography. Topography and geographic aspects of the vineyard lead to variation in the temperature and solar radiation and are proposed to directly impact the rotundone concentration in Shiraz grape berries.

This paper investigates the role of the temperature, solar exposure, and vine vigor on the berry rotundone concentration by studying the variation within individual vineyard, within individual vine, and within individual grape bunch. This study demonstrates that vineyard topography, vine vigor, vine orientation, and bunch orientation together influence the microclimate in the grape bunch zone and, therefore, influence grape quality parameters, including rotundone.

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Chemicals. Rotundone (135,8R,8S)-3,4,6,7,8-hexahydro-3,8-dimethyl-5-(prop-1-en-2-yl)-1(2H)-azulenone) and 1Hrotundone was synthesized as described previously.5,16 Working solutions of standards were prepared volumetrically in ethanol and stored at 4 °C until required. High-performance liquid chromatography (HPLC)-grade gradient-grade ethyl acetate, n-pentane, methanol, and ethanol were obtained from Rowe Scientific (Doveton, Victoria, Australia). Analytical-reagent-grade potassium i-tartrate monobasic, tartaric acid, and other chemicals were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Water was purified by the Milli-Q (Millipore, Australia). Vineyard Site. The study was conducted in a commercial vineyard (The Old Block, Mount Langi Ghiran, 37.31° S, 143.15° E) located in the Grampians wine region of Victoria, Australia. The vineyard is approximately 15.5 km east of the nearest Bureau of Meteorology (BOM) weather station at Ararat Prison (Australian BOM Station 08008S), where the long-term mean January temperature is 18.9 °C and annual mean January rainfall is 587.9 mm, and is classified as a cool-climate production region.17 The vineyard was planted in 1968 with Vitis vinifera cv. Shiraz on its own roots at 3.0 m between rows and 1.8 m between vines, with rows oriented northeast to southwest. Grapevines are trained to a vertical shooting position trellis (VSP). Grapevines are irrigated using drip irrigation when required at a rate of 5.76 l h⁻¹ vine⁻¹. Weather conditions and irrigation volume for both studied seasons are included in the Supporting Information. No significant pest or disease pressures were observed during the experimental season.

Experimental Design and Sampling. The experimental block has been previously divided into three vineyard zones based on plant cell density (PCD), electrical soil conductivity, and slope using the k-means procedure. The PCD is the mean infrared to red ratio and has been used as a surrogate measure of vine vigor.11 The values are provided for zone 1 (104, 63.5 mS/m, and 8.3%), zone 2 (174, 45.6 mS/m, and 5.9%), and zone 3 (134, 53.1 mS/m, and 3.4%) for mean PCD, electrical soil conductivity, and slope, respectively (Figure 1A). The three identified zones have different aspects, with zone 1 having a northern aspect, most of zone 2 having a southern aspect, and zone 3 being located along the ridge, having all four aspects (Figure 1B). In the southern hemisphere, a northerly aspect has the highest amount of sunlight exposure. Separating the vineyard into these zones is important to assess the involvement of vine vigor, vineyard soil conductivity, and topography on rotundone accumulation in Shiraz grapes.

Within-Vineyard Variability of Rotundone. Five random field replicates were selected and georeferenced from each of the three vineyard zones (described previously in the study by Scarlett et al.11) as the sampling points for bunches (Figure 1A). Each replicate consisted of six vines each distributed in two adjacent rows (three vines in each row). Bunches were sampled from the northwest side of canopies at the time near commercial harvest in two continuous seasons (2011–2012 and 2012–2013) on April 5, 2012 and April 10, 2013, respectively. Commercial harvest was performed between April 11 and 18 and between April 9 and 16, 2013 with selective hand-harvest first, followed by machine harvest at the later dates. Three bunches of grapes were sampled from each selected grapevine in zip-lock plastic bags, frozen at −20 °C, transferred to the University of Melbourne laboratories in styrofoam boxes on dry ice, and stored at −20 °C before conducting bunch sectionation and laboratory analysis detailed in the within-bunch variability study below. The rotundone concentration in grapes has previously been reported to be stable under normal ambient temperature conditions.6

Within-Vine Variability of Rotundone. To assess within-vine variability, shading treatments were applied on three adjacent rows in zone 1 (Figure 1A). This design was chosen to minimize variation between vines because large within-vine variation in the berry rotundone concentration was previously reported for this experimental block by Scarlett et al.18 Within these three rows, four replicates (blocks of six vines each) were used as six vines as buffers and were randomly distributed for each of the following artificial shading treatments for the 2012–2013 growing season: (i) naturally occurring shade (control (Ctrl)), (ii) fruit zone shaded vines from veraison to harvest (FS), and (iii) whole vine shaded from veraison to harvest (WS). Artificial shading was achieved using commercial shade cloth, which is ultraviolet (UV)-stabilized high-density polyethylene (HDPE) shade cloth blocking 56.94% of light and 59.83% of UV (Cooldar, Gale Pacific, Ltd., Australia). Within individual vines, grape bunches on two sides of the vineyard row were considered as separated groups because of differing degrees of incident solar exposure. For the FS treatment, the shade cloth was cut into 0.6 m widths and placed on both sides of the fruit zone. For the WS treatment, the whole vine was shaded with the shading cloth (see panels A and B of Figure S1 of the Supporting Information). Sampling was performed at commercial harvest (April 10, 2013). Grape bunches from both sides of the canopy wall were sampled separately. Samples from the northwest-facing side of Ctrl, FS, and WS were labeled as Ctrl-NW, FS-NW, and WS-NW, while samples from the southeast-facing side were labeled as Ctrl-SE, FS-SE, and WS-SE. Three bunches of grapes were sampled from each selected grapevine in zip-lock plastic bags, frozen at −20 °C, transferred to the laboratory in styrofoam boxes on dry ice, and stored at −20 °C before analysis. Total soluble solids (TSS), titratable acidity (TA), and pH of the grape samples were analyzed using a refractometer, an alkaline titration, and a pH meter, following published protocols.8 To monitor the fruit zone temperature on both sides of the canopy walls, temperature loggers (Tinytag transit 2, Gemini Data Logger, Ltd, Chichester, UK) were placed next to grape bunches on both sides of the canopy for each treatment group, and six temperature loggers were used in total. The loggers were shielded by bunch zone leaves to minimize direct solar exposure. These loggers have an operational range from −40 to 70 °C, with a sensitivity of 0.01 °C.11
The temperature was measured every 30 min from veraison (early February) to harvest, and the data recorded were analyzed with Tintyag Explorer software (version 4.7, Gemini Data Logger, Ltd., Chichester, U.K.), following the method described by Winter et al.15 Fruit zone temperature overtime was calculated as degree hours and reflected the heat accumulated in the fruit zone. Degree hours in different temperature ranges and their percentage to the total degree hours were calculated and associated with the rotundone concentration of grape berries.

**Within-Bunch Variability of Rotundone.** Grape bunches from the within-vineyard variability study were also used for the within-bunch variability study. All berries from each representative bunch collected were divided into four sectors: top front (TF), top back (TB), bottom front (BF), and bottom back (BB), dependent upon their position in the bunch and bunch orientation in relation to incident solar radiation (Figure 2A). The X and Y axes in Figure 2A represented the direction of sunlight and vineyard row, respectively.

![Illustration of grape bunch separation and surface temperature measurement](image)

**Figure 2.** Illustration of grape bunch separation and surface temperature measurement of the within-bunch study: (A) separation of the grape bunch based on the direction of sunlight and vineyard row, with the grape bunch surface temperature being obtained by taking thermal images on both sides of the grape bunch, and (B) separation of the grape bunch into top and bottom parts based on the ratio of the distance between end points of the bunch to separating point (mm/m = 4:6).

Infrared thermal images were obtained to assess the variability of berry surface temperatures and distribution within grape bunches from 24 randomly selected vines in the vineyard. The camera had a 0.3 megapixel (640 x 480) microbolometer sensor, sensitive in the temperature range from -40 to 2000 °C, with the thermal sensitivity of 0.04 °C, and a lens with an angular field of view of 25° (TG60 mod, FLIR System, Inc., Notting Hill, Australia). Thermal images were obtained between 15:30 and 16:00 h (Australian Eastern Daylight Time) on March 26, 2013 from both sides of the row with a distance of approximately 1 m between sampled bunches and the camera (Figure 3A). Thermal images were processed using the FLIR ResearchIR software (FLIR System, Inc., Notting Hill, Australia). Maximum, minimum, and average surface temperatures of the top and bottom parts of the bunch were analyzed separately, selecting regions of interest manually using the FLIR software.

**Preparation of Samples and Solid-Phase Microextraction—Gas Chromatography—Mass Spectrometry (SPME—GC—MS) Analysis of Rotundone.** Grape samples were prepared for rotundone analysis based on the protocol described by Siebert et al. Briefly, 100 g of destemmed grapes were sub-sampled before being homogenized using a hand-held blender. Sub-samples were centrifuged to separate the juice and solid parts. The solid parts were mixed with 30 ml of ethanol, 30 ml of water, and 100 μl of d₄-rotundone (516 ng/ml in ethanol) as the internal standard, then shaken for 24 h at 22 °C, and sonicated before adding the juice back. Sub-samples were then centrifuged (10,000 rpm for 20 min at 22 °C) and filtrated (1.6 μm glass fibre) to obtain berry extract filtrate, which was topped up to 200 ml with Milli-Q, deionized water before subjected to solid-phase extraction (SPE), performed as reported previously.7 The SPE residue supernatant collected was dried with nitrogen and reconstituted in 0.5 ml of ethanol and 9 ml of Milli-Q, deionized water. The sample was then analyzed and quantified at the Australian Wine Research Institute using a published SPME—GC—MS protocol.11

**Statistical Analysis.** The rotundone concentration, TSS, pH, and TA of the within-vine study samples were analyzed using one-way analysis of variance (ANOVA) at p < 0.05 (CoStat, version 6.4, CoHort Software, Monterey, CA). The relationship between the percentage of degree hours under the canopy and the grape rotundone concentration was calculated using MatLab software 2014a (The MathWorks, Inc., Natick, MA). The berry surface temperature, rotundone concentration, TSS, pH, TA, total anthocyanins, and total phenolics of the within-bunch study samples were analyzed using one-way and two-way ANOVA at p < 0.05 (CoStat, version 6.4, CoHort Software, Monterey, CA). Data were graphed into a spider plot using MatLab software 2014a (The MathWorks, Inc., Natick, MA).

### RESULTS AND DISCUSSION

In this study, we examined the berry rotundone concentration and other grape quality parameters as a function of the berry exposure and berry surface and bunch zone temperature over two growing seasons. Distinct within-vineyard, within-vine, and within-bunch variabilities have been observed in rotundone and other key grape quality attributes in this study. Dissection of the vineyard into distinctly different zones (as per Scarlett et al.11), vines into sides based on sunlight orientation, and grape bunches into sectors based on exposure to solar radiation has allowed for the influence of vine vigor, sunlight, and temperature to be considered for berry compositional parameters, including rotundone. Vineyard topography, vine vigor, vine orientation, and bunch orientation together influence grape berry surface/bunch zone temperature and, therefore, can influence grape quality parameters.

**Within-Vineyard Variability of Rotundone.** The differences in grape quality trait parameters and rotundone concentration between experimental vineyard zones in the 2011–2012 and 2012–2013 growing seasons were examined by two-way ANOVA (p < 0.01) (panels A and B of Figure 3). Overall, Shiraz grapes from zone 1 had a significantly lower concentration of rotundone (118.2 ng/kg) compared to zone 2 (196.9 ng/kg) and zone 3 (233.9 ng/kg), while the 2011–2012 growing season had a significantly higher concentration of rotundone overall (346.8 ng/kg) compared to the 2012–2013 growing season (192.2 ng/kg). In the 2011–2012 growing season, the rotundone concentration of Shiraz grapes from zone 2 (373.8 ng/kg) and zone 3 (447.3 ng/kg) was significantly higher compared to zone 1 (219.4 ng/kg) [Figure 3C]. However, no statistical significant differences in rotundone were observed among vineyard zones in the 2012–2013 growing season (Figure 3D).
Figure 3. Comparison of the rotundone concentration and quality parameters among vineyard zones in the 2011–2012 and 2012–2013 growing seasons. Two-way ANOVA was conducted considering (A) vineyard zones and (B) growing seasons as two ANOVA independent variables for each quality parameter, while one-way ANOVA was conducted to compare each quality parameter among three zones in individual growing season (C) 2011–2012 and (D) 2012–2013, where ns, *, **, *** and **** stand for no significant difference, no significant difference but 0.5 ≤ p < 0.1, p < 0.05, and p < 0.001, respectively. a, b, and c were used to label the differences between experimental groups.

The differences in the grape rotundone concentration between three vineyard zones were associated with variations in vine vigor and, therefore, natural shading. Although vine vigor of the experimental block was measured in 2012, it has been proven to be relatively stable across different growing seasons. The high vigor areas (zones 2 and 3) (Figure 1A, with a PCD index of zone 2 of 174 and zone 3 of 134) had more canopy coverage, mainly a southern, eastern, and western aspect (Figure 1B), and therefore, more natural bunch shading, receiving less direct solar radiation and, as a consequence, a relatively lower bunch zone/surface temperature compared to zone 1 (PCD index of zone 1 of 104), which were associated with the higher grape rotundone concentrations observed in zones 2 and 3, particularly in the 2011–2012 growing season (panels A and C of Figure 3). In the 2012–2013 growing season, where the temperature and solar exposure from veraison to harvest was high, the bunch zone temperature and surface temperatures under shading could still be too high for rotundone accumulation. As a result, no significant differences in the grape rotundone concentration were observed between grape samples from the three vineyard zones (zone 1, 17.0 ng/kg; zone 2, 20.5 ng/kg; and zone 3, 20.0 ng/kg) in the 2012–2013 growing season.

Large differences in the rotundone concentration were observed between the two studied growing seasons (2011–2012 and 2012–2013) (Figure 3B). The variation in the rotundone concentration between the two studied seasons (18-fold) is higher than the differences among vineyard zones (2-fold), the differences between shading treatments (1.7-fold), and the differences among bunch sectors (2-fold). This may be due to the large differences in weather patterns from veraison to harvest between the two studied seasons because rotundone mainly accumulates post-veraison. The 2012–2013 growing season has a higher mean ambient temperature (18.9 °C) and mean solar exposure (18.1 MJ/m²) from veraison to harvest but a lower total water precipitation (rainfall + irrigation) (208.4 mm) compared to the 2011–2012 growing season (16.7 °C, 16.1 MJ/m², and 259.8 mm). Higher temperatures in the 2012–2013 growing season led to higher evaporation and, therefore, more water loss. As a result, higher water deficiency in the 2012–2013 growing season may have decreased vine vigor and natural shading and, therefore, increased the berry surface temperature to influence the grape rotundone concentration. Importantly, the zones and vines that showed high rotundone in the 2011–2012 growing season also show high rotundone in the lower rotundone overall 2012–2013 growing season. Thus, the factors contributing to the high or low rotundone accumulation between seasons is constant, despite the overall averages being different in the two seasons studied.
Table 1. Comparison of the Rotundone Concentration and Grape Quality Parameters within-Vines in the 2012–2013 Growing Season Using Two-Way ANOVA Analysis (p < 0.05)

| two-way ANOVA | sample group | rotundone (mg/kg) | TSSa (°Brix) | pHa | TAb (g/l) | DHca (%)
<table>
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<tbody>
<tr>
<td>Ctrl-NW&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.5 ± 6.7</td>
<td>28.3 ± 1.7</td>
<td>3.86 ± 0.14</td>
<td>3.69 ± 0.11</td>
<td>13.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Ctrl-SE</td>
<td>34.6 ± 1.3</td>
<td>28.0 ± 2.1</td>
<td>3.87 ± 0.09</td>
<td>3.79 ± 0.11</td>
<td>8.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>FS-NW</td>
<td>20.4 ± 1.6</td>
<td>27.9 ± 1.1</td>
<td>3.88 ± 0.09</td>
<td>3.85 ± 0.09</td>
<td>10.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>FS-SE</td>
<td>33.0 ± 1.0</td>
<td>27.4 ± 1.5</td>
<td>3.90 ± 0.12</td>
<td>4.13 ± 0.21</td>
<td>6.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>WS-NW</td>
<td>24.8 ± 7.8</td>
<td>26.0 ± 1.0</td>
<td>3.87 ± 0.08</td>
<td>3.90 ± 0.22</td>
<td>7.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>WS-SE</td>
<td>37.9 ± 12.8</td>
<td>25.6 ± 3.3</td>
<td>3.83 ± 0.10</td>
<td>4.14 ± 0.19</td>
<td>6.9 ± 0.2</td>
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</tbody>
</table>

| factor 1 treatment groups | mean Ctrl | 29.4 ± 12.0 m<sup>a</sup> | 28.1 ± 1.8 a<sup>d</sup> | 3.86 ± 0.11 ns | 3.74 ± 0.12 a | n/a<sup>d</sup> |
| mean FS | 26.7 ± 9.5 m<sup>a</sup> | 27.8 ± 1.2 a | 3.84 ± 0.10 ns | 3.99 ± 0.21 b | n/a |
| mean WS | 33.3 ± 11.0 m<sup>a</sup> | 25.8 ± 0.7 b | 3.85 ± 0.09 ns | 4.02 ± 0.23 b | n/a |

| factor 2 vine orientation | mean NW | 24.4 ± 6.8 A | 27.4 ± 1.5 ns | 3.87 ± 0.10 ns | 3.81 ± 0.17 A | n/a |
| mean SE | 35.4 ± 11.3 B | 27.0 ± 1.7 ns | 3.83 ± 0.10 ns | 4.02 ± 0.23 B | n/a |

<sup>a</sup>TSS = total soluble solids. <sup>b</sup>TA = titratable acidity as tartaric acid equivalents. <sup>c</sup>DH<sub>ca</sub> = percentage of degree hours above 25 °C. <sup>d</sup>Ctrl, control; FS, fruit zone shading; WS, whole vine shading; NW, samples from the northwest side of the canopy; and SE, samples from the southeast side of the canopy. *<sup>a</sup> = no significant difference (p > 0.05); <sup>b</sup>, <sup>c</sup>, and <sup>d</sup> represent significant differences (p < 0.05) between treatment groups, while A and B represent significant differences (p < 0.05) between two sides of the vine. *<sup>a</sup> = not applicable.

Differences in grape quality trait parameters were observed among vineyard zones and between study seasons. Overall, significant differences were only observed in the total phenolics among the three zones (Figure 3A). Grapes from the 2012–2013 growing season had significantly higher total phenolics (1.45 au/g), pH (3.398), and TSS (27.0 °Brix) and lower total anthocyanins (1.68 mg/g) and TA (3.91 g/l) compared to the 2011–2012 growing season (1.06 au/g, 3.35, 25.6 °Brix, 1.93 mg/g, and 5.61 g/l) (Figure 3B). The differences in grape quality parameters between vineyard zones were further compared in individual seasons (panels C and D of Figure 3). In the 2011–2012 growing season, no significant differences were observed in grape quality parameters were observed among the three zones (Figure 3C). In the 2012–2013 growing season, zone 1 had significantly higher total anthocyanins, total phenolics, and TSS and lower TA compared to zone 2, while grapes from zone 3 had intermediate levels of most grape quality parameters (Figure 3D).

**Within-Vine Variability of Rotundone.** A range of grape quality parameters and rotundone concentration were compared between the artificial shading treatments and two sides of the vine canopy (two-way ANOVA; p < 0.05) (Table 1). No significant differences were observed between shade treatment groups in the rotundone concentration, while berries from the SE side of the canopy had a significantly higher rotundone concentration compared to those from the NW side. WS treatments had significantly lower TSS (25.8 °Brix) compared to Ctrl (28.1 °Brix) and FS treatments (27.6 °Brix), while no significant differences were observed between two sides of the canopy in TSS. Ctrl treatments had significantly lower TA (3.74) compared to FS (3.99) and WS (4.02) treatments, while the NW side (3.81) of the canopy has significantly lower TA compared to the SE side (4.02). No significant differences were observed between artificial shading treatments and two sides of the canopy in pH.

The relationship between rotundone and the fruit zone temperature was explored. The fruit zone temperature over time was calculated as degree hours. Degree hours in different temperature ranges and their percentages to the total degree hours were calculated and associated with the rotundone concentration of grape berries. Percentages of degree hours in different temperature ranges higher than 25 °C (above 35 °C, and between 30 and 35 °C, and between 25 and 30 °C) were found to have negative relationships with the grape rotundone concentration (details in the Supporting Information). Therefore, the percentage of degree hours above 25 °C (DH<sub>ca</sub>) was used to analyze the relationship between the fruit zone temperature and the grape rotundone concentration (Table 1), which was explored in Figure 4. A power curve between the grape rotundone concentration and the percentage of degree hours above 25 °C in fruit zone from veraison to harvest in the 2012–2013 growing season. The equation for the trend lines is y = 130.6x − 1009 (R<sup>2</sup> = 0.61; RMSE = 4.87; and p = 0.0065).
rotundone accumulation. No significant differences in the concentration of rotundone in grapes were observed among artificial shading treatments (Table 1), likely because of the large variation in the concentration of rotundone among vines. Another possible explanation is that the actual bunch zone solar radiation/temperature is determined not only by the artificial shading but also the natural canopy, which cause more variations in the actual light intensity in the canopy. Therefore, a better way to assess the impacts of shading is to directly study the relationship between the actual bunch zone temperature and the concentration of rotundone in grapes (Figure 4). While the relationship established in Figure 4 ($y = 130.6e^{0.067}$; $R^2 = 0.61$ and RMSE = 4.87) was not statistically significant ($p = 0.065$), likely because of the small sample size ($n = 6$ shading levels) and large variation in the concentration of rotundone, the data nevertheless suggest the importance of the daytime bunch zone temperature on the concentration of rotundone in berries. Thus, increasing the duration of bunch zone temperatures to above 25°C will result in a lower concentration of rotundone in grapes. The impacts of sunlight on grapevine can be separated into direct illumination and indirect solar-radiation-induced temperature increases, which should be investigated separately to test their importance for the accumulation of rotundone in grapes.

**Within-Bunch Variability of Rotundone.** Grape berry surface temperature data of both the forward and backward sides of the bunch were obtained from thermal images (Figure 2A) taken on March 26, 2013 between 3:30 pm and 4:00 pm, with a mean bunch zone air temperature of 31.4°C recorded in the vineyard during this period. Grape berry surface temperature data had a wide range, from 30.6 to 42.9°C with a coefficient of variation (CV) of 8.09% for the maximum surface temperature, from 29.3 to 35.9°C with CV of 4.57% for the minimum surface temperature, and from 30.05 to 39.05°C with CV of 6.14% for the average surface temperature (Table 2). As expected, the sun-facing TF and BF sectors of the bunch had significantly higher maximum, minimum, and average surface temperatures than backward-oriented TB and BB sectors (Table 2).

In both seasons, the TB sector (2011–2012, 493.5 ng/kg; 2012–2013, 259 ng/kg) had a significantly higher concentration of rotundone compared to all other sectors ($p < 0.05$) (Table 3). In the 2011–2012 season, the TF sector (339.9 ng/kg) had a significantly higher concentration of rotundone compared to the BF sector (250.2 ng/kg) and the BB sector (303.7 ng/kg) tended to have a higher rotundone concentration compared to the BF sector ($p = 0.091$). No statistically significant differences were observed in the berry rotundone concentration between the TF and BB sectors. In the 2012–2013 growing season, the TF sector (19.2 ng/kg) had a higher concentration of rotundone compared to both BF (15.3 ng/kg) ($p = 0.068$) and BB (16.2 ng/kg) ($p = 0.057$). However, no differences were observed between the BB and BF sectors for berry rotundone concentration. The pattern of rotundone concentration distribution within bunch sectors was consistent across the two seasons.

Three possible reasons may explain the higher concentration of rotundone of berries from the top sectors of bunches (Table 3). First, the variation in the concentration of rotundone might reflect differences in grape surface temperatures because more shading from proximal leaves or darker canopies or bunch architecture with a higher berry number within the top part of bunches could contribute to buffer the temperature of berries from these zones, lowering the surface temperature compared to fully exposed berries. On the contrary, the bottom part of bunches had less buffer capacity to maintain the temperature (in contact with fully exposed berries), which might explain higher temperatures in these sections (Table 2). Furthermore, the lower part of bunches is exposed to radiated transference of heat from the soil. Even though berry transpiration may lower the temperature of all berries, it cannot prevent the temperature increase caused by solar radiation. Therefore, the position of a berry in the bunch is crucial to its temperature, which is associated with its rotundone concentration. Notably, the position of a berry in the bunch might also result in increases in direct precursors to rotundone, such as guaiene, and increased concentrations in rotundone would then reflect the precursor concentrations. A second explanation is that the bottom sector of a bunch typically matures faster than the top sector. The maximum rotundone concentration in grape berries is reached after veraison and slowly decreases afterward in the lead up to harvest. As a result, a more mature bottom sector would have a lower rotundone concentration compared to the later ripening top sectors; i.e., rotundone concentrations would reflect the biochemical differences linked to a variation in maturity. A third possible explanation (the “transport theory”) is that a shorter distance from berries to leaves and stems affects rotundone accumulation, because it has been reported that leaves and stems may contain higher concentrations of rotundone than berries and are potentially having a role as a source or storage. This possibility requires an internal transportation mechanism for rotundone in grapevines. In other plant species, namely, *Asarum canadensis* (Scrophulariaceae) and *Catalpa speciosa* (northern catalpa), it has been reported that monoterpene glycosides, antirrhinioside, and catalpol can be translocated via phloem transport. However, translocation of aroma compounds has been studied in grapevines, with little evidence to show that it is a widespread phenomenon. For example, translocation of monoterpene glycosides and their precursors from other parts of the vine into berries is unlikely in Muscat, unless an active transport mechanism exists for a specific compound. In addition, the guaiaco glycosides can translocate between leaves and berries in Cabernet Sauvignon but to a very limited extent. Therefore, there is a possibility that rotundone or a precursor,

<table>
<thead>
<tr>
<th>Table 2. Comparison of the Grape Berry Surface Temperature among Four Bunch Sectors in the 2012–2013 Growing Season*</th>
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<tr>
<td>within-bunch sectors</td>
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<tr>
<td></td>
</tr>
<tr>
<td>TF</td>
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<td>TB</td>
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<tr>
<td>BF</td>
</tr>
<tr>
<td>BB</td>
</tr>
<tr>
<td>overall range</td>
</tr>
<tr>
<td>mean (CV)*</td>
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*The average surface temperature was calculated by taking the average of maximum and minimum temperatures. TF, top front sector of the bunch; TB, top back sector of the bunch; BF, bottom front sector of the bunch; and BB, bottom back sector of the bunch. a, b, c, and d represent significant differences ($p < 0.05$) between bunch sectors. *CV = coefficient of variation.
Table 3. Comparison of the Rotundone Concentration and Grape Quality Parameters among Four Bunch Sectors in the 2011–2012 and 2012–2013 Growing Seasons Using One-Way ANOVA Analysis (p < 0.05)*

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<tbody>
<tr>
<td></td>
<td>rottenone (ng/kg)</td>
<td>rottenone (ng/kg)</td>
</tr>
<tr>
<td>top front</td>
<td>339.9 ± 149.5 b</td>
<td>19.2 ± 6.5 b</td>
</tr>
<tr>
<td>top back</td>
<td>493.1 ± 167.2 a</td>
<td>23.9 ± 9.8 a</td>
</tr>
<tr>
<td>bottom front</td>
<td>290.2 ± 116.6 c</td>
<td>15.3 ± 5.6 b</td>
</tr>
<tr>
<td>bottom back</td>
<td>300.7 ± 103.6 bc</td>
<td>16.2 ± 4.7 b</td>
</tr>
</tbody>
</table>

*Grape berry parameters were not collected in 2011–2012. **TSS = total soluble solids. †TA = titratable acidity. *Bunch sectors are illustrated in Figure 2. a, b, c, and d represent significant differences (p < 0.05) between bunch sectors.

To some extent, may be formed elsewhere and is then translocated between berries, leaves, and stems, depending upon the availability of active transportation mechanisms, and this warrants further investigation.

In the 2012–2013 growing season, significant differences in grape quality parameters were observed (Table 3). Berries from the addition 2012–2013,” Brix, slightly reduced TSS compared to the BB sector (27.1 °Brix). Berries from the solar exposed side of the bunch (TF and BF) had significantly lower TA and higher pH compared to their corresponding shaded side (TB and BB). In addition, berries from the TB sector had significantly higher total anthocyanins (1.77 mg/g) and total phenolics (1.58 au/g) compared to the bunch TF sector (1.64 mg/g and 1.35 au/g).

The differences in grape quality parameters between studied seasons and among vineyard zones, shading treatment groups, and bunch sectors are associated with berry surface temperature and solar radiation. Higher TSS has been shown with greater sunlight exposure and temperature and may explain the variances between studied seasons (Figure 3B), among vineyard zones (Figure 3D), shading treatments (Table 1), and bunch sectors (Table 3).24,25 Higher temperatures stimulate respiration and modify organic acid metabolism, leading to decreased malate and citrate concentrations, and, therefore, a likely decrease in TA and increase in pH.26 As a result, berries from the warmer season (2012–2013) (Figure 3B) and the exposed sectors of bunch (Table 3) had higher pH and lower TA, while the unshaded treatment groups and the northwest side of the vine had lower TA (Table 1). A high temperature (>35 °C) has been shown to reduce the grape anthocyanin concentration, which explains the lower total anthocyanin concentration in the berries from the warmer season (2012–2013) (Figure 3B) and the exposed bunch sectors (Table 3).27 However, moderate solar exposure increases anthocyanin concentration in grape berries, which explains the relatively higher concentration of total anthocyanins in berries from the low vigor zone (zone 1) (Figure 3D).24,25 A previous study has demonstrated that the concentration of total phenolics increases gradually with increasing solar exposure and becomes relatively stable under higher solar exposure levels.25 This is consistent with the higher total phenolics observed in the berries from the higher solar intensity season (2012–2013) and lower vigor zone (zone 1) (panels A, B, and D of Figure 3). However, the shaded bunch sectors tend to have higher total phenolics than the exposed sectors (Table 3), which may be due to degradation of certain phenolics under a higher temperature, such as anthocyanins.25

In addition, grape quality parameters tend to be more sensitive at higher temperatures or solar exposure.25,27 This could be the reason that differences in grape quality parameters among zones were only observed in the 2012–2013 growing season (panels C and D of Figure 3).

Fruit zone temperature and berry surface temperature are correlated with the concentration of rotundone in Shiraz grapes at this site. Vineyard topography, vine vigor, location of bunches within the vine, and bunch orientation to incident solar radiation also influence rotundone accumulation in Shiraz grapes. These factors affect the shading of grape berries and, therefore, adjust bunch surface temperature and bunch zone temperatures. Sunlight interception and water availability may also influence rotundone production, which requires further investigation. Apart from climatic parameters, physiological parameters, such as the source–sink relationships between leaves/stems and bunch in rotundone, may also influence its concentration in grape berries, which warrants further study. This study demonstrates the importance of the vine growing micro-environment and, specifically, the importance of the temperature on the formation of a key sesquiterpene compound responsible for wine aroma.

ASSOCIATED CONTENT

Supporting Information

Weather record of the vineyard in 2011–2012 and 2012–2013 seasons (Table S1), comparison of the grape berry rotundone concentration (ng/kg) and fruit zone temperature between two sides of the row with different degrees of shading (Table S2), illustrations of grapevine shading treatments in the 2012–2013 growing season (Figure S1), relationship between the percentage of degree hours in fruit zone from veraison to harvest and the mean rotundone concentration in grape samples from the within-vine variability study (Figure S2), and comparison of the rotundone concentration and quality parameters among within-bunch sectors in an individual vineyard zone in the 2012–2013 growing season (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BOM, Bureau of Meteorology; VSP, vertical shooting positioning trellis; PCD, plant cell density; FS, fruit zone shaded; WS, whole vine shaded; NW, northwest facing side of the vines; SE, southeast facing side of the vines; TSS, total soluble solids; TA, titratable acidity; TF, top front sector of bunch; TB, top back sector of bunch; BF, bottom front sector of bunch; BB, bottom back sector of bunch; SPE, solid-phase extraction; au, absorbance units

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3.3 Addendum

Note: Abstract page 51 should be modified as below:

This study characterises the environmental factors driving rotundone concentrations in grape berries by quantifying rotundone variability and correlating it with viticultural parameters. Dissection of the vineyard into distinct zones (based on vigor, electrical soil conductivity and slope), vine into orientations to sun (shaded / unshaded) and grape bunches into sectors (top and bottom, front and back) shows the influence of vine vigor, sunlight and temperature on berry quality traits and rotundone concentration. Occurrence of the highest rotundone concentration was observed in shaded bunch sectors and vines, and from vines with higher vigor vines in the southern-facing areas of the vineyard. The highest concentration of rotundone was consistently found at the top and in shaded sectors of bunches, and this correlated to lower grape surface temperatures. Modelling showed that berry temperature exceeding 25°C negatively affects rotundone concentration in Shiraz. Both natural and artificial shading modulated the grape surface and air temperature at the bunch zone and increased rotundone concentration, without affecting other grape berry quality parameters. Thus temperature, and possibly sunlight interception is the main determinant of rotundone in grape berries. Vineyard topography, vine vigor, vine row and grape bunch orientation influence the level of berry shading, and can therefore adjust bunch surface and zone temperatures and influence berry rotundone concentration.

Note: Page 53 Right column, paragraph 2, line 11, ‘filtrated’ should be corrected as ‘filtered’.

Note: Page 56 Right column, paragraph 2, line 22, Re: "A second explanation is that the bottom sector of a bunch typically matures faster than the top sector". However, the results from the current study didn’t show faster maturity to the bottom sector of the grape bunch. Lower concentration of rotundone in bottom sector of bunch may due to less time to produce rotundone. As discussed in Chapter 2, rotundone mainly accumulates at a later stage of grape development, and less mature grapes may not have enough time for the rotundone level to reach the plateau.

Note: Page 57 Table 3. Paired t-test was used to analyse the differences between different bunch sectors. Even though the deviations were close to differences between means, statistical differences could still be established.
3.4 Supplementary data

Table S3.4.1. Weather record of the vineyard in 2011-12 and 2012-13 seasons (Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085 – located 15.5kms NW to the experimental site). The 2011-12 growing season is classified as ‘cool and wet’ compared with the 2012-23 growing season which is ‘warm and dry’. The date of 80% veraison is taken at 20\textsuperscript{th} Feb 2012 for 2011-12 and 19\textsuperscript{th} Feb 2013 for 2012-13 growing season. Commercial harvest date begins on 11\textsuperscript{th} Apr 2012 and 10\textsuperscript{th} Apr 2013.

<table>
<thead>
<tr>
<th>Weather record</th>
<th>2011-12</th>
<th>2012-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean January Maximum Temperature</td>
<td>28.3°C</td>
<td>28.8°C</td>
</tr>
<tr>
<td>Mean January Minimum Temperature</td>
<td>13.7°C</td>
<td>11.1°C</td>
</tr>
<tr>
<td>Mean January Temperature</td>
<td>21.0°C</td>
<td>20.0°C</td>
</tr>
<tr>
<td>Mean January Solar Exposure</td>
<td>26.7 MJ/m\textsuperscript{2}</td>
<td>29.1 MJ/m\textsuperscript{2}</td>
</tr>
<tr>
<td>Mean Maximum Temperature 80% veraison to harvest</td>
<td>23.2°C</td>
<td>26.3°C</td>
</tr>
<tr>
<td>Mean Minimum Temperature 80% veraison to harvest</td>
<td>10.2°C</td>
<td>11.5°C</td>
</tr>
<tr>
<td>Mean Temperature 80% veraison to harvest</td>
<td>16.7°C</td>
<td>18.9°C</td>
</tr>
<tr>
<td>Mean Solar Exposure 80% veraison to harvest</td>
<td>16.1 MJ/m\textsuperscript{2}</td>
<td>18.1 MJ/m\textsuperscript{2}</td>
</tr>
<tr>
<td>Total rainfall from Oct to Harvest</td>
<td>227.8 millimeter</td>
<td>124.1 millimeter</td>
</tr>
<tr>
<td>Total irrigation From Oct to Harvest</td>
<td>32.0 millimeter</td>
<td>84.3 millimeter</td>
</tr>
<tr>
<td>Total water precipitation (October to Harvest)</td>
<td>259.8 millimeter</td>
<td>208.4 millimeter</td>
</tr>
</tbody>
</table>
Table S3.4.2. Comparison of the grape berry rotundone concentration (ng/kg) and fruit zone temperature between two sides of the row with different degree of shading.

<table>
<thead>
<tr>
<th>Degree hours in different temperature range</th>
<th>Treatment Groups</th>
<th>Power relationship analysis</th>
<th>Correlation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Dhr *</td>
<td>29006.8</td>
<td>27551.1</td>
<td>27708.8</td>
</tr>
<tr>
<td>% of Dhr &gt;35°C</td>
<td>4.03</td>
<td>0.66</td>
<td>1.62</td>
</tr>
<tr>
<td>% of Dhr 30-35°C</td>
<td>3.93</td>
<td>2.74</td>
<td>3.20</td>
</tr>
<tr>
<td>% of Dhr 25-30°C</td>
<td>5.75</td>
<td>5.40</td>
<td>5.52</td>
</tr>
<tr>
<td>% of Dhr 20-25°C</td>
<td>8.80</td>
<td>9.05</td>
<td>8.75</td>
</tr>
<tr>
<td>% of Dhr 15-20°C</td>
<td>13.61</td>
<td>14.45</td>
<td>13.96</td>
</tr>
<tr>
<td>% of Dhr 10-15°C</td>
<td>18.58</td>
<td>19.86</td>
<td>19.48</td>
</tr>
<tr>
<td>% of Dhr &lt;10°C</td>
<td>45.29</td>
<td>47.84</td>
<td>47.48</td>
</tr>
</tbody>
</table>

*coefficient. *error sum of square. *root mean square deviation. *correlation coefficient. *degree hours. *T-test was conducted for correlation coefficients where ns, *, ** stands for no significant difference, no significant difference but 0.5≤P<0.1, P<0.05, respectively.
Figure S3.4.1. Experimental vine shading treatments in the 2012-13 growing season. Vines for within-vine variability study were shaded in (A) fruit zone on both side of the canopy wall with 1 meter length, 0.4 meter widths shading cloth or (B) fully shaded on both sides of the canopy wall with 9 meters length, 6 meters widths shading cloth.
Figure S3.4.2. The relationship between the percentage of degree hours (a1) above 35°C, (a2) between 30-35 °C, (a3) between 25-30°C, (b1) between 20-25°C, (b2) between 15-20°C and (b3) between 10-15°C in fruit zone from veraison to harvest and the mean rotundone concentration in grape samples from within vine variability study. The equation to the trend lines are (a1) y=27.2x^{-0.175} (R^2=0.6901, RMSE= 4.322, p=0.0984), (a2) y=48.3x^{-0.5487} (R^2= 0.5459, RMSE= 5.232, p= 0.0785), (a3) y=532.2x^{-1.777} (R^2=0.4769, RMSE=5.615, p=0.1215), (b1) y=0.09214x^{2.656} (R^2=0.0410, RMSE=7.603, p=0.7078), (b2) y=(6.49E-09)x^{8.368} (R^2=0.7643, RMSE=3.770, p=0.0269), (b3) y=(7.52E-06)x^{5.075} (R^2=0.5732, RMSE=5.072, p=0.0862).
Figure S3.4.3. Comparison of rotundone concentration and quality parameters among within-bunch sectors in individual vineyard zone (A) zone 1, (B) zone 2, (C) zone 3, in the 2012-13 growing season. One-way ANOVA was conducted for each parameter where ns, *, **, *** stands for no significant difference, no significant difference but 0.5≤P<0.1, P<0.05, P<0.01, respectively.
CHAPTER FOUR
Sunlight Exclusion Alters Rotundone and Sesquiterpenes in Vitis vinifera L. cv. Shiraz Grapes

ABSTRACT
The grapevine growing environment is known to determine rotundone concentrations in Shiraz grapes and wine. Here, we investigate the impact of sunlight exclusion at different stages of berry development on the concentration of rotundone and other sesquiterpenes in Shiraz grapes at harvest. Sunlight was excluded from Shiraz grape bunches at different grape development stages: six weeks after fruit set to harvest ($T_w$), six weeks after fruit set to 80% veraison ($T_v$), 80% veraison to intermediate-ripe ($T_i$), and intermediate-ripe to maturity ($T_h$). Nineteen sesquiterpenes, three norisoprenoids, and one monoterpane were identified and semi-quantified: sunlight exclusion significantly modified the concentrations of nine sesquiterpenes and one monoterpane in grape berries. Sunlight illumination at all berry development stages had an impact on rotundone concentrations in grapes at harvest, and the $\alpha$-guaiene to rotundone biosynthetic pathway may be particularly light sensitive.
4.1 Introduction

Sesquiterpenes are a class of terpenoids commonly found in *Vitis vinifera* wine grape cultivars (May and Wüst 2012). The majority of sesquiterpenes do not contribute flavour or aroma to grapes and wine, although one oxygenated bicyclic sesquiterpene, rotundone, confers a desirable black pepper character to grapes and wine (Siebert et al. 2008; Wood et al. 2008). This ‘peppery’ characteristic is stylistically important to some high-quality Australian Shiraz wines and is favoured by experienced wine consumers (Herderich et al. 2012; Williamson et al. 2012). The exact biochemical origin of rotundone is inconclusive, although a recent study reported rotundone synthesis by aerial oxidation of a precursor compound, α-guaiene (Huang et al. 2014). All grape berry sesquiterpenes, including rotundone and α-guaiene, are biosynthesised from the common C5 precursor isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) via the mevalonate (MVA) and methyl-erythritol-phosphate (MEP) pathways in the grape berry exocarp (skin) (Dunlevy et al. 2009; May et al. 2013). This is consistent with the observation that rotundone is only found in grape berry exocarp and not the mesocarp (flesh) (Siebert and Solomon 2011). Sesquiterpene biosynthesis is regulated by a terpenoid synthase gene subfamily (VvTPS), and the VvPNSeint gene is responsible for α-guaiene biosynthesis (Martin et al. 2010). However, the terpenoid synthase responsible for rotundone biosynthesis has yet to be identified.

The impact of the environment on sesquiterpene production in grapevines has not been fully investigated, although it is known that the vineyard microclimate
has an impact on rotundone production (Zhang et al. 2015a; Zhang et al. 2015c). Bunch zone air and grape surface temperature appear to be important factors influencing rotundone production, and temperatures exceeding 25°C reduce rotundone concentrations in grape berries (Zhang et al. 2015a; Zhang et al. 2015c). In vitro, temperatures higher than 40°C accelerate the aerial oxidation of α-guaiene to rotundone (Huang et al. 2014). Increased vineyard water availability and elevated irrigation during the veraison to harvest period have a positive impact on rotundone concentrations in the resulting wine (Geffroy et al. 2014; Zhang et al. 2015c).

It is uncertain how sunlight affects sesquiterpene production. Although sunlight was found to be inversely related to wine rotundone concentration in a vertical study of Shiraz wine produced from the same vineyard over fifteen vintages (Zhang et al. 2015c), it is unclear whether this was due to the direct effect of illumination or the indirect effect of the heat produced by solar radiation. Moderate sunlight exposure increases total potentially volatile terpene and monoterpenes concentrations at harvest in Traminette (Skinkis et al. 2010), while excessive sunlight reduces total terpene concentrations in Muscat (Belancic et al. 1997). Conversely, complete sunlight exclusion during grape ripening reduces total monoterpenes at harvest in Muscat (Zhang et al. 2014). Increased illumination stimulates the production of many plant secondary metabolites including sesquiterpenes (Bassman 2004). Importantly, ultraviolet-B (UV-B) radiation increases the concentration of a plant defence-related sesquiterpene, E-nerolidol, and upregulates terpene synthase activity in Malbec grapevine leaves (Gil et al. 2012). In addition, enhanced UV-B radiation increases
monoterpenes in intermediate-ripe Malbec grape berries (Gil et al. 2013). Therefore, sunlight exclusion might affect rotundone and sesquiterpene concentrations in grapes at harvest.

The influence of solar exposure on sesquiterpene production may vary according to the physiological stage of the grapevine, especially since some sesquiterpenes are known to develop during specific periods of berry development (May and Wüst 2012). Rotundone mainly accumulates a few weeks prior to harvest to reach relatively stable concentrations between 44 and 60 days after mid-veraison in Duras and Shiraz (Geffroy et al. 2014; Siebert and Solomon 2011; Zhang et al. 2015c). Sesquiterpenes have mainly been observed at the pre-veraison stages, decreasing to very low levels at veraison in Cabernet Sauvignon and Riesling (Kalua and Boss 2009, 2010). However, in the ‘Baga’ grape, sesquiterpenes, including α-guaiene, were only found at four weeks after half-veraison to post-maturation (Coelho et al. 2006). Another study reported that bicyclic sesquiterpene concentrations increase from half-veraison to harvest in Riesling, Lemberger, Shiraz, and Yellow Muscat, while acyclic sesquiterpene concentrations decreased (May and Wüst 2012). Therefore, the influence of solar exposure on sesquiterpene concentrations in grape berries at different development stages requires further, more detailed, investigation.

Here, we investigate the effect of sunlight exposure on sesquiterpene concentrations at different developmental stages of Shiraz grapes. We show that sunlight illumination has an important effect on rotundone production in Shiraz berries.
4.2 Materials and methods

4.2.1 Chemicals

Rotundone ((3S,5R,8S)-3,4,5,6,7,8-hexahydro-3,8-dimethyl-5-(prop-1-en-2-yl)-1(2H)-azulenone) and \(^2\text{H}_5\)-rotundone were synthesised as described previously (Siebert et al. 2008; Wood et al. 2008). A reference standard of \(\alpha\)-copaene was supplied by Sigma-Aldrich (Castile Hill, NSW, Australia). All working solutions of standards were prepared volumetrically in ethanol and stored at 4°C until required. High-performance liquid chromatography (HPLC)-grade ethyl acetate, n-pentane, methanol, and ethanol were obtained from Rowe Scientific (Doveton, Vic, Australia). Analytical-grade potassium L-tartrate monobasic, tartaric acid, and other chemicals were obtained from Sigma-Aldrich. Water was purified using the Milli-Q system (Millipore Australia, Bayswater, Victoria, Australia).

4.2.2 Vineyard site

The study was conducted in a commercial vineyard (The Old Block, Mount Langi Ghiran, 37.31°S, 143.15°E) located in the Grampians wine region of Victoria, Australia. The vineyard is approximately 15.5 km east of the nearest Bureau of Meteorology (BOM) weather station at Ararat Prison (Australian BOM Station No. 089085), where the long-term mean January temperature is 18.9°C, annual average rainfall is 587.9 mm, and mean January daily solar exposure is 26.7MJm\(^{-2}\) up to 2014: it is classified as a cool climate wine production region (Gladstones 2004). The mean January temperature, mean January daily solar exposure, and total rainfall from October to harvest for the studied growing season (2013-14) were 21.7°C, 27.0 MJm-2, and 140.9 mm, respectively (Table S-4.1). The vineyard was planted in 1968 with \textit{Vitis vinifera}, cv. Shiraz on its
own roots, 3.0 m between rows and 1.8 m between vines, with rows oriented northeast to southwest. Grapevines were trained to a vertical shoot positioned (VSP) trellis, and a dripping irrigation system was present along vineyard rows with a dripper spacing of 0.5 m and a dripper output of 1.5 litres per hour. Grapevines were irrigated when required at a rate of 5.76 L/(hr·vine). The total irrigation volume from October to harvest for the studied growing season was 60.8 mm. No significant pest or disease pressures were observed during the experimental season.

4.2.3. Experimental design and sampling

Shading treatments were applied to the shaded side (south facing side) of a single row to minimise variability between vines, since large within-vineyard and within-vine variations in berry rotundone concentration were previously reported for this experimental block (Zhang et al. 2015a; Scarlett et al. 2014). Within the selected row, seven vines were selected on the basis of canopy uniformity and cluster development. Selected vines were separated by at least two vines as a buffer. Shoots on selected vines were randomly distributed to receive each of the following artificial shading treatments in the 2013-14 growing season (Figure 4.1): i) naturally occurring shade (Ctrl); ii) bunches enclosed in an artificial shade box from pea-size (E-L 31) to harvest (E-L 38) (T_w); iii) bunches enclosed in an artificial shade box from pea-size (E-L 31) to 80% veraison (E-L 36) (T_v); iv) bunches enclosed in an artificial shade box from 80% veraison (E-L 36) to intermediate-ripe (E-L 37) (T_i); and v) bunches enclosed in an artificial shade box from intermediate-ripe (E-L 37) to harvest (E-L 38) (T_h). The phenological stages of grapevine growth were identified using the E-L system (Pearce and
Coombe 2004). In this study, pea-size grape, 80% veraison, intermediate-ripe, and harvest dates of grapes were taken at 23\textsuperscript{th} Jan, 28\textsuperscript{th} Feb, 31\textsuperscript{th} Mar, and 17\textsuperscript{th} Apr 2014, respectively. Treatments shoots were separated by at least 20 centimetres as buffer to avoid any influence of shading box on other treatment shoots.

**Fig. 4.1.** Experimental design of sunlight exclusion experiment. (a) The shading boxes used were the same boxes from a previous study (Downey et al. 2004), which has been demonstrated to eliminate light while maximising airflow in the bunch zone. (b) Grape bunches in the selected vines were shaded using artificial shading box at different phenological stages in the 2013-14 growing season.
For shading treatments, each bunch was shaded using a polypropylene shade box made from white polypropylene sheeting painted black on the inside as previously described (Downey et al. 2004). These boxes are designed to eliminate light while maximising airflow, thus reducing any temperature differences between bunches inside the boxes and the natural canopy. Light transmission through the shade box is less than 0.1% between wavelengths of 220 and 800nm and less than 0.01% below 400nm (Downey et al. 2004). In addition, a quantum sensor (Li6400, LI-COR, Lincoln, NE, USA) was used to measure photosynthetically-active radiation (PAR, 400 to 700nm) inside and outside the box. Illumination inside the box was measured with a lux meter (HI 97500 portable lux meter, Hanna instruments, Inc., Woonsocket, RI, USA). Light sensors for these instruments were placed inside the shade box in the same way as grape bunches during measurements.

Fruit zone temperature in the natural canopy and shade box were measured using temperature loggers (Tinytag Transit 2, Gemini Data Logger Ltd, Chichester, UK). One logger (L2014S) was placed in the shade box, and a second logger (L2014N) was placed next to the shade box covered by natural canopy. The LS2014S logger was hung inside the box to avoid any direct contact with the box. These loggers have an operational range of -40°C to 70°C and a sensitivity of 0.01°C. Temperature was measured every 30 min from flowering (E-L 25, 11 Dec 2013) until the last day of commercial harvest (E-L 38, 17 Apr 2014), and the data were analysed using Tinytag Explorer software (version 4.7, Gemini Data Logger Ltd, Chichester, UK).
Sampling was performed on the last day of commercial harvest (17th April 2014). Commercial harvest was performed between 8th and 17th April 2014 by selective hand harvest followed by machine harvest at later dates. Grape samples were collected in bunches using zip-lock plastic bags, frozen at -20°C, and transferred to the laboratory in Styrofoam boxes on dry ice for further storage at -20°C before analysis. Total soluble solids (TSS), titratable acidity (TA), pH, total anthocyanins, and total phenolics of bunch samples were analysed using a refractometer, alkaline titration, a pH meter, and spectrophotometric measurements at A520nm and A280nm according to published protocols (Iland and Patrick Iland Wine Promotions. 2004).

4.2.4. Preparation of samples and solid-phase microextraction-gas chromatography-multidimensional mass spectrometry (SPME-MDGC-MS) rotundone analysis

Grape samples were prepared for rotundone analysis based on the protocol described by (Scarlett et al. 2014). For each grape sample, 100 g of de-stemmed grapes were sub-sampled before being homogenised using a hand-held blender then centrifuged to separate solids and liquids. The solids were mixed with 30 ml of ethanol, 30 ml of water, and 100 μL d5-rotundone (516 ng/ml in ethanol) as internal standard, then shaken for 24 h at 22°C and sonicated prior to reintroducing the liquid component. Sub-samples were then centrifuged. The supernatant was combined with the liquid component and filtered (1.6 μm glass fibre) to obtain berry extract filtrate, which was topped up to 200 ml with deionised water prior to solid-phase extraction (SPE) as reported previously (Siebert et al. 2008). The SPE eluate was dried under nitrogen and
reconstituted in 0.5 ml ethanol and 9 ml deionised water. The samples were then analysed by SPME-MDGC-MS following published protocols (Geffroy et al. 2014).

4.2.5. Sesquiterpene analysis

Sesquiterpene analysis was based on a published protocol (Parker et al. 2007) with the following modifications. An Agilent Technologies 6890 gas chromatograph (GC; Agilent Technologies, Santa Clara, CA) was equipped with a Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5973 mass selective detector (MSD). The instruments were controlled using Agilent G1701EA MSD ChemStation software in conjunction with Gerstel Maestro software (version 1.4.20.0). The GC was fitted with a J&W DB-5ms capillary column measuring approximately 30 m × 0.25 mm, 0.25 μm film df. The carrier gas was helium (ultrahigh purity, BOC, Adelaide, SA, Australia), and the flow rate was 1.0 ml/min in constant flow mode. The GC inlet was fitted with a resilanised borosilicate glass SPME inlet liner (Supelco, 6.5 mm o.d., 0.75 mm i.d., 78.5 mm long) held at 220°C.

The SPME fibre was desorbed in the pulsed splitless mode and the splitter, at 50:1, was opened after 30 s. The fibre was allowed to bake in the inlet for 10 min. The oven was started at 50°C, held for 1 min, increased to 230°C at 3°C/min, then increased to 280°C at 20°C/min and held at 280°C for 5 min. The MS transfer line was held at 250°C. The temperatures of the MS source and quadrupole were 230°C and 150°C, respectively. The MS was operated in
positive EI mode at 70 eV with simultaneous selected ion monitoring (SIM) and scanning over a mass acquisition range of 35-280 m/z.

100 g of de-stemmed grapes were sub-sampled before being homogenised using a hand-held blender. 5 g of homogenised sample was transferred into a HS-SPME vial (Agilent Technologies, 20ml) and mixed with 500 μL α-copaene (200.64 μg/L in ethanol) as internal standard. The samples were then shaken for 24 h at 22°C before adding 2 ml saturated brine and being subjected to SPME-GC-MS analysis. The vial and its contents were heated to 45°C. A polydimethylsiloxane/divinylbenzene (PDMS/DVB, Agilent) 65μm SPME fibre was exposed to the headspace for 60 min with agitation. Sesquiterpenes were identified by comparing the mass spectra and retention indices with the terpenoids library in MassFinder (version 4.1, Dr Hochmuth Scientific Consulting, Hamburg, Germany). All compounds except α-guaiene were quantified as α-copaene equivalents. α-guaiene was determined by SIM with α-copaene as internal standard; the ions monitored were: m/z 105, 133, 147, 161, and 204; dwell time 25 ms each. The target ions were typically m/z 147 for α-guaiene and 161 for α-copaene with ions 105, 133, and 204 m/z used as qualifiers. Data were analysed using Agilent G1701DA MSD ChemStation software. α-guaiene was expressed as the ratio of m/z 147:m/z 161 multiplied by the concentration of α-copaene internal standard. The assay precision was validated by a series of standard additions of internal standard as described previously (Parker et al. 2007). Blank SPME runs and blank internal standards were checked regularly.
4.2.6. Statistical analysis

The relationship between the temperature in the natural canopy and shade box was calculated using MatLab® software 2014a (The MathWorks, Inc., Matick, MA, USA). TSS, TA, pH, total anthocyanins, total phenolics, and the concentration of terpenoids, including rotundone and α-guaiene, in grape samples from different shade treatments were compared using one-way analysis of variance (ANOVA) at \( p<0.05 \) (CoStat, version 6.4, CoHort Software, Monterey, USA).

4.3 Results and discussion

4.3.1. Effectiveness of artificial shading

The PAR inside the box was less than 0.5% of that outside the box, consistent with previous measurements (Downey et al. 2004). The light luminance inside the box was less than 0.5% of that outside the box in both cloudy and sunny conditions. In this study, half-hourly temperature data in the shade box from the beginning of shading to harvest were compared with temperatures in the natural canopy: there was a strong linear correlation between the data from the L2014N and L2014S temperature loggers (Fig. 4.2; \( y=0.9609x+0.3246, \ R^2=0.9965, \ RMSE=0.4545, \ p<0.0001 \)): compared to the logger (L2014N) positioned in the natural canopy, the artificially-shaded logger (L2014S) recorded slightly lower temperatures, with an average difference of ±0.44°C. This was consistent with previous studies reporting ±0.5°C and ±2.9% differences in temperature and relative humidity between the inside and outside of the shade box, respectively (Downey et al. 2004).
Fig. 4.2. Comparison of air temperature in the artificial shading box and in the natural canopy. A linear regression is established between temperature in shading box and canopy measured from 23 Jan to 17 Apr 2014 utilizing temperature data from logger L2014N and logger L2014S, $y=0.9609x+0.3246$ ($R^2=0.9965$, RMSE=0.4545, $p<0.0001$).
The shade box substantially reduced the light environment around the developing fruit. Due to the low light transmission through the shade box material, light in both PAR and UV ranges were blocked. Although some reflected light may have entered the shade box via small gaps in the box joints, measurements indicated that the light luminance inside the boxes was less than 0.5% of incident light. Although slight temperature differences were observed inside and outside the box (Fig 4.2), this has previously been reported to be insufficient to cause large differences in grape rotundone concentrations (Zhang et al. 2015a). Therefore, the boxes used in this study successfully prevented exposure of grape berries to sunlight without significantly modifying air temperature. Thus, it is reasonable to conclude that any differences observed in grape quality traits and sesquiterpene concentrations were due to light exclusion.

4.3.2. Berry weight and composition

Differences in grape berry weight and composition were observed between treatment groups (Table 4.1). Ctrl had significantly higher berry weight than Tw and tended to have a higher berry weight than Tv, Ti, and Th groups (Table 4.1). No statistically significant differences in pH were observed between Ctrl and any treatment group, although the pH of Tw was significantly higher than Th. In addition, Ti had significantly higher TA than all other groups. No statistically significant differences were observed in °Brix, total anthocyanins, and total phenolics between treatment groups.
Table 4.1. Comparison of berry weight and grape quality traits parameters among different shading treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Berry mass (g)</th>
<th>°Brix</th>
<th>pH</th>
<th>TA&lt;sup&gt;b&lt;/sup&gt; (g/L)</th>
<th>Total anthocyanins (mg/g)</th>
<th>Total phenolics (a.u./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>0.98±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7±0.5</td>
<td>3.64±0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.95±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.89±0.18</td>
<td>1.41±0.04</td>
</tr>
<tr>
<td>T&lt;sub&gt;w&lt;/sub&gt;</td>
<td>0.86±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.9±1.5</td>
<td>3.77±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.65±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.98±0.55</td>
<td>1.39±0.21</td>
</tr>
<tr>
<td>T&lt;sub&gt;v&lt;/sub&gt;</td>
<td>0.91±0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.3±0.7</td>
<td>3.68±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.70±0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.92±0.14</td>
<td>1.42±0.08</td>
</tr>
<tr>
<td>T&lt;sub&gt;i&lt;/sub&gt;</td>
<td>0.91±0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.3±0.8</td>
<td>3.65±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.64±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.70±0.19</td>
<td>1.42±0.14</td>
</tr>
<tr>
<td>T&lt;sub&gt;h&lt;/sub&gt;</td>
<td>0.90±0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.0±0.8</td>
<td>3.58±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.68±0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.78±0.33</td>
<td>1.34±0.12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Different letters in the column represent significantly different means ± standard error (n=7 replicates, p<0.05). <sup>b</sup>Titratable acidity.

The influence of sunlight exclusion on berry weight, pH, and TA varied according to period of cluster enclosure. Compared to naturally shaded grapes, sunlight-excluded clusters had a slightly lower berry weight, while the TSS was similar between groups. Previous studies have also reported no differences in berry weight and TSS between shaded and exposed clusters in *Vitis vinifera* Shiraz, Merlot, Pinot noir, and Jingxiu, a Chinese hybrid of Muscat and Panonia (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove et al. 2000; Li et al. 2013; Spayd et al. 2002). However, differences were reported in berry weights and TSS between the north and south sides of rows in Grenache and Cabernet Sauvignon (Bergqvist et al. 2001). In addition, a significantly lighter berry weight and lower TSS were reported in shaded Muscat clusters (Zhang et al. 2014). Another study observed lower TSS in naturally shaded compared to exposed Pinot noir bunches (Song et al. 2015). The small differences in berry weights and lack of difference in TSS in the current study suggest that cluster enclosure has a minimal effect on grape maturity. Thus, any changes in berry composition were likely to be due to sunlight exclusion rather than maturity.
A higher TA was only observed in clusters enclosed from veraison to intermediate-ripe ($T_i$), but not in other sunlight exclusion treatments. A slight increase in pH was noted in clusters enclosed for the entire duration from pea-size ($T_w$), while decreased pH was observed in clusters enclosed from intermediate-ripe to harvest ($T_h$). Previous studies have reported increased TA in all shaded clusters in Muscat, regardless of the time of shading (Zhang et al. 2014). An increased TA was also observed in samples from the north side of rows exposed to less sunlight in Cabernet Sauvignon (Bergqvist et al. 2001). However, no TA differences have been observed between shaded and exposed Merlot and Jingxiu grapes, although a slight decrease in TA was observed in shaded Pinot noir (Cortell and Kennedy 2006; Li et al. 2013; Spayd et al. 2002). For Grenache, grapes from the north side of rows with less sunlight had higher TA and pH than those from the south side, which had low PAR, but an inverse relationship was observed at high PAR (Bergqvist et al. 2001). In addition, a lower pH was observed in naturally-shaded bunches and grapes from the north side of rows receiving less sunlight in Cabernet Sauvignon and Pinot noir (Bergqvist et al. 2001; Song et al. 2015). The influences of sunlight exclusion on grape pH and TA are clearly not constant between *Vitis vinifera* cultivars. However, it is possible that the pH and TA differences observed here were due to sample variability. The influence of sunlight on berry pH and TA in Shiraz may also vary at different PAR levels, similar to the changes seen in Grenache and Cabernet Sauvignon (Bergqvist et al. 2001).

There were no statistically significant differences in total anthocyanins and phenolics between control and sunlight-exclusion treatments (Table 4.1).
Previous studies have reported higher anthocyanin levels in naturally-shaded Shiraz clusters compared to exposed clusters (Haselgrove et al. 2000), consistent with another study in which higher anthocyanins were found in sunlight-excluded clusters from fruit set to pre-veraison in Jingxiu grapes (Li et al. 2013). Higher total anthocyanins and phenolics were also observed in grapes from the north side of rows receiving less sunlight in Grenache and Cabernet Sauvignon (Bergqvist et al. 2001). In contrast, other studies have reported lower total anthocyanin levels in shaded clusters at harvest in Pinot Noir, Shiraz, and Merlot (Cortell and Kennedy 2006; Downey et al. 2004; Spayd et al. 2002). It is known that high temperatures degrade anthocyanins and reduce total anthocyanin accumulation in grapes (Mori et al. 2007; Yamane et al. 2006). Shading treatments effectively removed direct solar irradiation of grape bunches and, therefore, prevented high surface temperatures of grape berries. The influence of shading on bunch zone temperatures might be more pronounced in warmer climate wine regions due to the higher chance of heat waves, and might explain the observed increase in anthocyanins seen in some previous studies. However, a recent study showed that grape bunches shaded from UV resulted in significantly lower total anthocyanin and phenolic levels in the Pinot noir wine (Song et al. 2015). Therefore, the influence of shading on grape anthocyanins and phenolics may result from the balance between the temperature-reducing effects and UV-removing effects of shade.

4.3.3. Differences in rotundone and α-guaiene

The differences in rotundone concentration between experimental groups were compared using two scales, rotundone per kilogram grapes and rotundone per
100 grape berries, the results of which were similar (Fig. 4.3a). The highest concentrations of rotundone (273.2 ng/kg, 26.9 ng/100 berries) were observed in Ctrl, followed by Tₐ (192.9 ng/kg, 17.6 ng/100 berries) and Tᵢ (186.2 ng/kg, 17.0 ng/100 berries), which were significantly higher than Tₕ (91.2 ng/kg, 8.1 ng/100 berries) and Tₜ (26.0 ng/kg, 2.3 ng/100 berries). Tₜ had the lowest concentration of rotundone of all the groups studied.

Fig. 4.3. Comparison of different shading treatment groups in (a) rotundone concentration and (b) α-guaiene concentration in the 2013-14 growing season. (a) Grape rotundone concentration was calculated as (1) nanogram per kilogram grapes and (2) nanogram per 100 grape berries. Same statistical differences between treatment groups were observed using two scales, and a, b, c, d were used to label significant differences (p<0.05) between experimental groups. (b) Grape α-guaiene concentration was calculated as (1) nanogram α-copaene equivalents per kilogram grapes, and a, b was used to label significant
differences ($p<0.05$) between experimental groups; (2) nanogram $\alpha$-copaene equivalents per 100 grape berries, and A, B was used to label significant differences ($p<0.05$) between experimental groups.

Significantly lower rotundone concentrations were observed in all sunlight-removal treatments, indicating that an absence of sunlight effectively reduced rotundone concentrations in grapes at harvest. Sunlight removal from pea-size ($T_w$) dramatically reduced rotundone concentrations in grapes at harvest to less than 1/10 of that of natural shade (Ctrl) grapes. This is consistent with our previous findings of large rotundone concentration variability between different vintages, in line with the large solar exposure variations that exist between different growing seasons (Zhang et al. 2015a; Zhang et al. 2015c). Even though shading at all three grape development stages ($T_v$, $T_i$, and $T_h$) reduced rotundone levels at harvest, sunlight removal from intermediate veraison to harvest ($T_h$) reduced concentrations more effectively than at other stages. This may be due to the timing of rotundone production in grapes, which mainly accumulates a few weeks prior to harvest in Shiraz (Zhang et al. 2015b; Zhang et al. 2015c). We have previously shown that rotundone concentrations in wine of different vintages were negatively correlated with mean daily solar exposure from veraison to harvest. This suggests that lower levels of sunlight radiation exposure should increase rotundone concentrations in grapes, since wine rotundone originates from the grape berries (Siebert and Solomon 2011), consistent with the current results. The impact of sunlight exposure on grapes can be classified as direct (illumination effect) or indirect (solar radiation-induced temperature effect). Lower grape surface and air temperatures at the bunch zone have been associated with higher rotundone concentrations in
grapes (Zhang et al. 2015a). Here, the shade box slightly reduced air temperatures at the bunch zone (Fig. 4.2), which may either slightly increase rotundone concentrations or have no impact. Therefore, the lower rotundone concentrations observed in sunlight-excluded groups was likely to be due to the removal of illumination rather than a temperature effect. Sunlight illumination appears to be important for rotundone production in grapes, and the influence of sunlight exclusion depends on a balance between its temperature-reducing and illumination-removal effects.

As a component of sunlight illumination, UV radiation may contribute to the positive effects of sunlight on rotundone production in grapes. Previous studies have shown that UV-B radiation can influence abscisic acid, salicylic acid, jasmonic acid, and ethylene concentrations in plants, which play a central role in the control of plant immune response gene expression, especially terpenoid synthesis (Bassman 2004; Pieterse et al. 2012). Increases in the concentrations of some monoterpenes, diterpenes, a sesquiterpene (E-nerolidol), and terpene synthase activity were observed in Malbec leaves in response to UV-B radiation (Gil et al. 2012). Enhanced UV-B radiation also increased the concentration of some monoterpenes in intermediate-ripe Malbec grape berries (Gil et al. 2013). More recently, lower concentrations of two monoterpenes, Nerol and Geraniol, were observed in Pinot noir wine made from UV-shaded clusters compared to those made from unshaded clusters (Song et al. 2015). Therefore, it is possible that UV radiation may be the critical component of light that modulates rotundone concentration in grapes. This warrants further investigation.
The differences in α-guaiene concentrations between experimental groups were also compared using two scales: nanogram of α-copaene equivalents per kilogram grapes and per 100 grape berries, which produced slightly different results (Fig. 4.3b). Significantly lower α-guaiene concentrations were observed in all shading treatments (T_w: 328.7 ng α-copaene equivalents/kg; T_v: 364.5 ng α-copaene equivalents/kg; T_i: 264.8 ng α-copaene equivalents/kg; p<0.05) except T_h (410.7 ng α-copaene equivalents/kg) compared to Ctrl (519.4 ng α-copaene equivalents/kg). However, when berry weights were considered, the Ctrl group (51.4 ng α-copaene equivalents/100berries) had significantly higher berry weights compared to the shading treatments (T_w: 27.3 ng α-copaene equivalents/100berries; T_v: 33.1 ng α-copaene equivalents/100berries; T_i: 23.8 ng α-copaene equivalents/100berries; T_h: 37.9 ng α-copaene equivalents/100berries; p<0.05).

Overall, lower α-guaiene concentrations were observed in all sunlight-exclusion treatments, indicating that sunlight removal negatively affects grape α-guaiene concentrations at harvest. Similar to rotundone, which mainly accumulates a few weeks before harvest in Duras and Shiraz (Geffroy et al. 2014; Zhang et al. 2015c), α-guaiene was observed from four weeks after mid-veraison until post-harvest in *Vitis vinifera* L. cv. ‘Baga’ grapes (Coelho et al. 2006). However, we previously detected α-guaiene at all stages of grape ripening from pea-size in Shiraz (Zhang et al. 2015b). Thus, sunlight exclusion from bunches at different grape development stages had a similar impact on α-guaiene concentration. The impact of temperature on α-guaiene accumulation in grapes remains
unclear. However, temperatures higher than 40°C have been reported to accelerate the aerial oxidation of α-guaiene to rotundone (Huang et al. 2014). The temperature differences inside and outside the box were less than 0.5°C (Fig. 4.2), which was unlikely to have affected the oxidation of α-guaiene to rotundone. Similar to rotundone, UV radiation can modify terpenoid biosynthesis and affect α-guaiene production in grapes (Bassman 2004; Pieterse et al. 2012). Here, sunlight exclusion at intermediate-ripe to harvest (T_h) resulted in significantly lower rotundone levels and a tendency to higher α-guaiene levels in bunches compared to sunlight exclusion at other periods (T_v and T_i). It is highly likely that the α-guaiene to rotundone biosynthetic pathway is light/UV sensitive; therefore, sunlight exclusion might suspend or slow down this pathway, leaving more α-guaiene in T_h grapes. Studying the influence of sunlight on α-guaiene production is important for understanding rotundone biosynthesis and is worthy of further investigation.

4.3.4. Grape terpenoid analysis

This study investigated 41 known grape sesquiterpenes based on a commercial terpenoid library (Dr Hochmuth Scientific Consulting, Hamburg, Germany). Nineteen sesquiterpenes (including rotundone and α-guaiene), three norisoprenoids, and one monoterpene were detected in the grape samples (Table 4.2). T_w and T_i treatments tended to result in less total sesquiterpenes and terpenoids compared to controls. Although there were no statistically significant differences in total volatile compounds, total sesquiterpenes, or total terpenoids between the three treatment groups, most likely due to variability between experimental vines, the results nevertheless suggest the importance of
sunlight on total terpenoid and sesquiterpene production. epi-Zonarene, α-ylangene, theaspirane isomer 1, and γ-cadinene were the most abundant terpenoids, accounting for 15.71%, 14.57%, 8.46%, and 8.19% of the total terpenoids identified, respectively. This was consistent with a previous study that reported high α-ylangene, epi-zonarene, theaspirane isomer 1, and γ-cadinene concentrations in Shiraz at harvest (May and Wüst 2012; Zhang et al. 2015b). May and Wüst (2012) also reported the presence of (E, E)-α-farnesene, germacrene D, β-bourbonene, guaia-6,9-diene, α-humulene, and calamenene. However, only β-bourbonene, guaia-6,9-diene, α-humulene, and calamenene were detected in the current study. All terpenoids reported in Zhang et al. (2015b) at commercial harvest were observed in this study except 1,8-cineole and geranyl acetone.

Statistically significant differences in eight sesquiterpenes other than rotundone and α-guaiene were observed between sunlight-exclusion treatments. There was significantly less geraniol in T_h than T_w and T_v, but not between Ctrl and sunlight-exclusion treatments. A previous study reported that sunlight exclusion for five weeks until harvest significantly reduced total monoterpenes, with geraniol one of the five major monoterpenes detected (Skinkis et al. 2010). However, whether the total monoterpenes concentration changes were due to geraniol or other monoterpenes was not specified. Geraniol concentrations continuously decreased from early berry development until harvest. Therefore, sunlight exclusion may delay geraniol degradation during berry development.
Sunlight exclusion significantly increased clovene concentrations in grapes at harvest in all treatments compared to control (Table 4.2), suggesting that sunlight might impede clovene production, which is usually detected throughout berry development from pea-size until harvest (Zhang et al. 2015b). These are the first studies to report clovene production in Shiraz grapes. α-Humulene is produced in a similar biosynthetic pathway to clovene (Chappell and Coates 2010; Davis and Croteau 2000), and significantly lower α-humulene concentrations were observed in T_i compared to Ctrl, T_v, and T_h, suggesting that α-humulene production is sunlight sensitive (Table 4.2). Our previous research has shown that the α-humulene concentration continues to decrease from early berry development onwards (Zhang et al. 2015b). The current results indicate that shading at veraison may stimulate α-humulene degradation. Finally, T_h grapes contained significantly lower concentrations of β-bourbonene compared to Ctrl, T_v, and T_i, while no β-bourbonene was detected in T_w (Table 4.2); thus, only sunlight exclusion from intermediate-ripe to harvest influenced β-bourbonene concentrations in grapes at harvest. This is consistent with other studies showing that β-bourbonene is only produced after intermediate-ripe in ‘Baga’ and Shiraz (Coelho et al. 2006; Zhang et al. 2015b).
<table>
<thead>
<tr>
<th>Terpenes compounds and classes</th>
<th>Chemical structures</th>
<th>Concentration (µg α-copaene equivalents/kg) in different shading treatment groups</th>
<th>Percentage of individual terpenes to total terpenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ctrl</td>
<td>$T_w$</td>
</tr>
<tr>
<td><strong>Monoterpenes</strong></td>
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<tr>
<td>1,8-Cineole</td>
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<td>1.50±0.42</td>
<td>1.52±0.65</td>
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<tr>
<td>Geraniol</td>
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<td>5.85±1.45ab</td>
<td>7.44±1.09a</td>
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<tr>
<td><strong>Norisoprenoids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theaspirane isomer A</td>
<td><img src="image" alt="Theaspirane isomer A" /></td>
<td>9.53±1.77</td>
<td>10.51±3.18</td>
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<tr>
<td>Theaspirane isomer B</td>
<td><img src="image" alt="Theaspirane isomer B" /></td>
<td>1.54±0.24</td>
<td>2.04±0.63</td>
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<td>β-Ionone</td>
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<td>3.73±1.48</td>
<td>4.05±1.49</td>
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<td><strong>Sesquiterpenes</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Clovene</td>
<td><img src="image" alt="Clovene" /></td>
<td>0.12±0.09a</td>
<td>0.38±0.21b</td>
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<tr>
<td>α-Ylangene</td>
<td><img src="image" alt="α-Ylangene" /></td>
<td>20.19±6.66</td>
<td>13.80±7.18</td>
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Table 4.2. (Continued)

<table>
<thead>
<tr>
<th>Chemical structures</th>
<th>Concentration (µg α-copaene equivalents/kg) in different shading treatment groups</th>
<th>Percentage of individual terpenes to total terpenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>T&lt;sub&gt;w&lt;/sub&gt;</td>
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**Sesquiterpenes**

<table>
<thead>
<tr>
<th>Terpenes compounds and classes</th>
<th>H</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Bourbonene</strong></td>
<td>1.99±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>β-Copaene</strong></td>
<td>4.42±1.45</td>
<td>3.44±1.67</td>
</tr>
<tr>
<td><strong>Guaia-6,9-diene</strong></td>
<td>5.77±2.05</td>
<td>4.76±3.00</td>
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<tr>
<td><strong>Selina-4(15),6-diene</strong></td>
<td>7.04±2.16</td>
<td>5.65±2.99</td>
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<tr>
<td><strong>α-Humulene</strong></td>
<td>5.90±1.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.87±1.77&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td><strong>cis-muurola-4(15),5-diene</strong></td>
<td>2.49±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.16±1.32&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Terpenes compounds and classes</td>
<td>Chemical structures</td>
<td>Concentration (µg α-copaene equivalents/kg) in different shading treatment groups</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ctrl</td>
</tr>
<tr>
<td><strong>Sesquiterpenes</strong></td>
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<tr>
<td>γ-Murolene</td>
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<tr>
<td>δ-Selinene</td>
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<td>3.82%</td>
</tr>
<tr>
<td>epo-Zonarene</td>
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<td>15.99%</td>
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<tr>
<td>γ-Cadinene</td>
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<td>8.62%</td>
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<tr>
<td>δ-Cadinene</td>
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<td>6.14±2.22</td>
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<td></td>
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<td>4.63%</td>
</tr>
<tr>
<td>Cis/trans-Calamenene</td>
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<td>3.74±1.52</td>
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<tr>
<td></td>
<td></td>
<td>2.82%</td>
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Table 4.2. (Continued)

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<tr>
<th>Terpenes compounds and classes</th>
<th>Chemical structures</th>
<th>Concentration (µg α-copaene equivalents/kg) in different shading treatment groups</th>
<th>Percentage of individual terpenes to total terpenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ctrl</td>
<td>T_w</td>
</tr>
<tr>
<td><strong>Sesquiterpenes</strong></td>
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<td>7-epi-α-Selinene</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>4.09±1.51\textbf{ab}</td>
<td>3.43±2.57\textbf{a}</td>
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<tr>
<td>α-Cadinene</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>3.74±1.39</td>
<td>2.87±1.56</td>
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<td>α-Calacorene</td>
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<td>2.51±1.72</td>
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<td><strong>Total sesquiterpenes</strong></td>
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<td>110.14±37.58</td>
<td>76.16±41.10</td>
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<td><strong>Total terpenes</strong></td>
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<td>131.01±39.18</td>
<td>98.86±47.69</td>
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<td><strong>Total volatile compounds</strong></td>
<td></td>
<td>746.52±141.82</td>
<td>807.10±180.87</td>
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</tbody>
</table>

\(^{a}\)Different letters in the column represent significantly different means ± standard error (p<0.05). \(^{b}\)Not detected. \(^{c}\)The compound was detected only in 3 replicates.
cis-Muurola-4(15),5-diene and cis/trans-calamanene are both downstream products of δ-cadinene (Bülow and König 2000; Chappell and Coates 2010; Davis and Croteau 2000). δ-Cadinene and cis/trans-calamanene concentrations tended to be lower in T_i and T_w compared to control (Table 4.2). Significantly more cis-muurola-4(15),5-diene was observed in T_h, but not other shade treatments, compared to controls. δ-Cadinene and calamanene concentrations have been shown to decrease from early berry development onwards (Zhang et al. 2015b), and the current results indicate that shading near veraison accelerates this process. Shading increased cis-muurola-4(15),5-diene during the last two weeks prior to harvest (Table 4.2), while most δ-cadinene-derived compounds decreased to their minimum by harvest (Zhang et al. 2015b); it is, therefore, possible that shading during late ripening may activate or upregulate an alternative cis-muurola-4(15),5-diene biosynthetic pathway. 7-epi-α-selinene tended to be high at T_h compared to all other treatments, which may be due to the biosynthesis time of this compound, which has been demonstrated to only appear in grapes after intermediate ripeness (Zhang et al. 2015b).
4.4 Conclusion

Sunlight exclusion at three berry development stages slightly modified berry weight, pH, and TA, but had no effect on TSS, total anthocyanins, or total phenolics in grape berries at harvest. There were significant reductions in rotundone and $\alpha$-guaiene in grapes at harvest in sunlight-exclusion treatments at all three berry development stages. Rotundone and $\alpha$-guaiene concentrations in grape berries are sensitive to sunlight illumination, and UV radiation may be the most important mediator of their production. Sunlight exclusion also modified the concentration of other terpenoids, mainly sesquiterpenes, in grapes. The influence of sunlight on terpenoid concentrations varied between different berry development stages, which may be related to their specific production or degradation during berry development.

Acknowledgements

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4.5 References


4.6 Supplementary data

Table S-4.1. Weather record of the vineyard in 2013-14 growing seasons (Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085 – located 15.5kms NW to the experimental site). The dates of pea size grape, 80% veraison, intermediate ripe and harvest are taken at 23\textsuperscript{rd} Jan, 28\textsuperscript{th} Feb, 31\textsuperscript{st} Mar and 17\textsuperscript{th} Apr 2014, respectively.

<table>
<thead>
<tr>
<th>Weather record</th>
<th>2013-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean January Temperature</td>
<td>21.7°C</td>
</tr>
<tr>
<td>Mean January Daily Solar Exposure</td>
<td>27.0 MJ/m(^2)</td>
</tr>
<tr>
<td>Mean Daily Solar Exposure from pea-size grape to harvest</td>
<td>18.8 MJ/m(^2)</td>
</tr>
<tr>
<td>Mean Daily Solar Exposure from pea-size grape to 80% veraison</td>
<td>24.6 MJ/m(^2)</td>
</tr>
<tr>
<td>Mean Daily Solar Exposure from 80% veraison to intermediate ripe</td>
<td>16.4 MJ/m(^2)</td>
</tr>
<tr>
<td>Mean Daily Solar Exposure from intermediate ripe to harvest</td>
<td>11.3 MJ/m(^2)</td>
</tr>
<tr>
<td>Total rainfall from Oct to Harvest</td>
<td>140.9 millimeter</td>
</tr>
<tr>
<td>Total irrigation From Oct to harvest</td>
<td>60.8 millimeter</td>
</tr>
<tr>
<td>Total water precipitation (October to Harvest)</td>
<td>201.7 millimeter</td>
</tr>
</tbody>
</table>
CHAPTER FIVE
Terpene evolution during the development of Vitis vinifera
L. cv. Shiraz grapes

Abstract
Grape berry terpene concentrations may vary at different stages of berry development. This study aimed to investigate terpene evolution in grape berries from four weeks post-flowering (4 wpf) to maturity (17 wpf). Grape bunches were sampled at fortnightly intervals over two vintages (2012-13 and 2013-14). In total, five monoterpenes, 24 sesquiterpenes, and four norisoprenoids were detected in grape samples. The highest concentrations of total monoterpenes, total sesquiterpenes, and total norisoprenoids in grapes were all observed at pre-veraison. Terpenes derived from the same biosynthetic pathway had a similar production pattern during berry development. Terpenes in grapes at harvest might not necessarily be synthesised at post-veraison, since the compounds or their precursors may already exist in grapes at pre-veraison, with the veraison to harvest period functioning to convert these precursors into final products.

Keywords: rotundone, terpene, sesquiterpene, grape ripening

Highlights
- Pre-veraison berries contain the highest total terpenoid concentrations
- Berries at different developmental stages have different terpene profiles
- Terpene biosynthesis pathways dictate production patterns during berry development
- Rotundone was present in Shiraz grapes at both pre-veraison and post-veraison
5.1. Introduction

Grape berries contain hundreds of compounds that contribute to wine flavour and aroma. Most of these grape compounds exist as odourless, glycosidically-bound forms that are hydrolysed into active aroma compounds during fermentation or storage (Hjelmeland and Ebeler 2015). However, some compounds in grapes, such as certain terpenes, may undergo minimal or no alteration and are directly linked to wine aroma (Dunlevy et al. 2009). The majority of identified terpenes that contribute to grape and wine flavour and aroma belong to the monoterpene sub-family, and only one sesquiterpene sub-family member is reported to be important for wine aroma and flavour (Dunlevy et al. 2009). The oxygenated bicyclic sesquiterpene, named rotundone, gives grapes and wine a ‘black pepper’ character (Wood et al. 2008) and is stylistically important to the ‘terroir’ of some cool climate Australian wine regions (Herderich et al. 2012). Rotundone can be formed by aerial oxidation of a precursor compound, α-guaiene (Huang et al. 2014). Rotundone’s discovery has directed attention to the importance of sesquiterpene flavours, and there is only limited research on sesquiterpene production in grapes.

Grape terpene production varies at different berry developmental stages: pre-veraison, the lag phase, and post-veraison (Kalua and Boss 2009). In west Victoria (Australia), Shiraz grapevines start their annual cycle around late September/October, when dormant vines undergo bud burst and start to grow. In this region, Shiraz fruit set typically occurs from early to middle December, when berries experience a period of rapid cell division and slight cell expansion (pre-veraison) before entering the lag phase of minimal berry growth during early January. At the end of the lag phase (late January to early February in this region), berries go
through veraison with cell expansion, berry softening, and colour change (Gladstones 2004). For Shiraz, harvest ripeness typically occurs from the middle of March to early April in this region, but usually in middle to late April in our study vineyard (Zhang et al. 2015b; Zhang et al. 2015a). The post-veraison to harvest period is critical for quality grape production, since grape berries rapidly accumulate sugar, change colour, shift metabolism, and accumulate flavour compounds (Gladstones 2004).

Most previous studies on the development period-specific evolution of grape volatiles and terpenes have focused on the post-veraison to harvest period. A previous study reported that the total monoterpane concentration reaches its maximum at intermediate-ripe at 6-8° Baumé in Airen, and at around 11° Baumé in Chardonnay and Macabeo (García et al. 2003). However, maximum norisoprenoid and monoterpane concentrations occur at harvest in Pinot noir grapes (Fang and Qian 2006). In Vitis vinifera L. cv ‘Fernão-Pires’ grapes, 16 monoterpenes and two norisoprenoids were detected in grapes from veraison to harvest, which reached maximum concentrations at intermediate-ripe (Coelho et al. 2007). Another study reported that bicylic sesquiterpene concentrations increased from half-veraison to harvest in Riesling, Lemberger, Shiraz, and Yellow Muscat, while acyclic sesquiterpene concentrations decreased (May and Wüst 2012). However, only 13 sesquiterpenes were identified in this study, with nine observed in Shiraz. A more comprehensive study on sesquiterpene composition in Vitis vinifera L. cv. Baga found that most sesquiterpenes, including α-guaiene, continuously increased from intermediate-ripe to post-maturation (Coelho et al. 2006). In Cabernet Sauvignon, two monoterpenes and seven sesquiterpenes were identified at pre-veraison, but no
Terpenoids were observed at veraison and post-veraison (Kalua and Boss 2009, 2010). The same study reported monoterpenes and sesquiterpenes in Riesling grapes at both pre-veraison and post-veraison, but not at veraison. Studies that have investigated rotundone accumulation in grapes after veraison demonstrated that it mainly accumulates after intermediate ripeness (Geffroy et al. 2014; Zhang et al. 2015b). An understanding of sesquiterpene accumulation during berry development, especially prior to veraison, is lacking, and a detailed study on sesquiterpene evolution throughout the berry development stages is required.

Here, we fill this information gap and explore terpene evolution, especially the less well-studied but important sesquiterpenes, in grapes. Terpene profiles in Shiraz grapes were measured from early berry formation to late ripening. These data are useful for manipulation of compound production, such as rotundone, to improve wine aroma.

5.2. Materials and methods

5.2.1. Chemicals

Rotundone \( ((3S,5R,8S)-3,4,5,6,7,8\text{-hexahydro-3,8\text{-dimethyl-5-(prop-1-en-2-yl)}-1(2H)}\text{-azulenone}) \) and \( ^2\text{H}_5\text{-rotundone} \) were synthesised as previously described (Siebert et al. 2008; Wood et al. 2008). A reference standard of \( \alpha\text{-copaene} \) was supplied by Sigma-Aldrich (Castile Hill, NSW, Australia). Working solutions of standards were prepared volumetrically in ethanol and stored at 4°C until required. High-performance liquid chromatography (HPLC)-grade ethyl acetate, \( n\text{-pentane} \), methanol, and ethanol were obtained from Rowe Scientific (Doveton, Vic, Australia). Analytical-grade potassium L-tartrate monobasic, tartaric acid, and other chemicals
were obtained from Sigma-Aldrich. Water was purified using the Milli-Q system (Millipore Australia, Bayswater, Victoria, Australia).

5.2.2. Vineyard site

The study was conducted in a commercial vineyard (The Old Block, Mount Langi Ghiran 37.31°S, 143.15°E) located in the Grampians wine region of Victoria, Australia. The vineyard is approximately 15.5 km east of the nearest Bureau of Meteorology (BOM) weather station at Ararat Prison (Australian BOM Station No. 089085). The long-term mean January temperature (MJT) recorded at this weather station is 19.1°C, with annual average rainfall of 584.2 mm by February 2015: it is classified as a cool climate wine region (Gladstones 2004). The MJT and total rainfall from October to harvest for the two studied growing seasons (2012-13 and 2013-14) were 20.0°C, 124.1 mm and 21.7°C, 140.9 mm, respectively (Table S-5.1). The vineyard was planted in 1968 with *Vitis vinifera*, cv. Shiraz on its own roots, 3.0 m between rows and 1.8 m between vines, with rows oriented northeast to southwest. Grapevines were trained to a vertical shoot positioned (VSP) trellis, and a dripping irrigation system was installed along vineyard rows with a dripper spacing of 0.5 m and dripper output of 1.5 litres per hour. Grapevines were irrigated when required at a rate of 5.76 L/(hr-vine). The total irrigation volumes from October to harvest in the two studied growing seasons (2012-13 and 2013-14) were 84.3 mm and 60.8 mm, respectively. No significant pest or disease pressure was observed during the experimental seasons.

5.2.3. Evaluation of rotundone evolution during grape development
Shiraz grapes were sampled in triplicate (n=30 random sampling points, 2 kg per field replicate) at fortnightly intervals from four weeks post-flowering (wpf) until commercial harvest in two consecutive growing seasons (2012-13 and 2013-14). The wpf was counted from the time of a minimum of 80% cap-fall, grapevine growth stage E-L 25-26 (Pearce and Coombe 2004). Grape bunch samples were randomly collected from different grapevines across the vineyard (n>30 grapevines) on each sampling date. In the 2013-14 growing season, samples were also collected at 80% cap-fall (E-L25) on 11 December 2013. Final sampling was conducted at the beginning of commercial harvest on 10 April 2013 and 8 April 2014, respectively. Commercial harvest was performed between 9 and 16 April 2013 and between 8 and 17 April 2014 with selective hand harvest first followed by machine harvest at later dates. Grape samples were collected in zip-lock plastic bags, frozen at -20°C, transferred to the laboratory in Styrofoam boxes on dry ice, and stored at -20°C before analysis. Total soluble solids (TSS), titratable acidity (TA), and the pH of bunch samples were analysed using a refractometer, alkaline titration, and a pH meter, respectively, following published protocols (Iland 2004).

5.2.4. Preparation of samples and solid-phase microextraction-gas chromatography-multidimensional mass spectrometry (SPME-MDGC-MS) rotundone analysis
Grape samples were prepared for rotundone analysis based on a published protocol (Geffroy et al. 2014). For each grape sample, 100 g of de-stemmed grapes were sub-sampled before being homogenised with a hand-held blender. Sub-samples were centrifuged to separate solids and liquids. The solids were mixed with 30 ml ethanol, 30 ml water, and 100 μl d5-rotundone (516 ng/ml in ethanol) as internal standard, then shaken for 24 h at 22°C and sonicated before reintroducing the juice.
Sub-samples were then centrifuged and filtered (1.6 μm glass fibre) to obtain berry extract filtrate, which was topped up to 200 ml with deionised water prior to solid phase extraction (SPE) performed as reported previously (Siebert et al. 2008). The SPE residue supernatant was air dried with nitrogen and reconstituted in 0.5 ml ethanol and 9 ml deionised water. The samples were then analysed by SPME-GC-MS in the Australian Wine Research Institute (AWRI) following established protocols (Geffroy et al. 2014).

5.2.5. Sesquiterpene analysis

Sesquiterpene analysis was based on a published protocol (Parker et al. 2007) with the following modifications. An Agilent Technologies 6890 gas chromatograph (GC; Agilent Technologies, Santa Clara, CA) was equipped with a Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5973 mass selective detector (MSD). The instruments were controlled using Agilent G1701EA MSD ChemStation software in conjunction with Gerstel Maestro software (version 1.4.20.0). The GC was fitted with a J&W DB-5ms capillary column measuring approximately 30 m × 0.25 mm, 0.25 μm film df. The carrier gas was helium (ultrahigh purity, BOC, Adelaide, SA, Australia), and the flow rate was 1.0 ml/min in constant flow mode. The GC inlet was fitted with a resilanised borosilicate glass SPME inlet liner (Supelco, 6.5 mm o.d., 0.75mm i.d., 78.5 mm long) held at 220°C.

The SPME fibre was desorbed in the pulsed splitless mode and the splitter, at 50:1, was opened after 30 s. The fibre was allowed to bake in the inlet for 10 min. The oven was started at 50°C, held for 1 min, increased to 230°C at 3°C/min, then increased to 280°C at 20°C/min and held at 280°C for 5 min. The MS transfer line
was held at 250°C. The temperatures of the MS source and quadrupole were 230°C and 150°C, respectively. The MS was operated in positive EI mode at 70 eV with simultaneous selected ion monitoring (SIM) and scanning over a mass acquisition range of 35-280 m/z.

100 g of de-stemmed grapes were sub-sampled before being homogenised using a hand-held blender. 5 g of homogenised sample was transferred into a HS-SPME vial (Agilent Technologies, 20ml) and mixed with 500 μL α-copaene (200.64 μg/L in ethanol) as internal standard. The samples were then shaken for 24 h at 22°C before adding 2 ml saturated brine and being subjected to SPME-GC-MS analysis. The vial and its contents were heated to 45°C. A polydimethylsiloxane/divinylbenzene (PDMS/DVB, Agilent) 65μm SPME fibre was exposed to the headspace for 60 min with agitation. Sesquiterpenes were identified by comparing the mass spectra and retention indices with the terpenoids library in MassFinder (version 4.1, Dr Hochmuth Scientific Consulting, Hamburg, Germany). All compounds except α-guaiene were quantified as α-copaene equivalents. α-guaiene was determined by SIM with α-copaene as internal standard; the ions monitored were: m/z 105, 133, 147, 161, and 204; dwell time 25ms each. The target ions were typically m/z 147 for α-guaiene and 161 for α-copaene with ions 105, 133, and 204 m/z used as qualifiers. Data were analysed using Agilent G1701DA MSD ChemStation software. α-guaiene was expressed as the ratio of m/z 147:m/z 161 multiplied by the concentration of α-copaene internal standard. The assay precision was validated by a series of standard additions of internal standard as described previously (Parker et al. 2007). Blank SPME runs and blank internal standards were checked regularly.
5.2.6. Statistical analysis

TSS, TA, pH, and terpenoids in grape samples from different berry developmental stages were compared using one-way analysis of variance (ANOVA) at $p<0.05$ (CoStat, version 6.4, CoHort Software, Monterey, USA). Terpenoid concentrations at different berry developmental stages were analysed by discriminant analysis using SPSS v.21 (SPSS Inc., Chicago, IL. USA).

5.3. Results and discussion

5.3.1. Berry development pattern differentiation

Different grape berry developmental stages have previously been characterised using grape berry weight, °Brix, pH, and titratable acidity measurements (Table 5.1) (Coombe and Iland 2004; Kalua and Boss 2009). In this study, veraison occurred between 10 and 11 wpf in both growing seasons. The 2013-14 harvest was brought forward due to heavy rain forecast in late April 2014 (harvest at 17 wpf) and, as a result, the grapes were less ripe than in the 2012-13 growing season (harvest at 18wpf). As expected, °Brix and pH were lower and TA was higher in 2013-14 grapes. Initial measurements showed that there were fewer terpenes in grape samples at veraison compared to both pre- and post-veraison (Fig. 5.1).
Table 5.1.

<table>
<thead>
<tr>
<th>wpf</th>
<th>Berry mass (g)</th>
<th>TSS(^b) (°Brix)</th>
<th>pH</th>
<th>TA(^d) (g/L)</th>
<th>Phenological stages(^e)</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.27</td>
<td>6.0±0.2(^a)</td>
<td>2.37±0.02(^a)</td>
<td>31.90±0.80(^a)</td>
<td>E-L 31</td>
<td>Green pea size berries</td>
</tr>
<tr>
<td>6</td>
<td>0.40</td>
<td>5.4±0.2(^a)</td>
<td>2.39±0.01(^a)</td>
<td>33.58±0.95(^b)</td>
<td>E-L 32</td>
<td>Green pea size berries</td>
</tr>
<tr>
<td>8</td>
<td>0.45</td>
<td>5.8±0.0(^a)</td>
<td>2.52±0.01(^b)</td>
<td>34.40±0.45(^b)</td>
<td>EL 33-34</td>
<td>Most still hard and green</td>
</tr>
<tr>
<td>11</td>
<td>0.98</td>
<td>14.5±0.2(^b)</td>
<td>3.05±0.03(^c)</td>
<td>10.38±0.47(^c)</td>
<td>E-L 35-36</td>
<td>80% veraison</td>
</tr>
<tr>
<td>13</td>
<td>1.02</td>
<td>20.1±0.3(^c)</td>
<td>3.40±0.02(^d)</td>
<td>5.51±0.30(^d)</td>
<td>E-L 36</td>
<td>Light purple berries</td>
</tr>
<tr>
<td>14</td>
<td>1.12</td>
<td>22.1±0.4(^d)</td>
<td>3.73±0.02(^c)</td>
<td>4.05±0.31(^e)</td>
<td>E-L 37</td>
<td>Purple berries</td>
</tr>
<tr>
<td>16</td>
<td>1.01</td>
<td>24.5±0.3(^e)</td>
<td>3.81±0.03(^f)</td>
<td>4.12±0.10(^e)</td>
<td>E-L 37-38</td>
<td>Dark purple berries</td>
</tr>
<tr>
<td>18</td>
<td>0.99</td>
<td>26.3±1.0(^f)</td>
<td>3.90±0.07(^g)</td>
<td>4.00±0.15(^e)</td>
<td>E-L 38</td>
<td>Some berries slightly shrank</td>
</tr>
</tbody>
</table>

The 2013-14 growing season

<table>
<thead>
<tr>
<th>wpf</th>
<th>Berry mass (g)</th>
<th>°Brix</th>
<th>pH</th>
<th>TA (g/L)</th>
<th>Phenological stages</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0049</td>
<td>n/a(^g)</td>
<td>n/a</td>
<td>n/a</td>
<td>E-L 25-26</td>
<td>Flowering 80% cap-fall</td>
</tr>
<tr>
<td>4</td>
<td>0.18</td>
<td>6.3±0.2(^a)</td>
<td>2.82±0.08(^a)</td>
<td>30.58±0.26(^a)</td>
<td>E-L 31</td>
<td>Green pea size berries</td>
</tr>
<tr>
<td>6</td>
<td>0.28</td>
<td>5.3±0.2(^b)</td>
<td>2.76±0.03(^a)</td>
<td>32.45±0.84(^b)</td>
<td>E-L 32</td>
<td>Green pea size berries</td>
</tr>
<tr>
<td>9</td>
<td>0.41</td>
<td>7.1±0.5(^c)</td>
<td>2.90±0.03(^a)</td>
<td>26.70±0.36(^c)</td>
<td>EL 34-35</td>
<td>Begin to colour and enlarge</td>
</tr>
<tr>
<td>11</td>
<td>0.69</td>
<td>15.9±0.7(^d)</td>
<td>3.17±0.10(^b)</td>
<td>11.62±2.33(^d)</td>
<td>E-L 35-36</td>
<td>80% veraison</td>
</tr>
<tr>
<td>13</td>
<td>0.85</td>
<td>19.9±0.2(^e)</td>
<td>3.46±0.05(^c)</td>
<td>6.45±0.52(^e)</td>
<td>E-L 36</td>
<td>Light purple berries</td>
</tr>
<tr>
<td>15</td>
<td>0.95</td>
<td>22.9±0.6(^f)</td>
<td>3.72±0.05(^d)</td>
<td>4.87±0.35(^ef)</td>
<td>E-L 37</td>
<td>Purple berries</td>
</tr>
<tr>
<td>17</td>
<td>0.90</td>
<td>24.9±0.3(^g)</td>
<td>3.74±0.09(^d)</td>
<td>4.20±0.32(^f)</td>
<td>E-L 38</td>
<td>Dark purple berries</td>
</tr>
</tbody>
</table>

\(^a\)Different letters in the column represent significantly (p<0.05) different mean ± standard error (n=3 field replicates). \(^b\)weeks post-flowering. \(^c\)total soluble solid. \(^d\)titratable acidity. \(^e\)E-L system was used to determine phenological stages (Pearce and Coombe 2004). \(^f\)flower samples collected at around 80% cap-fall, composition not compared with grape samples. \(^g\)not applicable.
Fig. 5.1. Chromatograms showing the differences in terpenoids at different berry development stages: (a) pre-veraison, 4 weeks post flowering, E-L 31; (b) 80% veraison, 11 weeks post flowering, E-L 35-36; (c) post-veraison, 17 weeks post flowering, E-L 38. Peaks: (1) Limonene; (2) 1,8-Cineole; (3) Geraniol; (4) Theaspirane isomer A; (5) Theaspirane isomer B; (6) Clovene; (7) α-Ylangene; (8) α-copaene (internal standard); (9) (E)-β-Damascenone; (10) β-Bourbonene; (11) (E)-β-Caryophyllene; (12) β-Copaene; (13) α-Guaiene; (14) Guaia-6,9-diene; (15) Selina-4(15),6-diene; (16) Geranyl acetone; (17) α-Humulene; (18) γ-Muurolene; (19) β-ionone; (20) δ-Selinene; (21) epi-Zonarene; (22) α-Muurolene; (23) γ-Cadinene; (24) δ-Cadinene; (25) Cis/trans-Calamenene; (26) Zonarene; (27) Citronellol; (28) 7-
epi-α-Selinene; (29) ω-Cadinene; (30) α-Cadinene; (31) α-Calacorene; (32) 1-epi-Cubenol; (33) Cubenol

The differences in terpene profiles at different berry developmental stages were analysed using discriminant analysis to identify terpene production patterns and the most important compounds present at each developmental stage. Discriminant analysis biplots (Fig. 5.2) were used to visualise terpene concentration patterns during berry development, and most of the variance (91.2%) was explained by the first two discriminant functions. Grape berries at 4 and 17 wpf were clearly separate from the other developmental stages, while those as 9, 13, and 11 wpf and 6 and 15 wpf clustered into two separate groups. These two clusters were very close to each other. Terpene profiles changed rapidly and markedly after fruit set between 4 and 6 wpf, but only slightly from 6 wpf to peri-veraison (9, 11, and 13 wpf). Terpene profiles changed slightly from veraison to intermediate-ripe (15 wpf), but rapidly towards 17 wpf. Thus, discriminant analysis allowed us to categorise berry developmental stages into early, pre-veraison, near veraison, intermediate-ripe, and harvest, a useful frame of reference for understanding terpene evolution during berry development.
Fig. 5.2. Discriminant analysis biplots illustrating the pattern of terpenoids production at different berry development stages in Shiraz grapes. Numbers in the biplots represent the weeks post-flowering (wpf) for Shiraz grapes in the 2013-14 growing season.

5.3.2. Berry developmental stages and representative compounds

A visual inspection of terpenoid profiles indicated that berries contain different terpenoids at different developmental stages (Fig. 5.1). Grape berries are terpene-rich, and peaks were well separated at 4 wpf (Fig. 5.1a). The number and peak size dropped dramatically towards veraison (11 wpf) (Fig. 5.1b). However, there were more compounds at relatively low abundance at harvest (17 wpf; Fig. 5.1c). It is, therefore, reasonable to explore terpene evolution in grapes based on their developmental stages.
Fig. 5.3. Heat map of terpenoids compounds in grape berries at different grape development stages in the 2013-14 growing season, and heat map legends show the relative concentration range of (a) all terpenoids detected and (b) selected sesquiterpenoids. Number in front of each compound represents the compound number in the Fig. 5.4. The Biosynthesis of cubenol is unclear, therefore number was not assigned to this compound.

5.3.2.1. Early berry developmental stage terpenoid profile (4 wpf)

At the early developmental stage, berries appeared to be very capable of terpene production, as evidenced by the large numbers and high concentrations of terpenoids (Table S-5.2, Fig. 5.3a). Berries at this stage contained the highest concentration of total volatiles and terpenoids compared to all other stages, with the
total terpenoid concentration accounting for 24.22% of the total volatiles detected (Table S-5.2). The highest total monoterpenes were also observed at this stage, with geranyl acetone the most abundant monoterpene. Limonene was only observed at this stage, while 1,8-cineole (eucalyptol) and geraniol concentrations were significantly higher at this stage compared to other stages. The major sesquiterpenes observed at this stage were α-humulene and β-caryophyllene, accounting for 33.88% and 29.95% of the total terpenoids, respectively. This is consistent with a previous study that reported the highest concentrations of these two sesquiterpenes at early berry development in Cabernet Sauvignon (Kalua and Boss 2009). In addition, γ-muurolene, α-muurolene, δ-cadinene, zonarene, ω-cadinene, and α-calacorene concentrations were also significantly higher at this stage than at all other stages. However, in Cabernet Sauvignon, only γ-muurolene was observed at early-stage berry development (Kalua and Boss 2009). Furthermore, epi-cubenol and cubenol were only observed at this stage. Four isoprenoids were observed at this stage and accounted for 8.44% of the total terpenoids.

5.3.2.2. Pre-veraison terpenoid profile (6 wpf)

At this stage, the terpenes were distant from those at 4 wpf in discriminant analysis (Fig. 5.2), indicating that the terpenoid profiles had significantly changed between these two time points. Significant decreases in total volatiles and terpenoid concentrations were observed at this stage (Table S-5.2). The total monoterpenes slightly decreased, while geranyl acetone remained the most abundant monoterpene. All monoterpane concentrations decreased except citronellol, which slightly increased. The total sesquiterpene concentration decreased dramatically to less than 1/6 that of the previous stage, with α-humulene and β-caryophyllene accounting for
the majority of the decrease but remaining the most abundant sesquiterpenes. The concentrations of γ-muurolene, α-muurolene, δ-cadinene, zonarene, ω-cadinene, and α-calacorene all markedly decreased at this stage, while the concentration of α-guaiene slightly increased and calamenene started to appear in the berries. The highest total norisoprenoid concentrations were observed at this stage and accounted for 37.7% of the total terpenoids. Theaspirane isomer A was the most abundant norisoprenoid and accounted for 19.1% of the total terpenoids at this stage. Compared to the previous stage, the concentration of all norisoprenoids increased except β-ionone, which slightly decreased.

5.3.2.3. Veraison terpenoid profile (9-13 wpf)

This developmental stage was characterised by lower concentrations of total volatiles and terpenes (Table S-5.2), suggesting that the berries at this stage might have lost the ability to produce volatile compounds or that previously formed compounds had been converted into non-volatile forms. This was consistent with previous observations in Cabernet Sauvignon, in which the fewest and lowest concentrations of volatiles were observed at similar stages (Kalua and Boss 2009). The 1,8-cineole, geraniol, and geranyl acetone concentrations continued to decrease and reached their lowest concentrations at wpf 11, while citronellol was not detected from wpf 11. Geraniol and geranyl acetone were previously observed at veraison in Vitis vinifera L. cv. Baga grapes (Coelho et al. 2006) and, in the same study, limonene and citronellol were also detected at veraison. Geraniol, geranyl acetone, limonene, and citronellol were also observed in Vitis vinifera L. cv. ‘Fernão-Pires’ grapes at veraison (Coelho et al. 2007). However, no monoterpenes were detected near veraison in Cabernet Sauvignon and Riesling (Kalua and Boss 2009, 2010).
The total sesquiterpene concentration decreased to its lowest level during this period, accounting for only 5.75% of the total terpenoids at wpf 13. β-Caryophyllene was not detected from wpf 11, while clovene re-appeared at wpf 11. The concentrations of α-guaiene, α-humulene, δ-cadinene, and calamenene decreased to very low levels, while γ-muurolene, α-muurolene, and ω-cadinene were not detected. The concentrations of zonarene and α-calacorene continued to decrease to zero by the end of this period. Sesquiterpenes were not detected at veraison in Baga, Cabernet Sauvignon, and Riesling grapes (Coelho et al. 2007; Kalua and Boss 2009, 2010). The norisoprenoid concentration also decreased at this stage, but not as much as monoterpenes and sesquiterpenes. As a result, total norisoprenoids accounted for 71.54% of the total terpenoids at 11 wpf. All four norisoprenoids were also detected in Baga grapes, while only β-damascenone and β-ionone were observed in ‘Fernão-Pires’ grapes at this stage (Coelho et al. 2007; Coelho et al. 2006).

5.3.2.4. Intermediate ripeness terpenoid profile (15 wpf)

At this stage, the total volatile and terpenoid concentrations slightly increased but remained relatively low (Table S-5.2). The concentrations of all three monoterpenes, 1,8-cineole, geraniol, and geranyl acetone, slightly increased. A slight increase in geraniol was also observed in Baga and ‘Fernão-Pires’ grapes at intermediate ripeness (Coelho et al. 2007; Coelho et al. 2006). The sesquiterpene concentrations, including clovene, α-guaiene, α-humulene, δ-cadinene, and calamenene, remained low. α-ylangene and epi-zonarene first appeared at this stage, paralleling a previous report in Baga (Coelho et al. 2006). The total norisoprenoid concentration slightly decreased, with β-damascenone not detected from this stage onwards. However,
relatively higher norisoprenoid concentrations were observed at this stage in Baga and ‘Fernão-Pires’ grapes (Coelho et al. 2007; Coelho et al. 2006).

5.3.2.5. Harvest terpenoid profile (17 wpf)

The terpene profile at harvest was distant from intermediate ripeness in discriminant analysis (Fig. 5.2), indicating that the terpenoid profile significantly changed from 15 to 17 wpf. The total volatile concentration slightly decreased, while total terpenoids increased during this period (Table S-5.2). This increase was mainly due to the sesquiterpenes, since total monoterpenes and norisoprenoids slightly decreased at this stage compared to 15 wpf. This suggests that the late ripening period, rather than the whole post-veraison period, is important for sesquiterpene development at harvest. β-bourbonene, β-copaene, guaia-6,9-diene, selina-4(15),6-diene, δ-selinene, γ-cadinene, 7-epi-α-selinene, and α-cadinene first appeared, while α-calacorene reappeared, at this stage. The concentration of clovene, α-ylangene, α-guaiene, rotundone, and calamenene significantly increased, while α-humulene, epi-zonarene, and δ-cadinene tended to increase. This was consistent with previous observations in Baga grapes, in which all sesquiterpenes were only observed at later stages of ripening (Coelho et al. 2006). In another study in Shiraz, higher α-ylangene, β-bourbonene, γ-cadinene, and α-humulene concentrations were observed at harvest compared to veraison (May and Wüst 2012), although α-humulene remained low in the present study. α-muurolene was observed in Riesling grapes, but not the current study, at harvest (Kalua and Boss 2010).
Fig. 5.4. Biosynthesis of selected monoterpenoids, sesquiterpenoids and norisoprenoids. Arrow line indicates the biosynthesis pathways, while the biosynthesis pathways labelled by dash line may not be activated in the present study. Compounds detected in this study are highlighted in red box.
5.3.3. Terpenoid evolution

All plant terpenoids are biosynthesised from their universal C5 precursor isopentenyl diphosphate (IPP) (1) and its allylic isomer, dimethylallyl pyrophosphate (DMAPP) (2), via the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways (Fig. 5.4a) (A list of compounds for Figure 5.4 is provided after Table S-5.2). All terpenes are synthesised by repetitive addition of C5 isoprenoid units, with isoprene (3) being the smallest compound in this family. (1) and (2) elongate to form geranyl diphosphate (GPP) (4), while farnesyl diphosphate (FPP) (5) and geranylgeranyl diphosphate (GGPP) (6) are the precursors of monoterpenes (C10), sesquiterpenes (C15), and norisoprenoids (C13), respectively. We discuss terpenoid biosynthesis in this study according to terpene sub-families and pathways.

5.3.3.1. Evolution of monoterpenes

The total free monoterpene concentration decreased from early berry development onwards (Fig. 5.3a). The four detected free monoterpenes are biosynthesised via different pathways from (4), while geranyl acetone (7), also a monoterpene, is derived from (5) via nerolidol (8) (Boland et al. 1998) (Fig. 5.4b). Geraniol (9), limonene (10), and linalool (11) are synthesised directly from (4) (Davis 2010; Davis and Croteau 2000; Hsiao et al. 2006; Luan et al. 2005). 1,8-cineole (12) is synthesised via the α-terpinyl cation (13) and α-terpineol (14), while citronellol (15) is synthesised via (9) (Davis and Croteau 2000; Davis 2010; Hsiao et al. 2006). Biosynthesis of the four detected monoterpenes are regulated by different terpenoid synthase genes (VvTPS), with some overlap (Martin et al. 2010).
The production of (10) and (12) is similar (Fig. 5.3a) due to the common VvTPS genes responsible for their biosynthesis. (10) is the product of VvGwaPhe, VvPNaPin, and VvCsbOciM, with the first two genes also responsible for the biosynthesis of (14) and, therefore, related to (12) (Martin et al. 2010). One VvPNaPin isoform, VvPNaPin1, is mainly expressed during flowering, early berry development, and maturity, but not around veraison in Moscato Bianco (Matarese et al. 2014). This partially explains the detection of (10) and (12) at wpf 4 in the present study. Biosynthesis of (14) is also related to another gene, VvTer, which is mainly expressed at flowering and early berry development in Moscato Bianco (Matarese et al. 2014), which also explains the relatively higher concentration of (12) at wpf 4.

The production of (9) and (15) is also similar (Fig. 5.3a), which may due to their biosynthetic relationship (Fig. 5.4b). (9) is regulated by VvGwGer, VvCSGer, and VvPNGer of the VvTPS-g subfamily of VvTPS, and VvPNGer is likely to be the main enzyme responsible for the biosynthesis of (9) (Martin et al. 2010; Matarese et al. 2013). VvPNGer is mainly expressed in flowers and berries in early development (Matarese et al. 2014), which explains the higher concentration of (9) at wpf 4. Since (15) is derived from (9), VvGwGer, VvCSGer, and VvPNGer may also be related to the biosynthesis of (15). In addition, (15) can be produced from (9) and (11) during fermentation (Luan et al. 2005). (11) is a product of many VvTPS genes including VvPNaPin, VvPNRLin, VvPNLinNer, and VvPNLNGI1, of which VvPNRLin has the highest transcription levels in Moscato Bianco and Aleatico (Matarese et al. 2013). However, no (11) was detected in the present study. The production of (7) is slightly different from the other four monoterpenes (Fig. 5.3a), which may be due to its synthesis from (5) rather than (4). It is unknown which enzymes are responsible for
the biosynthesis of (7). Overall, the production of (9), (10), (12), and (15) have similar trends of evolution during berry development, which reflects their possible genetic interactions.

5.3.3.2. Sesquiterpene evolution

The total sesquiterpene concentration decreased from early berry development to veraison and slightly increased at harvest. Although present at significantly lower total concentrations, the number of sesquiterpenes at wpf 17 was similar to that at wpf 4. However, sesquiterpene profiles at these two stages were different, which may be due to the activation of different biosynthetic pathways at different berry developmental stages. All sesquiterpenes are synthesised from (5) via farnesyl carbocation (16) (Fig. 5.4). The downstream biosynthetic pathways of the compounds detected in this study can be classified into five categories: the humulyl carbocation pathway (17-20) (Fig. 5.4c), the germacrene A pathway (21-26) (Fig. 5.4d), the germacrene C pathway (27-29) (Fig. 5.4e), the germacrene D pathway (32-54) (Fig. 5.4f), and the nerolidol diphosphate pathway (55-58) (Fig. 5.4g) (Bülow and König 2000; Chappell and Coates 2010; Davis and Croteau 2000; Davis 2010)

5.3.3.2.1. The humulyl carbocation pathway

The humulyl carbocation pathway starts with humulyl carbocation (17), which is derived from (16) (Fig. 5.4c) (Chappell and Coates 2010; Davis and Croteau 2000). The main pathway products include β-caryophyllene (18), clovène (19), and α-humulene (20), which were most abundant at the early stage of berry development (Table S-5.2). The biosynthesis of (18) and (20) is regulated by three common VvTPS genes, VvGwECar, VvPNECar, and VvPNaHum, which explains their similar
production trends during berry development (**Fig. 5.3a**) (Martin et al. 2010). Previous study have demonstrated that VvGwECar and VvPNaHum are mainly expressed in flower buds and open flowers in Moscato Bianco (Matarese et al. 2014). This is consistent with the current observations, since high concentrations of (18) and (19) observed at wpf 4 could have been synthesised during flowering. In addition, (18) is also regulated by a fourth gene, VvPNEb2epi Car, which helps to explain their different production patterns after veraison. The concentration of (19) was extremely low compared to (18) and (20) (**Fig. 5.3a**), and the gene responsible for its production has yet to be identified. (19) is only remotely related to (18), and therefore its production pattern is different (Chappell and Coates 2010; Davis and Croteau 2000).

5.3.3.2.2. The germacrene A pathway

The germacrene A pathway starts with germacrene A (21), which is derived from (16) (**Fig. 5.4d**). The primary pathway products include α-guaiene (22) and α-selinene (23). A very recent study reported aerial oxidation of (22) into rotund-2-ol (24) and rotundone (25) via hydroperoxy-guaiene (26) (Huang et al. 2014; Huang et al. 2015). It is possible that (22) was released by grape berries and oxidised into (25) via this pathway (**Fig. 5.4d**). However, this chemical synthesis does not explain the mechanism of storage of (25). In the present study, (22) and (25) had similar production patterns, suggesting that (25) is highly likely to be related to (22) in terms of their biosynthesis.
Fig. 5.5. Comparison of grape berry rotundone concentration (ng/100 berries) at different phenological stages of ripening in the 2012-13 and 2013-14 growing seasons. (a) Two-way ANOVA (p<0.05) was conducted to compare grape rotundone concentration between ripening stages and between growing season. (b) One-way ANOVA (p<0.05) was conducted to compare grape rotundone concentration between different ripening stages in each growing seasons separately. a, b, c, d were used to label significant differences (p<0.05) between ripening stages; A, B were used to label significant differences (p<0.05) between growing seasons; α, β were used to label significant differences (p<0.05) between flower and grape rotundone concentration in the 2013-14 growing season.

The production of (25) at different berry developmental stages was further investigated in detail in two growing seasons (Fig. 5.5). Overall, grapes from the 2012-13 growing season contained significantly higher concentrations of (25) (2.06 ng/100 berries) compared to those from the 2013-14 growing season (0.90 ng/100
berries), while grapes at harvest (E-L38) had a significantly higher concentration of (25) (3.19 ng/100 berries) than all other groups, followed by E-L 31 (2.12 ng/100 berries), E-L 32 (1.28 ng/100 berries), E-L 37 (1.10 ng/100 berries), E-L 36 (0.72 ng/100 berries), and E-L 35 (0.44 ng/100 berries) (two-way ANOVA, p<0.05) (Fig. 5.5a). In the 2012-13 growing season, the grape concentration of (25) gradually decreased from pre-veraison (E-L 31: 3.24 ng/100 berries, E-L 32: 1.52 ng/100 berries) to 80% veraison (E-L 35: 0.44 ng/100 berries), and then gradually increased until harvest (E-L36: 1.04 ng/100 berries, E-L 37:1.61 ng/100 berries, E-L: 4.48 ng/100 berries) (Fig. 5.5b). A similar trend was observed in the 2013-14 growing season. The pre-veraison groups (E-L31: 1.00 ng/100 berries, E-L 32: 1.04 ng/100 berries) contained significantly higher concentrations of (25) compared to E-L 35 (0.45ng/100 berries), E-L36 (0.41 ng/100 berries), and E-L 37 (0.59 ng/100 berries). Grapes at harvest (E-L 38: 1.89 ng/100 berries) had significantly higher concentrations of (25) than all other groups. The concentration of (25) in flower caps were also analysed in the 2013-14 growing season. Flower caps (1.62 ng/100 flowers) contained significantly higher concentrations of rotundone than grape berries at all stages before harvest, and were similar to those in harvested grapes.

Overall, the production of (25) was ‘U’ shaped in both seasons, consistent with that of (22) (Table S-5.2). VvPNSeint is responsible for the production of (22), δ-selinene (27), and 7-epi-α-selinene (28) (Martin et al. 2010), and the two latter compounds were only observed at wpf 17. This indicates that VvPNSeint may only be activated at a late stage of berry development, and other enzymes might be responsible for the production of (22) and (25) during early berry development. This warrants further investigation.
5.3.3.2.3. The germacrene C pathway

The germacrene C pathway starts with germacrene C (29), which is derived from (16) (Fig. 5.4e). Major pathway products include guaia-6,9-diene (30) and (27), which had a similar production pattern (Fig. 5.3b). It is unknown exactly how (28) and (+)-valencene (31) are synthesised, but they appear to be derived from (16) (Lücker et al. 2004). However, they may be derived from the germacrene A and/or C pathways, since (28) is a product of the activity of the same VvPNSeint enzyme as (22) and (27) (Martin et al. 2010). It has previously been shown that VitisM4670 cDNA expression is mainly confined to late-stage berry development, and encodes the VvVal VvTPS responsible for the biosynthesis of (28) and (31) (Lücker et al. 2004). This is consistent with our finding that (28) is only present at wpf 17.

5.3.3.2.4. The germacrene D pathway

The germacrene D pathway is the most complicated pathway implicated in the synthesis of the products detected in the present study, and is thought to be a major sesquiterpene synthesis pathway (Fig. 5.4f) (Bülow and König 2000; Chappell and Coates 2010; Davis and Croteau 2000). Germacrene D (32) is mainly regulated by VvGwGerD and VvPNGerD and is also a by-product of VvGwECar and VvPNECar (Martin et al. 2010). Three major sub-pathways could explain our current findings: the cadinenyl cation pathway (33-36), the muurolenyl cation pathway (37-49), and the amophenyl cation pathway (50-51).

The cadinenyl cation pathway starts with the cadinenyl cation (33), which is derived from (32) (Bülow and König 2000; Chappell and Coates 2010; Davis and Croteau
Its major products are γ-cadinene (34) and α-cadinene (35), both of which were only observed at wpf 17. (34) is a major product of VvGwgCad and a minor product of VvPNCuCad (Martin et al. 2010). It is uncertain how (35) is synthesised. ω-cadinene (36) may also be derived from (35) (Bülow and König 2000). In the present study, (36) showed a different pattern of production during berry development compared to (34) and (35) (Fig. 5.3b). Therefore, the pathway converting (35) to (36) might not be activated at the berry development stages studied, and the latter may be produced via an alternative pathway, as discussed below.

The muurolenyl cation pathway is important to discuss in the context of the present study, and starts with the muurolenyl cation (37), derived from (32) (Bülow and König 2000; Chappell and Coates 2010; Davis and Croteau 2000). The direct products of (37) are α-muurolene (38), δ-cadinene (39), γ-muurolene (40), and α-copaene (41). α-calacorene (42), (36), and cis-muurola-4(15),5-diene (43) are synthesised directly from (39). Two intermediates (A: 44 and B: 45) are also synthesised from (39), then converted into epi-zonarene (46) and zonarene (47), respectively. (46) and (47) can be further converted into different calamenenes ((R): 48, (S): 49), which can also be synthesised from (43). In the present study, (43) was not detected at any developmental stages, indicating that the pathway between (43) and (48, 49) may not be activated in Shiraz grapes at the berry developmental stages studied.

The majority of sesquiterpenes produced by the muurolenyl cation pathway had highly similar production patterns (Fig. 5.3a) and were predominantly produced at early berry developmental stages before veraison (wpf 4-11). The production of (39)
was highly similar to (47) (Fig. 5.3a), suggesting that production of (47) is more related to (39), and (47) could be the major downstream product of (39). (46) and (47) had completely different production patterns (Fig. 5.3a), indicating that these two sesquiterpenes were likely to be regulated by different mechanisms. (36), (38), (40), and (42) also had similar production patterns (Fig. 5.3b). The VvPNCuCad gene product is responsible for the production of (39) and (41), but no other enzymes have been identified as responsible for the production of sesquiterpenes from the muurolenyl cation pathway (Martin et al. 2010).

The amophenyl cation pathway starts with the amophenyl cation (50) and is mainly responsible for the biosynthesis of α-amorphene, γ-amorphene, δ- amorphene, and ω-amorphene (Bülow and König 2000). However, none of these compounds were detected in the present study, the only product detected being α-ylangene (51). In addition, the VvTPS gene responsible for amophenyl cation production remains elusive.

Apart from the cadinenyl cation, muurolenyl cation, and amophenyl cation sub-pathways, the germacrene D pathway is also responsible for the biosynthesis of (27), selina-4(15),6-diene (52), β-bourbonene (53), and β-copaene (54) (Bülow and König 2000); these compounds were only observed at harvest in the present study (Table S-5.2). (52) can be further modified into (27), which is also a product of the germacrene C pathway, as discussed earlier. Therefore, it is unclear which pathway produces (27).
Previous studies have reported that the VvTPS gene responsible for the production of (32), VvGwGerD, is highly expressed in flowers but has low expression in grape berries (Matarese et al. 2014). However, no (32) was observed in this study. (32) might have already been converted to downstream products, such as those in the muurolenyl cation pathway, after flowering. It is also reasonable to conclude that the cadinenyl cation and amophenyl cation pathways are regulated by different VvTPS genes than the muurolenyl cation pathway, since their end-product production patterns were completely different during berry development (Fig. 5.3).

5.3.3.2.5. The nerolidol diphosphate pathway

The nerolidol diphosphate pathway significantly overlaps with the germacrene D pathway (Fig. 5.4g). (37), (51), and (54) may be synthesised from (16) through the nerolidol diphosphate pathway via nerolidol diphosphate (NPP) (55) and intermediate C (56), rather than from the germacrene D pathway (Chappell and Coates 2010; Davis and Croteau 2000). Since (37) was the precursor for the muurolenyl cation pathway, the whole pathway may not necessarily be derived from the germacrene D pathway but from the nerolidol diphosphate pathway. Furthermore, the starting point of the germacrene D pathway, (32), may actually be derived from (56) (i.e., the nerolidol diphosphate pathway). 1-epi-cubenol (57) is another nerolidol diphosphate pathway product via intermediate D (58). This compound and its isomer, cubenol, had similar production patterns (Fig. 5.3b), and their biosynthesis may, therefore, be related. (57) was only observed at wpf 4, which was consistent with timing of production of most compounds derived from the muurolenyl cation pathway (37-49). Since both (57) and muurolenyl cation pathway sesquiterpenes were mainly detected at wpf 4, it would be reasonable to assume that (56) was abundant at the
same time. In the current study, (51) and (54) were only observed at late stages of berry development. Thus, the pathway producing (51) and (54) from (56) might not be activated at early berry developmental stages.

5.3.3.3 Norisoprenoid evolution

The total norisoprenoid concentration reached a maximum at wpf 6 and decreased thereafter until wpf 17. All norisoprenoids detected in the present study are synthesised from (6) via β-carotene (59) (Fig. 5.4h) (Mendes-Pinto 2009). Theaspirane (60) and β-ionone (61) are derived from (59) via different pathways, while β-damascenone (62) is derived from (59) via neoxanthin (63) (Baumes et al. 2002; Mendes-Pinto 2009). In grape berries, the genes encoding carotenoid biosynthesis and catabolism are skin-specific (Grimplet et al. 2007). Carotenoids are mainly synthesised during pre-veraision and degrade after veraison to produce norisoprenoids (Grimplet et al. 2007). However, the norisoprenoids formed from carotenoids are mainly in glycoconjugated forms (Wirth et al. 2001). Another study reported that the concentration of total glycoconjugated norisoprenoids gradually increases from veraison to harvest in Shiraz, with no free norisoprenoids produced at any point (Mathieu et al. 2005). Clearly, the free norisoprenoids observed in the present study followed a similar trend to that of the carotenoids, and no significant increase in norisoprenoids was observed after wpf 11. This suggests that, despite the accumulation of glycoconjugated norisoprenoids after veraison, the glycosidase enzyme may not be available in Shiraz to release free norisoprenoids during post-veraision stages. In addition, abscisic acid (64), a sesquiterpene, can be biosynthesised from (63) rather than (5) (Taylor et al. 2000). It is well known that biosynthesis of this compound is related to plant water availability (Christmann et al.
A recent study reported that the biosynthesis of (25) was closely related to vineyard water availability (Zhang et al. 2015b; Geffroy et al. 2014). Therefore, the biosynthesis of (64) may be used as an indicator of (25).
5.4. Conclusions

Monoterpenes, sesquiterpenes, and norisoprenoids have different production patterns during berry development from pea-size to commercial harvest. Clear differences in berry terpene profiles at different berry developmental stages indicate that the mechanisms of terpene biosynthesis may be different at different ripening stages. The decrease in the number and concentration of terpenes from pre-veraison to veraison suggests that a number of their biosynthetic pathways could be inactivated. Alternatively, terpenes produced during early berry development may be degraded into other compounds or converted into non-volatile forms. Sharp increases in sesquiterpene concentrations during the last two weeks of berry development indicate that the later ripening stages, rather than the whole veraison to maturity period, is more critical in defining the final concentration of sesquiterpenes, such as rotundone, in harvested grapes and, therefore, wine.

Our analysis of terpene compound evolution during berry development suggests that terpene biosynthesis is more dependent on the activation of the pathway it belongs to, and terpenes synthesised via similar pathways tend to appear at similar berry developmental stages. Categorisation of terpenes based on their biosynthetic pathway, especially sesquiterpenes, helps to understand pathway activation and regulation and can be used to predict the production patterns for other, less well-characterised compounds in the same pathway. Furthermore, highly similar production patterns in the same pathway will help future molecular classification studies that investigate the genes that regulate the whole pathway rather than a single product, and more pathway interactions are likely to be present. Even though
some compounds can be synthesised by multiple pathways, not all pathways are active simultaneously.

Acknowledgements
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5.5 References


5.6 Supplementary data

Table S-5.1. Weather record of the vineyard in 2012-13 and 2013-14 seasons (Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085 – located 15.5kms NW to the experimental site). The date of 80% veraison is taken at 19th Feb 2013 and 28th Feb 2014 and commercial harvest begins on 10th Apr 2013 and 8th Apr 2014.

<table>
<thead>
<tr>
<th>Weather record</th>
<th>2012-13</th>
<th>2013-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean January Maximum Temperature</td>
<td>28.8°C</td>
<td>30.0°C</td>
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<tr>
<td>Mean January Minimum Temperature</td>
<td>11.1°C</td>
<td>13.4°C</td>
</tr>
<tr>
<td>Mean January Temperature</td>
<td>20.0°C</td>
<td>21.7°C</td>
</tr>
<tr>
<td>Mean January Solar Exposure</td>
<td>29.1 MJ/m²</td>
<td>27.0 MJ/m²</td>
</tr>
<tr>
<td>Mean Maximum Temperature</td>
<td>26.3°C</td>
<td>24.7°C</td>
</tr>
<tr>
<td>80% veraison to harvest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Minimum Temperature</td>
<td>11.5°C</td>
<td>10.1°C</td>
</tr>
<tr>
<td>80% veraison to harvest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Temperature</td>
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<td>17.4°C</td>
</tr>
<tr>
<td>80% veraison to harvest</td>
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<tr>
<td>Mean Solar Exposure</td>
<td>18.1 MJ/m²</td>
<td>15.4 MJ/m²</td>
</tr>
<tr>
<td>80% veraison to harvest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total rainfall from Oct to Harvest</td>
<td>124.1 millimeters</td>
<td>140.9 millimeters</td>
</tr>
<tr>
<td>Total irrigation From Oct to Harvest</td>
<td>84.3 millimeters</td>
<td>60.8 millimeters</td>
</tr>
<tr>
<td>Total water precipitation (October to Harvest)</td>
<td>208.4 millimeters</td>
<td>201.7 millimeters</td>
</tr>
</tbody>
</table>
Table S-5.2. Comparison of common and abundant terpenes at different grape development stages in the 2013-14 growing season$^\text{a}$

<table>
<thead>
<tr>
<th>GC Peak number</th>
<th>Compound number</th>
<th>Terpenes compounds and classes</th>
<th>Chemical structures</th>
<th>Odour quality$^{bc}$</th>
<th>Concentration (µg $\alpha$-copaene equivalents/kg) at different weeks post-flowering (wpf)</th>
<th>Percentage of individual terpenoids to total terpenoids</th>
</tr>
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<tbody>
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<td>wpf 4</td>
<td>wpf 6</td>
<td>wpf 9</td>
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</tr>
<tr>
<td><strong>Monoterpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>Limonene</td>
<td>lemon, orange</td>
<td>6.25±0.76</td>
<td>1.12%</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1,8-Cineole</td>
<td>mint, sweet</td>
<td>7.94±0.66$^a$</td>
<td>1.43%</td>
<td>2.21±1.6b</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>Geraniol</td>
<td>rose, geranium</td>
<td>9.31±6.33$^a$</td>
<td>1.67%</td>
<td>5.57±3.16ab</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>Geranyl acetone</td>
<td>magnolia, green</td>
<td>27.18±5.86$^a$</td>
<td>4.89%</td>
<td>22.39±7.31a</td>
</tr>
<tr>
<td>27</td>
<td>15</td>
<td>Citronellol</td>
<td>rose,</td>
<td>1.47±0.47$^a$</td>
<td>0.26%</td>
<td>2.88±1.33b</td>
</tr>
<tr>
<td><strong>Sesquiterpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>Clovene</td>
<td>NA$^e$</td>
<td>0.55±0.09$^a$</td>
<td>0.10%</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>$\alpha$-Ylangene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>53</td>
<td>$\beta$-Bourbonene</td>
<td>herb</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$From a paper by J. Smith and colleagues.

$^b$Odour quality: lemon, mint, rose, magnolia, herb.

$^c$Concentration in µg $\alpha$-copaene equivalents/kg.

$^d$ND: not detected.

$^e$NA: not applicable.
<table>
<thead>
<tr>
<th>Peak number</th>
<th>Compound number</th>
<th>Terpenes compounds and classes</th>
<th>Chemical structures</th>
<th>Odour quality$^{bc}$</th>
<th>Concentration (µg α-copaene equivalents/kg) at different weeks post-flowering (wpf)</th>
<th>Percentage of individual terpenoids to total terpenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>18</td>
<td>(E)-β-Caryophyllene</td>
<td>wood, spice</td>
<td>166.65±24.10a</td>
<td>29.95%</td>
<td>27.12±4.52b</td>
</tr>
<tr>
<td>12</td>
<td>54</td>
<td>β-Copaene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>22</td>
<td>α-Guaiene$^g$</td>
<td>wood, balsamic</td>
<td>0.09±0.03ab</td>
<td>0.02%</td>
<td>0.26±0.04c</td>
</tr>
<tr>
<td>14</td>
<td>30</td>
<td>Guaia-6,9-diene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>52</td>
<td>Selina-4(15),6-diene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>20</td>
<td>α-Humulene</td>
<td>wood</td>
<td>188.50±33.58a</td>
<td>33.88%</td>
<td>18.12±5.48b</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>γ-Muurolene</td>
<td>herb, wood, spice</td>
<td>3.67±1.20a</td>
<td>0.66%</td>
<td>0.86±0.23b</td>
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<td>Peak number</td>
<td>Compound number</td>
<td>Terpenes compounds and classes</td>
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<td>Odour quality&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>Concentration (µg α-copaene equivalents/kg) at different weeks post-flowering (wpf)</td>
<td>Percentage of individual terpenoids to total terpenoids</td>
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<td>wpf 4</td>
<td>wpf 6</td>
</tr>
<tr>
<td>20</td>
<td>27</td>
<td>δ-Selinene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td>46</td>
<td>epi-Zonarene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22</td>
<td>38</td>
<td>α-Muurolene</td>
<td>wood</td>
<td>6.44±0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.16%</td>
<td>3.29±0.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>23</td>
<td>34</td>
<td>γ-Cadinene</td>
<td>wood</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>39</td>
<td>δ-Cadinene</td>
<td>thyme, medicine, wood</td>
<td>40.28±5.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.24%</td>
<td>11.08±0.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>48/49</td>
<td>Cis/trans-Calamenene</td>
<td>herb, spice</td>
<td>ND</td>
<td>2.47±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.50%</td>
</tr>
<tr>
<td>26</td>
<td>47</td>
<td>Zonarene</td>
<td>NA</td>
<td>29.52±2.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.31%</td>
<td>2.19±0.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>Compound number</td>
<td>Terpenes compounds and classes</td>
<td>Chemical structures</td>
<td>Odour quality</td>
<td>Concentration (µg α-copaene equivalents/kg) at different weeks post-flowering (wpf)</td>
<td>Percentage of individual terpenoids to total terpenoids</td>
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<td>wpf 6</td>
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<tr>
<td><strong>Sesquiterpenes</strong></td>
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</tr>
<tr>
<td>28</td>
<td>28</td>
<td>7-epi-α-Selinene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>29</td>
<td>36</td>
<td>ω-Cadinene</td>
<td>NA</td>
<td>6.24±0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12%</td>
<td>1.79±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>35</td>
<td>α-Cadinene</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>31</td>
<td>42</td>
<td>α-Calacorene</td>
<td>wood</td>
<td>4.44±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80%</td>
<td>3.01±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>32</td>
<td>57</td>
<td>Epi-Cubenol</td>
<td>NA</td>
<td>5.47±0.19</td>
<td>0.98%</td>
<td>ND</td>
</tr>
<tr>
<td>33</td>
<td>NA</td>
<td>Cubenol</td>
<td>spice, herb, green tea</td>
<td>5.43±0.63</td>
<td>0.98%</td>
<td>ND</td>
</tr>
<tr>
<td>Peak number</td>
<td>Compound number</td>
<td>Terpenes compounds and classes</td>
<td>Chemical structures</td>
<td>Odour quality(^{bc})</td>
<td>Concentration (µg (\alpha)-copaene equivalents/kg) at different weeks post-flowering (wpf)</td>
<td>Percentage of individual terpenoids to total terpenoids</td>
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</tr>
<tr>
<td>4</td>
<td>60</td>
<td>Theaspirane isomer A</td>
<td>fruits, peach, honey</td>
<td>21.14±1.78a</td>
<td>3.80%</td>
<td>14.30±0.62c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32.16±4.57b</td>
<td>19.51%</td>
<td>43.19%</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>Theaspirane isomer B</td>
<td>camphor, mint, wood, eucalyptus</td>
<td>8.36±1.23a</td>
<td>1.50%</td>
<td>5.39±0.08c</td>
</tr>
<tr>
<td>9</td>
<td>62</td>
<td>(E)-(\beta)-Damascenone</td>
<td>apple, rose, honey</td>
<td>4.61±0.86a</td>
<td>0.83%</td>
<td>5.01±1.07a</td>
</tr>
<tr>
<td>19</td>
<td>61</td>
<td>(\beta)-Ionone</td>
<td>seaweed, violet, flower, raspberry</td>
<td>12.85±2.16a</td>
<td>2.31%</td>
<td>11.23±1.46b</td>
</tr>
<tr>
<td>Total monoterpenes</td>
<td></td>
<td></td>
<td></td>
<td>52.15±10.74a</td>
<td>9.37%</td>
<td>33.06±11.62b</td>
</tr>
<tr>
<td>Total norisoprenoids</td>
<td></td>
<td></td>
<td></td>
<td>46.95±4.76a</td>
<td>8.44%</td>
<td>61.60±9.44b</td>
</tr>
<tr>
<td>Total sesquiterpenes</td>
<td></td>
<td></td>
<td></td>
<td>465.27±66.87a</td>
<td>82.19%</td>
<td>70.19±10.62b</td>
</tr>
<tr>
<td>Total terpenes</td>
<td></td>
<td></td>
<td></td>
<td>11.14±2.54a</td>
<td>35.64%</td>
<td>164.84±30.14b</td>
</tr>
<tr>
<td>Total volatile compounds</td>
<td></td>
<td></td>
<td></td>
<td>2297.5±495.00a</td>
<td>24.22%</td>
<td>1564.11±514.75</td>
</tr>
</tbody>
</table>

\(^{a}\)Different letters in the column represent significantly different means ± standard error (p < 0.05). \(^{b}\)Odour of the compound taken from Flavornet by Terry acre and Heinrich Arn, http://www.flavornet.org © Datu Inc., 2014. \(^{c}\)Odour of the compound taken from Leffingwell & Associates, http://www.leffingwell.com © Leffingwell & Associates, 2014. \(^{d}\)Not detected. \(^{e}\)Not available. \(^{f}\)The compound was detected only in 2 replicates. \(^{g}\)The compound was detected only in 1 replicate. \(^{h}\)\(\alpha\)-Guaiene was expressed as the ratio of m/z 147: m/z 161 multiply by the concentration of internal standard, \(\alpha\)-copaene.
Compounds list of Fig. 5.4:

1. Isopentenyl diphosphate (IPP)
2. Dimethylallyl pyrophosphate (DMAPP)
3. Isoprene
4. Geranyl diphosphate (GPP)
5. Farnesyl diphosphate (FPP)
6. Geranyl geranyl biphosphate (GGPP)
7. Geranyl acetone
8. Nerolidol
9. Geraniol
10. Limonene
11. Linalool
12. 1,8-cineole
13. α-Terpinyl cation
14. α-Terpineol
15. Citronellol
16. Farnesyl carbocation
17. Humulyl carbocation
18. β-caryophyllene
19. Clovène
20. α-Humulene
21. Germacrene A
22. α-Guaiaine
23. α-selinene
24. Rotund-2-ol
25. Rotundone
26. Hydroperoxoy-guaiene
27. δ-Selinene
28. 7-epi-α-Selinene
29. Germacrene C
30. Guai-6,9-diene
31. (+)-Valencene
32. Germacrene D
33. Cadinenyl cation
34. γ-Cadinene
35. α-Cadinene
36. ω-Cadinene
37. Muurolenyl cation
38. α-Muurolene
39. δ-Cadinene
40. γ-Muurolene
41. α-Copaene
42. α-Calacorene
43. cis-Muurola-4(15),5-diene
44. Intermediate A
45. Intermediate B
46. epi-Zonarene
47. Zonarene
48. (R)-Calamenene
49. (S)-Calamenene
50. Amophenyl cation
51. \( \alpha \)-Ylangene
52. Selina-4(15),6-diene
53. \( \beta \)-Bourbonene
54. \( \beta \)-Copaene
55. Nerolidol diphosphate (NPP)
56. Intermediate C
57. epi-Cubenol
58. Intermediate D
59. \( \beta \)-Carotene
60. Theaspirane
61. \( \beta \)-Ionone
62. \( \beta \)-Damascenone
63. Neoxanthin
64. Abscisic acid
CHAPTER SIX
Rotundone production and translocation in *Vitis vinifera* L. cv. Shiraz grapevines

Abstract

Rotundone is an important grape-derived aroma compound that is also present in different plant tissues, such as leaves, petioles and peduncles. To establish if rotundone is translocated between non-grape tissues and grape berries, the concentration of rotundone was quantified in different vine parts throughout grapevine development. In addition, the most likely mode of rotundone translocation - via the phloem – was studied by stable isotope feeding-GC/MS. Finally, the influence of herbivores attack on rotundone concentrations in grapevine leaves was assessed. Different grapevine tissue samples showed a similar pattern of rotundone production from pre-veraison to harvest, and in individual vine shoots non-grape tissues contained higher concentrations and amounts of rotundone compared to berries. Potted grapevine leaves were placed in contact with aqueous d$_5$-rotundone, and subsequently different tissues were collected and analysed by GC-MS. However, no d$_5$-rotundone could be detected in tissues without direct contact to d$_5$-rotundone and phloem exudates from leaf petioles. In addition, the monoterpane geraniol and six sesquiterpenes (clove, α-ylangene, β-copaene, α-murolene, δ-cadinene, and cis/trans-calamenene), but not the rotundone precursor alpha-guaiene could be detected in phloem exudates, indicating that phloem transport of rotundone is unlikely to occur. Similar to other herbivore-induced terpenes, rotundone might be produced by grapevines in response to herbivore attack. Hence, potted
grapevines were subjected to light brown apple moth larval feeding, simulated herbivorous physical damage, and exogenous phytohormone treatment. The effect of herbivorous activity on rotundone concentrations in grapevine leaves was limited. Although rotundone is unlikely to be transported via the phloem in grapevines, other sesquiterpenes could be transported by this mechanism, with potential implications for grape aroma.

**Keywords:** rotundone, sesquiterpenes, phloem composition, translocation, herbivore induced terpene synthesis

### 6.1 Introduction
Rotundone has been reported to be the main compound responsible for the “peppery” aroma in Shiraz grapes and wine, and is the first sesquiterpene compound shown to contribute to grape and wine aroma (Siebert et al. 2008; Wood et al. 2008). Rotundone is thought to be derived from a sesquiterpene precursor, α-guaiene, by aerial oxidation and biosynthetic transformation (Huang et al. 2014; Zhang et al. 2015b). Rotundone levels vary in grape skin of different Shiraz clones, and rotundone is extracted into juice and, ultimately, the wine during fermentation (Herderich et al. 2012; Herderich et al. 2013). Rotundone is also present at high levels in grape leaves and stems, as fermentation with added leaves and stems results in an up to six-fold increase in wine rotundone concentrations (above 200 ng L⁻¹) (Capone et al. 2012). Previous studies have shown that high-vigour grapevines, seasons with high water availability, and grape bunches located closer to leaves have higher rotundone concentrations in the grape berries (Zhang et al. 2015a; Zhang et al.
Therefore, there is a potential source-sink relationship between grape berries and grapevine vegetative tissues.

In vascular plants, the phloem can transport nutrients, defence compounds, chemical signals, and a number of secondary metabolites from source to sink tissues (Turgeon and Wolf 2009). Rotundone is a secondary plant metabolite that may be transported between grapevine tissues via the phloem pathway. Previous studies have shown that two monoterpane derivatives were transported via the phloem in *Asarina scandens* (Scrophulariaceae) and *Catalpa speciosa* (Northern Catalpa) (Gowan et al. 1995; Turgeon and Medville 2004). Conversely, monoterpane glycosides and their precursors were not translocated into berries from other parts of the vine in Muscat grapevines unless an active transport mechanism for a specific compound was present (Gholami et al. 1995). To the best of our knowledge, phloem sesquiterpene transport has not been investigated in any plant, although Hampel et al. (2005) did demonstrate that sesquiterpenes could be biosynthesised in the phloem of carrot roots.

The origin of rotundone biosynthesis in grapevines remains unclear and could be related to a response to the surrounding environment. Rotundone belongs to the terpene chemical family, many of which are considered herbivore-induced plant volatiles (Arimura et al. 2005; Howe and Jander 2008). It is possible that rotundone or its precursors are produced as defensive compounds, especially at the high concentrations found in leaves, to protect grapevines from herbivores (Zakir et al. 2013). The two main impacts of herbivorous insects on
plants are physical damage and the effects of their oral elicitor secretions (Howe and Jander 2008). In simulations of mechanical damage to mimic physical herbivore damage, monoterpenes biosynthesis was increased (Bricchi et al. 2010; Mithöfer et al. 2005). Phytohormones, including salicylic acid (SA), jasmonic acid (JA), methyl jasmonate (MeJA), ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), played important roles in regulating plant responses to herbivores by activating or suppressing terpenoid biosynthesis genes (Arimura et al. 2005; Bari and Jones 2009; Howe and Jander 2008; Pieterse et al. 2012; Gómez-Plaza et al. 2012). Therefore, phytohormones may be a useful and valid surrogate of herbivore elicitors when assessing grapevine terpene synthesis.

Here, we examine the distribution of rotundone in different grapevine tissues during grape maturity, and study the potential transport of rotundone, \( \alpha \)-guaiene, and other sesquiterpenes between different grapevine tissues in the phloem. The regulation of rotundone production in grapevines was also investigated in response to: i) native Australian grapevine pests (light brown apple moth larvae; \textit{Epiphyas postvittana}); ii) simulated herbivorous physical damage; and iii) spraying leaves with exogenous phytohormones. We show that, non-grape tissues contain higher concentrations and amounts of rotundone compared to grape berries at both veraison and harvest. Although rotundone is unlikely to be transported via the phloem in grapevines, other sesquiterpenes may be transported by this mechanism, with potential implications for grape aroma. The effect of herbivorous activity on rotundone concentrations in grapevine leaves
was limited, and rotundone is unlikely to be a major component of herbivore-induced grapevine volatiles.

6.2 Materials and methods

6.2.1 Chemicals
Rotundone ((3S,5R,8S)-3,4,5,6,7,8-hexahydro-3,8-dimethyl-5-(prop-1-en-2-yl)-1(2H)-azulenone) (Supplementary Fig. 6.1A) and ²H₅-rotundone (Supplementary Fig. 6.1B) were synthesised as previously described (Siebert et al. 2008; Wood et al. 2008). A reference standard of α-copaene was purchased from Sigma-Aldrich (Castile Hill, NSW, Australia) for phloem sesquiterpene analysis. Working solutions of standards were prepared volumetrically in ethanol and stored at 4 °C until required. Analytical-grade potassium L-tartrate monobasic, tartaric acid, SA, JA, MeJA, ACC, and other chemicals were also obtained from Sigma-Aldrich. HPLC-grade solvents for rotundone extraction, including ethyl acetate, n-pentane, methanol, and ethanol, were supplied by Rowe Scientific (Doveton, Vic, Australia). Water was purified using the Milli-Q system (Millipore Australia, Bayswater, Victoria, Australia).

6.2.2 Vineyard Site
Distribution of rotundone among grapevine tissues within individual shoot was investigated in a commercial vineyard (The Old Block, Mount Langi Ghiran 37.31°S, 143.15°E) located in the Grampians wine region of Victoria, Australia. The vineyard is approximately 15.5 km east of the nearest Bureau of Meteorology (BOM) weather station at Ararat Prison (Australian BOM Station
No. 089085). At this weather station, the weather data of the two studied growing seasons (2012-13 and 2013-14) were recorded and specified in Supplementary Table 6.1. The vineyard was planted in 1968 with *Vitis vinifera*, cv. Shiraz on its own roots at 3.0 m between rows and 1.8 m between vines, with rows oriented northeast to southwest. Grapevines are trained to a vertical shoot positioned (VSP) trellis, and irrigated by a drip-irrigation system when required at a rate of 5.76 L/(hr-vine). No significant pest or disease pressure was observed during the experimental seasons and management was performed following the company’s guidelines.

6.2.3 Rotundone content in vine shoots

Ten vines from a single row of the experimental vineyard were selected to study the rotundone production in individual vine shoot as large within-vineyard in berry rotundone concentration was previously reported for this experimental block (Scarlett et al. 2014; Zhang et al. 2015a). The vines were selected on the basis of uniformity of canopy and cluster development. Since adjacent vines in the same row always intertwined, at least two vines served as buffer in between target vines. One representative shoot of each selected vine was collected at around 80% veraison (E-L 35, 28<sup>th</sup> of Feb 2014) and the beginning of commercial harvest (E-L 38, 8<sup>th</sup> of Apr 2014) in the 2013-14 growing season, as the period from veraison to harvest has been proven the key stage rotundone development in grape berries (Zhang et al. 2015c). In addition to sampling from specific vines, grape bunches and leaves with petioles were also sampled randomly from different grapevines (n>30 grapevines at each sampling date) across the whole vineyard block in triplicates at fortnightly intervals from pea-
size berry (E-L 31, 9\textsuperscript{th} of Jan 2014) until the beginning of commercial harvest (E-L 38, 8\textsuperscript{th} of Apr 2014) in the 2013-14 growing season. The sampled shoots were separated into grape berries, peduncles/rachises, leaves, petioles and canes, weighted, packed in zip-lock plastics bags, and stored at -20°C before analysis.

### 6.2.4 Glasshouse Plant materials

Two-year-old *Vitis vinifera* L.cv. Shiraz grapevines on their own roots were used for translocation and herbivore simulation experiments. Plants were grown in 20L pots and maintained at 20 °C for night and day times in a glasshouse (System Garden, Parkville, The University of Melbourne). An automatic drip irrigation system was installed with a set irrigation volume of 0.85 L per vine per day. A commercial NPK fertiliser (Osmacote, Scotts Australia Pty Ltd, NSW, Australia) was used to provide general nutrients. No significant pest or disease pressure was observed during the experimental period.

### 6.2.5 Stable isotope-feeding assay

Two experimental groups were prepared with three potted vines per treatment as replicates. Control group grapevines had no contact with d\textsubscript{5}-rotundone solution. On each treatment vine, fifteen randomly selected, fully expanded, attached mature leaves were partially dipped in 10 ml aqueous d\textsubscript{5}-rotundone solution (12.9 ng ml\textsuperscript{-1}) in plastic zip-lock sandwich bags. No surfactant was used. d\textsubscript{5}-Rotundone solutions were applied one week prior to berry harvest, and no contact was made with other leaves on the grapevine. Treated leaves with petioles, untreated leaves with petioles, and grape bunches from each
grapevine were sampled after seven days. Treated leaves and petioles were rinsed with Milli-Q water to remove any residual d$_5$-rotundone solution. Grape leaves and petioles were separated at sampling and stored in separate zip-lock bags. For grape bunches, grape berries and grape peduncles/rachises were separated after sampling and stored in separate zip-lock bags. Leaf and petiole samples were collected from each control vine on the same day. All samples were stored at -20 °C until chemical analysis.

6.2.6 Phloem extraction

Three experimental groups were prepared with three potted vines per treatment as replicates. Control vines had no contact with d$_5$-rotundone solution (control-phloem). For the “whole leaves” treatment, 15 whole, fully expanded, attached mature leaves from each treatment vine were placed in contact with 10 ml aqueous d$_5$-rotundone solution (25.8 ng ml$^{-1}$) in plastic zip-lock sandwich bags. For the “cut leaves” treatment, 15 fully expanded and attached mature leaves from each treatment vine were cut in half under MilliQ water then dipped into 10 ml aqueous d$_5$-rotundone solution (25.8 ng ml$^{-1}$) in plastic zip-lock sandwich bags. Higher concentration of d$_5$-rotundone solution was used in this experiment (25.8 ng ml$^{-1}$) compared to stable isotope-feeding assay (12.9 ng ml$^{-1}$) in order to increase the possibility of phloem transportation. Leaf samples were collected four days after treatment and immediately transferred to the laboratory for phloem exudate analysis as described below.

Phloem exudate extraction was performed using the EDTA method (Gowan et al. 1995). For this purpose, fifteen leaves were cut at the petiole base,
immediately re-cut under MilliQ water to avoid cavitation, and subsequently rinsed to avoid contamination of the cellular fluid. The leaves were then placed in a 10 ml plastic screw cap tube, and the base of the petioles was immersed in 0.2 ml of a 20 mM EDTA in 20 mM PIPES buffer (pH 6.8) solution. The leaves were then removed from the petiole. The tubes were capped and placed in a humidified chamber in complete darkness at 22 °C for 8 h. The solutions were then prepared for GC-MS analysis. Phloem exudate samples were prepared for each treatment replicate. Five control-phloem samples were prepared: 100 μL d5-rotundone (516 ng ml⁻¹ in ethanol) was added to the first as “positive control-phloem”, no d5-rotundone to the second as “negative control-phloem”, and the rest were used for sesquiterpene analysis.

6.2.7 Herbivore treatment

_E. postvittana_ larvae were cultured and supplied by the Bio21 Molecular Science & Biotechnology institute, University of Melbourne. _E. postvittana_ larvae were prepared as in (Singh et al. 1985). Briefly, _E. postvittana_ eggs collected from adult moths were placed in plastic cups and maintained in a growth chamber under controlled conditions (21±2 °C, 50±5 % relative humidity, 14 h light and 10 h dark) until larvae emerged.

Three experimental groups were prepared with six potted vines per treatment as replicates. Around 20 leaves on each vine were treated with: i) no _E. postvittana_ larvae treatment (control [Ctrl]); ii) low-density _E. postvittana_ larvae treatment (LD, larvae number: 1 per leaf); iii) high-density _E. postvittana_ larvae treatment (HD, larvae number: 6 per leaf). In the two larval treatment groups,
seven-day-old larvae were gently tapped onto vine foliage between 4 and 5 pm, and larvae were allowed to feed for 7 days. After removal of the larvae, vine leaves were harvested and stored at -20 °C until subsequent analysis. In the LD and HD treatment groups, leaves were partially damaged after 7 days. The damage and undamaged parts of the leaves were separated, and the damaged parts were labelled “LD-T” or “HD-T”, while the undamaged parts were labelled “LD-C” and “HD-C”.

6.2.8 Physical wounding treatment

Four experimental groups were conducted with six potted vines per treatment as replicates, and experimental groups were separated with plastic film to prevent interactions. Mechanical wounding was only conducted on fully expanded leaves: i) no physical damage (control); ii) each leaf on the vine randomly punched 10-15 times per day with a 1 mm diameter hole puncher (1mmLD); iii) each leaf on the vine randomly punched 30-45 times per day with a 1mm diameter hole puncher (1mmHD); (iv) each leaf on the vine randomly punched 10-15 times per day with 5 mm diameter hole puncher (5mmLD). Treated leaves were sampled in zip-lock plastic bags on day seven and stored at -20°C until analysis.

6.2.9 Phytohormone treatment

Seven experimental groups were conducted with six potted vines per treatment as replicates, and experimental groups were separated by plastic film to prevent interactions. All chemicals used in the experiment were prepared in deionized Milli-Q water and stored at 4 °C until required: i) Control (Milli-Q water); ii) ACC
(1mM); iii) ACC+JA (1mM+1mM); iv) JA (1mM); v) JA+SA (1mM+1mM); vi) SA (1mM); and vii) MeJA (1mM). For each treatment, 10 ml of the liquid was evenly sprayed on all leaves of each vine three times on the 1\textsuperscript{st}, 4\textsuperscript{th}, and 7\textsuperscript{th} day of treatment. Treated leaves were sampled in zip-lock plastic bags on the 8\textsuperscript{th} day of the experiment and stored at -20 °C until analysis.

6.2.10 Preparation of samples for d\textsubscript{5}-rotundone identification in stable isotope treatments

Stable isotope treatment samples were prepared for d\textsubscript{5}-rotundone analysis using a previously described rotundone analysis protocol (Siebert et al. 2008; Wood et al. 2008). For grape samples, 50 g of de-stemmed grapes were homogenised with a hand-held blender and centrifuged to separate solids and liquids. The solid parts were mixed with 30 ml ethanol and 30 ml water then shaken for 24 h at 22 °C and sonicated before reintroducing the juice. Samples were then centrifuged and filtered (1.6 μm glass fibre) to obtain berry extract filtrate, which was topped up to 200ml with Milli-Q water prior to solid-phase extraction (SPE) as previously described (Siebert et al. 2008). SPE residue supernatant was air dried with nitrogen and reconstituted in 0.5 ml ethanol and 9ml Milli-Q water. Samples were analysed in the Australian Wine Research Institute using a published solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) protocol (Geffroy et al. 2014). d\textsubscript{5}-Rotundone was identified at m/z 208 and 223 as previously described (Siebert et al. 2008). For grape peduncles/rachises, 3 g of finely cut up leaf and petiole samples were mixed with 30 ml ethanol and 30 ml water in 250 ml screw cap bottles. No internal standard was added to the treatment samples; however, two
control samples were prepared: i) an additional 100 μL d₅-rotundone (516 ng ml⁻¹ in ethanol) was added to the bottle as internal standard (positive control); ii) without d₅-rotundone (negative control). Samples were shaken for 24 h at 22 °C, filtered (1.6 μm glass fibre), and topped up to 100 ml with Milli-Q water prior to SPE and SPME-GC-MS analyses, performed as above. For phloem exudates, the phloem exudates in EDTA and PIPES buffer solution were topped up to 100 ml with Milli-Q water and then subjected to SPE and SPME-GC-MS analyses as above.

6.2.11 Preparation of samples for rotundone quantification

Grape berries were prepared for rotundone analysis based on the published protocol (Geffroy et al. 2014). Non-grape materials samples were prepared for rotundone quantification based on a previously published protocol (Wood et al. 2008) with the following modification: 3 g of finely cut up grape peduncles/rachises, leaves, petioles and canes samples were soaked in mixture of 24 ml ethanol, 24 ml Milli-Q water, and 100 μL of d₅-rotundone (516 ng ml⁻¹ in ethanol as internal standard) for 72 h at 22 °C, then topped up to 200 ml with Milli-Q water and filtered prior to SPE and SPME-GC-MS analysis at the Australian Wine Research Institute following established protocols (Geffroy et al. 2014).

6.2.12 Preparation of phloem exudate samples for sesquiterpene analysis

Grape petiole phloem exudates were prepared for sesquiterpene analysis following established protocols (Parker et al. 2007). Phloem exudates were transferred into an HS-SPME vial (Agilent Technologies, Santa Clara, CA, USA;
20 ml) and mixed with 2 ml saturated brine and 500 μL α-copaene (200.64 μg L⁻¹ in ethanol) as internal standard and shaken for 24 h at 22 °C. Samples were analysed by SPME-GC-MS at the Australian Wine Research Institute following published protocols (Parker et al. 2007; Zhang et al. 2015b).

6.2.13 Statistical analysis

The rotundone concentration in different grapevine tissues at veraison and harvest were compared using one-way analysis of variance (ANOVA; p<0.05) in CoStat software to analyse statistical differences of means (veraison 6.4, CoHort Software, Monterey, USA). Grapevine leaf rotundone concentrations in herbivore, physical wounding, and phytohormone treatment groups were also compared using one-way ANOVA ( p<0.05) (CoStat, version 6.4, CoHort Software, Monterey, USA). The percentage physical damage on physical wounding leaf samples was analysed using customized code written in MatLab® ver. 2014a (The MathWorks, Inc., Matick, MA, USA). Pearson's test was used to assess correlations between grapevine leaf rotundone concentrations and physical damage (CoStat). A p-value <0.05 was considered statistically significant.
6.3 Results and discussion

6.3.1 Distribution of rotundone in different grapevine tissues

The distribution of rotundone in different vine tissues was studied by investigating the rotundone concentrations in individual vine shoots (Fig. 6.1). Significant increases in the concentration of rotundone in berries, leaves petioles and shoots were observed at harvest compared to veraison (Fig. 6.1a). Berries tended to have lower concentration of rotundone compared to non-grape tissues (Fig. 6.1a). Large variation in rotundone concentration was observed among grape and non-grape tissue samples across the vineyard (Supplementary Fig. 6.2), which was consistent with the previous report of large within-vineyard variation in rotundone (Zhang et al. 2015a). As a result, statistically significantly differences could hardly be established between grape tissues at different grapevine phenological stages, nevertheless, different grape tissues showed a similar rotundone production pattern across the whole ripening period (E-L 31 to E-L 38) (Supplementary Fig. 6.2).
Fig. 6.1. Comparison of rotundone production in non-grape materials and berries at a specific vineyard location in the 2013-14 growing season. (a) Concentration of rotundone among different non-grape materials and berries at veraison and harvest, where a, b, c, d and A, B, were used to label significant differences ($p<0.05$) among grapevine materials, while $\alpha$, $\beta$, $\gamma$ were used to label significant differences ($p<0.05$) between veraison and harvest. (b) Comparison of total rotundone content in individual vine shoot between veraison and harvest, where a, b were used to label the significant differences ($p<0.05$) between veraison and harvest, A, B were used to label the significant differences ($p<0.05$) between total rotundone content in leaves, petiole and peduncles/rachises and that of berries.
The total rotundone content was calculated for individual vine shoot together with the rotundone content of each grapevine material per shoot (Fig. 6.1b). The total rotundone content per shoot significantly increased from veraison to harvest, and was mainly contributed by the significantly increase of rotundone content in berries \( (p<0.05) \). The total content of rotundone from leaves, petioles and peduncles/rachises was significantly higher \( (p<0.05) \) than that of berries at both veraison (17.5-folds) and harvest (2.8-fold) (Fig. 6.1b). The rotundone content in these non-grape tissues could be especially important for wineries conducting whole-bunch fermentation, as extraction of peduncles/rachises, leaves and petioles during the fermentation process might lead to higher concentration of rotundone in resulted wine (Capone et al. 2012).
Fig. 6.2. GC-MS chromatograms of d$_5$-rotundone ($m/z$ 208 and 223) present in (A) positive control, (B) negative control, (C) treated leaves, (D) treated petioles, (E) untreated leaves, (F) untreated petioles, (G) grape berries and (H) grape peduncles/rachises sample. The positive control graph (A) used a different
scale of intensity compared to the rest graphs. The peaks near 43.1 mins and 43.3 mins are irrelevant to this study.

6.3.2 Translocation of rotundone between grapevine tissues

The positive d$_5$-rotundone control in stable isotope feeding trials showed abundant intensity peaks (Fig. 6.2A), while no peaks were observed in the negative control, as expected (Fig. 6.2B). There were no other compounds in grapevines with peaks around $m/z$ 208 and 223 at similar retention times; therefore, $m/z$ 208 and 223 are valid indicators of d$_5$-rotundone. In all samples, two small peaks near times 43.12 and 43.31 represented other compounds irrelevant to the current study, as these peaks were of different retention time compared to d$_5$-rotundone (43.22) (Fig. 6.2). Very small peaks corresponding to d$_5$-rotundone were observed in treated leaves (Fig. 6.2C), but not in the treated petioles (Fig. 6.2D). The d$_5$-rotundone detected in the treated leaf samples may correspond to residual d$_5$-rotundone solution used for leaf treatment or d$_5$-rotundone diffusion into the leaf tissues. No d$_5$-rotundone was detected in untreated parts of the grapevine (Fig. 6.2E, F, G, H), showing that d$_5$-rotundone translocation in the grapevine did not occur. Since only very small peaks corresponding to d$_5$-rotundone were detected in leaves in direct contact with high-concentration d$_5$-rotundone solution (Fig. 6.2C), it is possible that d$_5$-rotundone was not absorbed in whole leaf experiments. Therefore, leaves were cut to facilitate d$_5$-rotundone uptake in phloem extraction experiments.
6.3.3 Phloem exudate composition

As expected, the phloem exudate positive control samples exhibited abundant d$\textsubscript{5}$-rotundone peaks (Fig. 6.3A), while the negative control did not (Fig. 6.3B); the peak at time 42.78 represented another compound irrelevant to the current study, as the peak was of a different retention time compared to d$\textsubscript{5}$-rotundone (42.9) irrelevant to rotundone/d$\textsubscript{5}$-rotundone. Furthermore, no significant peaks corresponding to d$\textsubscript{5}$-rotundone were observed in both whole and cut leaf phloem exudates. For phloem translocation, it is essential that the compound is mobilised from the source tissue. The translocation process may involve the
passive symplastic pathway or the active apoplastic pathway (Turgeon and Wolf 2009). For a secondary metabolite like rotundone, active transport may be necessary (Gholami et al. 1995). Grapevines might not possess the latter mechanism, and therefore, rotundone translocation via the phloem may not be possible in Shiraz grapevines or the phenomenon was not detectable by the techniques used here.

Fig. 6.4. Chromatograms showing the terpenoids in phloem sap extracted from the petiole: (1) geraniol; (2) clovene; (3) α-ylangene; (4) α-copaene (internal standard); (5) β-copaene; (6) α-muurolene; (7) δ-cadinene; (8) cis/trans-calamenene.

The sesquiterpene composition of phloem exudates was further investigated (Fig. 6.4). Neither rotundone nor its precursor α-guaiene was detected, while one monoterpen, namely geraniol, and six sesquiterpenes (clovene, α-ylangene, β-copaene, α-muurolene, δ-cadinene, and cis/trans-calamenene) were observed. These results further confirm that rotundone is not translocated via the phloem, since rotundone was detectable at high levels in the leaves of potted vines (225.2 ± 7.2 ng kg\(^{-1}\)). Rotundone was also detected in petiole tissues (25.0±4.1 ng kg\(^{-1}\)), suggesting that rotundone may be produced in the
petiole but cannot be translocated. Most of the sesquiterpenes detected in this study (except cloven) are products of germacrene D, which is biosynthetically related to a large group of plant sesquiterpenes (Zhang et al. 2015b; Bülow and König 2000). The sesquiterpenes detected may be biosynthesised by phloem cells as suggested previously (Hampel et al. 2005) or translocated from other grapevine tissues, such as the leaves. This requires further investigation.

![Fig. 6.5.](image)

**Fig. 6.5.** Influence of herbivore and mimic herbivore attack on the concentration of rotundone in Shiraz grape leaves. Comparison of rotundone concentration in grape leaves among (A) light brown apple moth larvae treatment groups; (B) plant hormones treatment groups; (C) physical wounding treatment groups. (D) The relationship between rotundone concentration in leaves and the percentage of leaf area damage based on results of physical wounding treatments. The equation for the trend line is \( y = 10.26x + 78.38 \) (\( R^2 = 0.58 \), RMSE = 15.59, \( p = 0.0004 \)).

6.3.4 *Influence of herbivores, physical wounding, and phytohormones*
No significant differences in leaf rotundone concentrations were observed between herbivore treatments and controls (Fig. 6.5A). This might be related to large variations in rotundone concentrations between leaves. Nevertheless, samples from leaves that had been attacked by light brown apple moth larvae showed a tendency to have higher rotundone concentrations compared to control samples. Even though terpenoids are major secondary metabolites induced by herbivore attacks in other plant species (Arimura et al. 2005; Howe and Jander 2008), in *V. vinifera* rotundone may not be produced as a consequence of feeding by light brown apple moth larvae.

Slight increases in leaf rotundone concentration were only observed with phytohormone treatment with both ACC and JA, but not any other treatment group (Fig. 6.5B). This may be explained by the known synergistic effect of ACC and JA signalling, which has been shown to induce higher expression of genes involved in plant herbivore defences (Bari and Jones 2009). Furthermore, application of JA and MeJA individually did not significantly alter leaf rotundone concentrations, consistent with a previous report in field-grown *V. vinifera* L. cv. Duras vines (Geffroy et al. 2014). The SA signalling pathway is different from that of ACC and JA in regulating herbivore-induced terpene synthesis (Pieterse et al. 2012). A previous study in grape cell culture showed that SA and JA may be antagonistic (D’Onofrio et al. 2009). Here, exogenous SA did not significantly alter leaf rotundone concentrations. Our results suggest that herbivores may induce rotundone biosynthesis via activation of ACC and JA together, but not via other signalling pathways.
All three physical wounding conditions were associated with lower leaf rotundone concentrations, with high-density wounding resulting in the lowest rotundone concentration (Fig. 6.5C). In addition, the percentage of damaged leaf area was negatively correlated to leaf rotundone concentrations (Fig. 6.5D). There are two possible explanations for these observations: i) mechanical wounding might induce the biosynthesis of other terpenoids at the expense of rotundone (Bricchi et al. 2010; Mithöfer et al. 2005). ii) mechanical damage to the leaves could increase rotundone degradation, since this compound can be readily oxidized from its precursor α-guaiene (Huang et al. 2014).

Overall, rotundone concentrations in grapevine leaves depend on physical wounding and herbivore-associated elicitors. Physical wounding might reduce rotundone concentrations in grapevine leaves, while ACC and JA elicitors can increase it. As a result, the rotundone concentration in leaves could be under complex regulatory control and may not necessarily be modified by herbivore attack, explaining the observed results of herbivore treatment (Fig. 5.5A).
6.4 Conclusion

In this study, we investigated rotundone distribution in different grapevine tissues, and confirmed that non-grape tissues contain significantly higher concentration and total amounts of rotundone than the berries. This suggested that non-grape tissues can be a major source of rotundone, which is of particular interest for wineries conducting whole-bunch fermentation and wishing to maximise this aroma compound in wine. This study further examined rotundone transport between different grapevine tissues via the phloem, and concluded that under these study conditions no phloem-mediated translocation of rotundone was detectable. Phloem exudate analysis of sesquiterpenes further excluded the possibility that its precursor, α-guaiene, was translocated in the phloem. For the first time, geraniol, cloven, and five germacrene D-derived sesquiterpenes were detected in phloem exudates, indicating possible interactions between different grapevine tissues in the production of these compounds. Two aspects of herbivore attack, physical damage and the effect of chemical elicitors, had opposing effects on rotundone concentrations in grapevine leaves. As a result, the overall influence of herbivores on leaf rotundone concentrations is uncertain. It is possible that rotundone constitutes a member of herbivore-induced terpenes, but not the major one.

Author contribution PZ, SF, EWB, MK, MH and KH conceived and designed research. PZ, YW and RD conducted experiments. MH contributed analytical tools. PZ, KH, YW, RD and SF analyzed data. PZ and KH wrote the manuscript. All authors read and approved the manuscript.
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6.5 References


### 6.6 Supplementary data

#### Supplementary Table 6.1. Weather record of the vineyard in 2012-13 and 2013-14 seasons

(Data from Ararat Prison weather station, Australian Bureau of Meteorology Station No. 089085 – located 15.5kms NW to the experimental site). The date of 80% veraison is taken at 19th Feb 2013 and 28th Feb 2014 and commercial harvest begins on 10th Apr 2013 and 8th Apr 2014.

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<td>Mean January Minimum Temperature</td>
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Supplementary Fig. 6.1. Chemical structure of (A) (-)-rotundone and (B) d5-rotundone.
Supplementary Fig. 6.2. Comparison of rotundone concentration among non-grape materials and berries at different grapevine phenological stages in the 2013-14 growing season. The grape materials were collected in the vineyard, and were the average of the area. a, b, c, d and A, B were used to label significant differences ($p<0.05$) among different phenological stages, while $\alpha$, $\beta$, $\gamma$ were used to label significant differences ($p<0.05$) among different grapevine materials.
CHAPTER SEVEN

General Discussion and Conclusion

In this research, the concentration of rotundone in wine produced from different growing seasons were compared to historical weather data obtained from Australian Bureau of Meteorology weather stations and Australian Water Availability Projects climate maps. Results showed that fruit zone air temperature, daily solar exposure and vineyard water balance were identified as the most important environmental factors affecting rotundone concentration in wine. The importance of the fruit zone air temperature was further analysed by studying the variations in rotundone within the same vineyard, the same vine and the same bunch. Furthermore, berry surface temperature and fruit zone air temperatures were negatively correlated to the concentration of rotundone in Shiraz grapes at harvest. Higher concentration of rotundone were found in grapes from the following locations: less exposed canopy side to the sun of the vineyard; south facing side of rows and more shaded bunch sectors. The percentage of degree hours above 25 °C within the fruit zone was negatively correlated to the concentration of rotundone in grape berries. The influence of solar exposure on grape rotundone was also investigated by excluding sunlight from grape bunches. Significantly reduced concentrations of rotundone and α-guaiene were observed in grapes at harvest for sunlight exclusion treatments. The impacts of sunlight exposure can be divided into two parts: i) direct illumination and ii) indirect solar radiation induced temperature increase. Solar radiation inducing increments in temperature would negatively affect the concentration of rotundone in grape berries, while higher direct illumination could increase the concentration of rotundone due to the contrary effect. It is highly possible that the biosynthesis pathway from α-guaiene to rotundone is light sensitive, and UV radiation could be the active factor determining this process. Apart from rotundone and α-guaiene, sunlight exclusion also significantly modified the concentration of other 7 sesquiterpenes and 1 monoterpene in grape berries.
Our preliminary study showed that vineyard environmental factors from the veraison to harvest period of grape development were critical to the final concentration of rotundone in grapes at harvest. This project investigated the concentration of rotundone and other terpenoids in grape berries at different phenological stages of grapevine, and observed relatively higher concentration of rotundone and α-guaiene at the fruit setting and harvest stages. However, a significantly lower concentration at veraison was also found. The period from veraison to harvest was identified as the most important time for rotundone accumulation in grape berries, and therefore they are very important for the final rotundone concentration at harvest. The production patterns from different terpenoids during berry development were linked to their biosynthetic pathway. Terpenoids derived from similar biosynthetic pathway had a similar production patterns. Therefore, it is highly likely that rotundone could be produced from a similar biosynthesis pathway as α-guaiene.

The production pattern of rotundone during berry development in non-grape organs was similar as that in grape berries. However, production of rotundone in other grape organs may not be directly related to that in grape berries, as rotundone could not be actively transported from non-grape organs into grape berries via the phloem. Nevertheless, within a shoot, non-grape organs contained higher amount of rotundone compared to berries and could be extracted during primary fermentation, and this could contribute to the rotundone concentration in resultant wine, especially for wineries doing whole-bunch fermentation and machine harvest. The concentration of rotundone in grapevine leaves could also be affected by herbivore behaviours in two ways: i) leaf damage, which could reduce the concentration of rotundone in leaves; ii) elicitors secreted by the herbivores, which could slightly increase the concentration of rotundone in leaves. However, results were inconclusive for rotundone been produced by grapevines as herbivore-induced terpenoids, since only small changes in rotundone concentration were observed in this study.

This project has demonstrated that environmental factors, including grape surface temperature and bunch zone air temperature, irrigation and solar exposure, affect the concentration of rotundone in grape berries at harvest. Vineyard vigor, aspect, vine row direction, bunch exposure and canopy
morphology also affect the temperature and sunlight exposure, and therefore also determine the concentration of rotundone in grape berries indirectly. Rotundone in grapes and other non-grape organs is produced at both, pre-veraison and post-veraison periods of berry development. However, post-veraison is more critical to the concentration of rotundone in berries at harvest. Even though rotundone could not be translocated from non-grape organs to grape berries, higher concentration of rotundone in non-grape organs could still contribute to the rotundone content in wine by conducting machine harvest and whole bunch fermentation practices. Vineyard herbivore activity may also affect the concentration of rotundone in grape berries, although the impacts are less important compared to other parameters studied in this project.

**Strategies Designed for Grape Growers**

Based on this study, a management strategy has been designed to help grape growers to achieve more peppery Shiraz grapes and wines:

1. Vineyard management
   a) Increase the irrigation volume before veraison to stimulate vine canopy development, and reducing bunch zone air temperature. This needs to be performed taking in consideration the balance between the vegetative and reproductive organs from vines.
   b) Spring pruning should be avoided for the low vigor vineyard areas, especially for vineyard with northerly aspect in southern atmosphere.

2. Harvest
   a) Harvest time should be as late as possible without affecting other quality attributes of grape berries.
   b) Differential harvest could be conducted for grape bunches from the shaded side of the row in the high vigor and/or southerly aspect area of the vineyard, which could be used to produce premium high peppery Shiraz wines.
   c) Machine harvest should harvest grapes from the high vigor vineyard area separately from the low vigor vineyard area.

3. Fermentation
a) Hand-picked grape bunches described above should be fermented as whole bunch to maximize rotundone concentration in wine.
b) Wine made from machine harvest grapes may also contain relatively high concentration of rotundone.
CHAPTER EIGHT
List of References


Appendix

A. Potted vine establishment

Dormant *Vitis vinifera* cv. Shiraz grapevine cane cuttings were collected randomly from a commercial vineyard (the Old block, Mount Langi Ghiran) in July 2012. The vine cuttings were propagated following the protocol described by Nicholas et al. (1992). Briefly, the cuttings were shortened to 3 nodes in length, sanitized using 30% ethanol, treated with indolebutyric acid (IBA) at the base, and placed in sterilized propagation mix (Table A.1). Propagation trays were then placed in an incubator equipped with head-bed and mist propagator, and maintained at 24 °C and 95% relative humidity for 4 weeks (Figure A.1).

Table A.1 Composition of propagation mix

<table>
<thead>
<tr>
<th>Composition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>25%</td>
</tr>
<tr>
<td>Perlite</td>
<td>62.5%</td>
</tr>
<tr>
<td>Peat</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

Figure A.1 Shiraz vine cuttings in the propagation hood

4 weeks later, vine cuttings with more than 20 roots were planted in 4.5 litre pots and maintained at 20°C in a glasshouse (System Garden, Parkville, The
University of Melbourne). A commercial NPK fertilizer (Osmacote, Scotts Australia Pty Ltd, NSW, Australia) was used to provide general nutrients. An automatic drip irrigation system was installed for the potted vines with a set irrigation volume of 0.85 L per vine per day.

Every 8 months, the potted vines were placed in a cool room (4°C) for 6 weeks, and forced into dormancy. The dormant potted vines were then pruned and brought back to the glasshouse to start a new growth cycle. By doing this, the grapevine phenological cycle was shorten, which facilitated glasshouse experiments.

Reference
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