An examination of malate metabolism in *Vitis vinifera* during fruit ripening and in response to elevated vineyard temperature

by

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Abbreviations

ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ANGIS	Australian National Genomic Information Service
AOX	Alternative oxidase
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
CH/BL	Chamber / blower heat treatment
CoA	Coenzyme A
COX	Cytochrome oxidase
Ct	Cycle threshold
DEDTC	Diethyldithiocarbamate
DFCI	Dana Farber Cancer Insitute
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
KCN	Potassium cyanide
MDH	Malate dehydrogenase
ME	Malic enzyme
MES	2-(*N*-morpholino)ethanesulfonic acid
MPA	Metaphosphoric acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NAD(P)	NAD and NADP
NAD(P)H	NADH and NADPH
NCBI	National Centre for Biotechnology Information

OAA	Oxaloacetate
OG	Octyl gallate
OTC	Open top chamber heat treatment
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PEG	Polyethylene glycol
PEP	Phospho*enol*pyruvate
PEPC	Phospho*enol*pyruvate carboxylase
PEPCK	Phospho*enol*pyruvate carboxykinase
PEPCkin	Phospho*enol*pyruvate carboxylase kinase
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
mRNA	Messenger RNA
RT-PCR	Reverse transcriptional PCR
qRT-PCR	Quantitative real-time PCR
SA	Surface area
T_a	PCR cycle annealing temperature
T_m	PCR cycle melting temperature
TCA	Tricarboxylic acid
TES	3-(*N*-morpholino)propanesulfonic acid
TMPD	*N*,*N*,*N*',*N*'-tetramethyl-p-phenylenediamine
WGS	Whole genome shotgun
bp / kb	Base pairs / kilobases
˚C	Degrees, Celsius
g/mg/µg/ng	Gram / milligram / microgram / nanogram
L/ml/µl	Litre / millilitre / microlitre
M	Molar (moles.litre^{-1})
mM/µM/nM	Millimolar / micromolar / nanomolar
v/v	volume / volume
w/v	weight / volume

Summary

Acidity is an important characteristic for many fruits, particularly in aspects of fruit biochemistry and sensory quality. In grapes (*Vitis vinifera*), one of the most important agricultural fruiting crops, the use of fruit with low acidity and high pH in winemaking can increase risk of microbial spoilage and undesirable fermentative outcomes. Levels of the organic acid malate can determine acidity and pH in many fruits, through species- and cultivar-specific regulation of developmental and environmental responses. Malate is also extensively involved in primary and secondary metabolic pathways that can be critical to fruit development and ripening. The grape berry demonstrates particularly striking changes in malate accumulation and degradation during development and in response to vine temperature, likely driven by changes in metabolic pathways involving the acid. If harvested late in the season, or if ripening berries are exposed to unusually warm temperatures, the fruit are likely to contain less-than-optimal acid due to reduced levels of malate. Despite the considerable influence that berry acidity imparts on berry and wine quality, the mechanisms of regulation for malate metabolism in response to changes in grape berry development and vine temperature are still largely unknown. This thesis contains an evaluation of the activities and transcripts of enzymes in *V. vinifera* fruit that are involved in processes such as glycolysis, gluconeogenesis, CO_2 assimilation, respiration and fermentation, each involving malate either directly or indirectly. Enzymes from purified grape berry mitochondrial preparations were also explored, providing evidence for two activities that were, until now, undetermined in grapes: NAD-dependent malic enzyme and alternative oxidase. A developmental series of grapes from fruit set until harvest maturity displayed the expected pattern of pre-véraison malate accumulation, and post-veraison malate loss. Activities or transcripts that were differentially regulated with development included NADP-dependent malic enzyme, PEP carboxylase, PEP carboxykinase, pyruvate, orthophosphate dikinase, alcohol dehydrogenase and the terminal oxidases of the mitochondrial transport chain. To test the effect of elevated vine temperature on grape berry malate, field-based heat trials were established at numerous developmental stages. Temperature experiments were set up as mild, long-term heating trials to mimic a climatic shift, as well as shorter, more intense trials to represent heat-wave events. Effects of raising bunch temperature during the day and night were also investigated. Heating of grapevines at the post-set developmental stage (i.e. malate accumulation phase) led to higher berry malate,

particularly when bunches were heated at night. Heating of grapevines around the véraison and pre-harvest stages (i.e. malate degradation phase) generally led to lower berry malate, unless bunches were also heated at night, or if the majority of malate had already been lost from the fruit before the time of treatment. Data indicated that the temperature-sensitive changes in grape berry malate during the day and/or night at different developmental stages could be linked to alterations in PEP carboxylase, PEP carboxykinase, pyruvate, orthophosphate dikinase and NAD-dependent malic enzyme. Implications of such shifts in metabolism are discussed in detail within the text.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Statement of the contributions of jointly authored papers

1. **Sweetman, C., Deluc, L.G., Cramer, G.R., Ford, C.M., Soole, K.L.** Regulation of malate metabolism in grape berry and other developing fruits. Review published in *Phytochemistry* on 15th September, 2009.

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Author Contributions: CS designed and conducted all research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and design, and the editing of the manuscript.

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Author Contributions: CS designed and conducted all research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and design, and the editing of the manuscript.

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Author Contributions: CS assisted with experimental design of the CH/BL field experiment, collected materials, conducted all laboratory research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and the editing of the manuscript. VOS designed field experiments and assisted with data analysis and editing of the manuscript.

The following authors agree that the Statement of the contributions of jointly authored papers accurately describes their contribution to research manuscripts 1., 2., 3., and 4. and give consent to their inclusion in this thesis.

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Regulation of malate metabolism in grape berry and other developing fruits

Regulation of malate metabolism in grape berry and other developing fruits

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1.1. Abstract

Organic acids are present in all plants, supporting numerous, and varied facets of cellular metabolism. The types and levels of plant organic acids are extremely variable between species, developmental stages and tissue types. Furthermore, acidity plays important roles in the organoleptic properties of plant tissues, where examples of both enhanced and reduced palatability can be ascribed to the presence of specific organic acids. In fruits, acids such as citrate, malate, oxalate, quinate, succinate and tartrate are responsible for sour tastes, which may be particularly noticeable in un-ripened produce. Acidity imposes a strong influence on crop quality, and is an important factor in deciding the harvest date, particularly for fruits where acidity is important for further processing, as in wine grapes. In the grape, as for many other fruits, malate is one of the most prevalent acids, and is an important participant in numerous cellular functions. Accumulation of malate is thought to be due in large part to *de novo* synthesis in fruits such as the grape, through metabolism of assimilates translocated from leaf tissues, as well as photosynthetic activity within the fruit itself. During ripening, the processes through which malate is catabolised are of interest for advancing metabolic understanding, as well as for potential crop enhancement through agricultural or molecular practices. A new body of literature describes research that has begun to unravel the regulatory mechanisms of enzymes involved in malate metabolism during fruit development, through exploration of protein and gene transcript levels. Datasets derived from a series of recent microarray experiments comparing transcript levels at several stages of grape berry development have been re-visited, and presented here with a focus on transcripts associated with malate metabolism. Developmental transcript patterns for enzymes potentially involved in grape malate metabolism have shown that some flux may occur through pathways that are less commonly regarded in ripening fruit, such as aerobic ethanol production. The data also suggest pyruvate as an important intermediate during malate catabolism in fruit. This review will combine an analysis of microarray data with information available on protein and enzyme activity patterns in grapes and other fruits, to explore pathways through which malate is conditionally metabolised, and how these may be controlled in response to developmental and climatic changes. Currently, an insufficient understanding of the complex pathways through which

malate is degraded, and how these are regulated, prevents targeted genetic manipulation aimed at modifying fruit malate metabolism in response to environmental conditions.

1.2. Introduction

1.2.1. Fruit acidity and malate

Fruits contain a wide variety of compounds, including organic acids that dictate acidity throughout development. Malate is the predominant acid in many fruits, both climacteric, including plum (Singh and Singh, 2008), tomato (Kortstee et al., 2007), peach (Wu et al., 2005), apple (Beruter, 2004), kiwifruit (Walton and De Jong, 1990), mango (Selvaraj and Kumar, 1989), banana (Agravante et al., 1991), pear (Chen et al., 2007), fig (Shiraishi et al., 1996) and peach (Moing et al., 1998) and non-climacteric, including pineapple (Saradhuldhat and Paull, 2007), lime, orange, lemon (Albertini et al., 2006), cherry (Usenik et al., 2008), strawberry (Moing et al., 2001) and grape (Kliewer et al., 1967). While organic acids are chiefly in the ionised form at cellular pH, for the purposes of this review the term “malate”, which technically describes the conjugate base, will be used to refer to all physiological forms of the compound. This nomenclature will be maintained for all organic acids mentioned in the text.

Malate can play a variety of roles in plants, from controlling stomatal aperture, improving plant nutrition, and increasing resistance to heavy metal toxicity (Fernie and Martinoia, 2009; Schulze et al., 2002), to other processes more intricately linked with metabolic pathways, some of which are represented in Figure 1.1. Patterns of malate accumulation differ between plant species, and even cultivars (Kliewer et al., 1967; Selvaraj and Kumar, 1989). In fruit, patterns of malate accumulation and degradation cannot be explained by the classification of species as climacteric or non-climacteric, nor can they be attributed to changes in overall respiration rates. Some climacteric fruit such as plum and tomato appear to utilize malate during the respiratory burst (Goodenough et al., 1985; Kortstee et al., 2007; Singh and Singh, 2008), while others such as mango and banana continue to accumulate malate throughout ripening, even at the climacteric stage (Agravante et al., 1991; Selvaraj and Kumar, 1989). Non-climacteric fruits also display widely varying malate

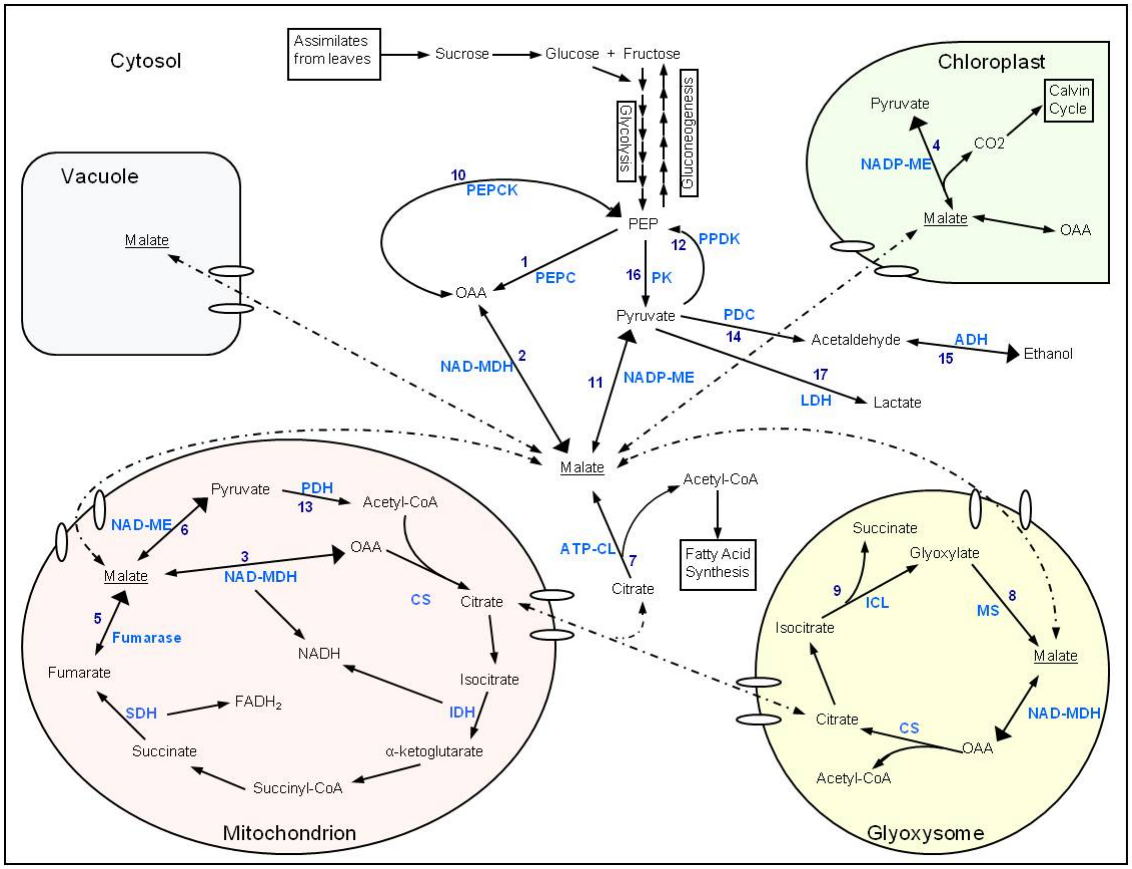

Figure 1.1: Potential Metabolic Pathways Involving Malate in Fruit Cells. ADH, alcohol dehydrogenase; ATP-CL, ATP-citrate lyase; CS, citrate synthase; ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MS, malate synthase; NAD-ME, NAD-linked malic enzyme; NAD-MDH, NAD-linked malate dehydrogenase; NADP-ME, NADP-linked malic enzyme; NADP-MDH, NADP-linked malate dehydrogenase; PDC, pyruvate decarboxylase; PDH; pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PPDK, pyruvate orthophosphate dikinase; SDH, succinate dehydrogenase. Favoured direction for reversible reactions is indicated by larger arrowhead.

accumulation and degradation events (Albertini et al., 2006; Moing et al., 2001; Saradhuldhat and Paull, 2007). However, it should be noted that most of the studies listed above have depicted malate accumulation in fruits as various concentration units or percentages, rather than on a whole fruit basis. It is difficult to determine whether malate losses shown in these data are due to active metabolism, or dilution as the fruits become larger. Both situations will result in a decrease in malate concentration (but not total fruit content), as demonstrated by Walton and De Jong (1990) in kiwifruit and Famiani *et al*. (2005) in numerous other fruits. Fruit malate levels can also be dramatically affected by exposure of the plant to various environmental conditions (Lakso and Kliewer, 1978; Richardson et al., 2004; Ruffner et al., 1976).

1.2.2. Malate in fruit development

In the non-climacteric fruit of *Vitis vinifera* (grape), malate metabolism has been a strong focus of research, as the balance of acids in winegrape must (juice) is central for supporting desirable growth (and preventing undesirable growth) of microorganisms responsible for wine fermentation. Malate concentration can also affect final wine characteristics through involvement in secondary processes such as carbonic maceration and malolactic fermentation, and can even alter the growth capabilities of malolactic bacteria (Kunkee, 1991). Unlike many other fruits, grapes do not contain large amounts of citrate, and the large quantity of tartrate present in the fruit is not used in primary metabolic pathways. Therefore malate is the only high-proportion organic acid that is actively metabolised throughout ripening of grapes. Combined with the ever-improving density of genetic information available for *V. vinifera*, grapes make an ideal system for studying the metabolism of malate during ripening of non-climacteric fruit. A large body of work has also been reported for tomato acid metabolism, which is used to exemplify a climacteric equivalent to grapes in this review.

Loss of grape berry malate is due to metabolic degradation during ripening, which occurs after an earlier period of accumulation. The switch from net accumulation to degradation of malate occurs just before veraison, or the inception of ripening (Ruffner and Hawker, 1977). Pre-veraison grapes accumulate malate mostly through the metabolism of sugars that have been translocated to the berry, but also potentially through fruit photosynthesis (Hale,

1962). In post-veraison fruit, malate is liberated from the vacuole and becomes available for catabolism through various avenues, including the TCA cycle and respiration, gluconeogenesis, amino acid interconversions, ethanol fermentation, and the production of complex secondary compounds such as anthocyanins and flavonols (Famiani et al., 2000; Farineau and Laval-Martin, 1977; Ruffner, 1982; Ruffner and Kliewer, 1975). With the accumulation of sugars and inhibition of glycolysis in ripening grapes (Ruffner and Hawker, 1977), malate is likely a vital source of carbon for these pathways.

Some fruits, including mango, kiwifruit and strawberry may rely less on malate, and more on the hydrolysis of accumulated starch as a source of carbon for biosynthesis and energy metabolism during ripening (Han and Kawabata, 2002; Moing et al., 2001; Selvaraj and Kumar, 1989). These particular species' display no net loss of malate throughout ripening (Moing et al., 2001; Ruffner, 1982; Selvaraj and Kumar, 1989; Walton and De Jong, 1990). For this reason, the metabolism of malate will be further explored with a focus on grape and tomato fruits, in which the acid plays a more metabolically active role (Goodenough et al., 1985; Ruffner, 1982).

1.3. Pathways of malate synthesis in fruit

In pre-veraison grape berries, sucrose transported from the leaves is broken down to glucose and fructose, which can enter glycolysis for use in respiration. Therefore translocated sucrose acts as the major fuel for ATP synthesis, and also enables synthesis of malate in the fruit. Enzymes responsible for this synthesis are present and active in grapes (Hawker, 1969; Taureilles-Saurel et al., 1995a, b). Photosynthesis is another means of synthesising malate in the berry (Hale, 1962), and reaches a peak rate approximately one week before veraison, before decreasing rapidly throughout the remainder of berry development (Pandey and Farmahan, 1977).

Despite the focus of numerous radiolabelling experiments on pathways of sugar and organic acid metabolism in grapevine leaves and berries (Beriashvili and Beriashvili, 1996; Hardy, 1968; Kliewer, 1964), it is yet to be absolutely determined whether labeled berry analytes such as malate are exclusively synthesised *in situ*, or if some proportion is transported in

this form from other tissues. For the purposes of this review, it is assumed that most of the malate in fruit comes from biosynthesis within the fruit cells, whether this occurs through photosynthetic assimilation, or through catabolism of sugars translocated from the leaves. This may be a fair assumption, as phloem disruption by girdling did not alter malate content in grapes at any stage of development (Hunter and Ruffner, 2001), despite the potential for organic acid translocation from leaves to fruits (Hardy, 1969).

During grape ripening, milimolar concentrations of malate were shown to accumulate in the berry apoplast (Wada et al., 2008), coinciding with a switch of phloem unloading from a symplasmic route (via plasmodesmata) to an apoplasmic route (Zhang et al., 2006). The accumulation of malate in the apoplast, along with other acids and sugars, may be related to the control of fruit turgor at veraison (Wada et al., 2008), rather than metabolism.

1.3.1. Glycolysis: PEPC-MDH reaction (pyruvate kinase bypass)

Pyruvate is generally the major product of glycolysis, whereby phosphoenolpyruvate (PEP) is directly converted to pyruvate through the activity of pyruvate kinase. However plant cells can alternatively produce malate from PEP through the activities of phosphoenolpyruvate carboxylase (PEPC; Figure 1.1, indicated by reaction “1”) and malate dehydrogenase (MDH; Figure 1.1, “2”) as discussed by (Givan, 1999). In potato tuber, glycolytic intermediates accumulated when NAD-dependent malic enzyme activity (Section 1.4.2.3) was reduced (Jenner et al., 2001), suggesting that the conversion of malate to pyruvate can influence glycolytic flux in plants. This alternate route bypasses pyruvate kinase activity and may be necessary in situations where ADP becomes limiting (Borsani et al., 2009; Plaxton, 1996).

1.3.1.1. Phosphoenolpyruvate carboxylase (PEPC)

PEPC (E.C. 4.1.1.31) isoforms can be found in multiple plant tissues, some of which are more specific to fruit (Guillet et al., 2002). The developmental regulation of fruit PEPC activity occurs at different levels. At the transcriptional level, PEPC gene isoforms tend to be more favourably expressed early in development (Guillet et al., 2002; Or et al., 2000b; Terrier et al., 2005), as malate levels increase. However in peach, one or more PEPC genes were also upregulated during the ripening phase, coinciding with a drastic accumulation of citrate and loss of malate (Moing et al., 2000). Recent data from Deluc *et al*. (2007), have been

reorganised with a focus on malate metabolism for the purposes of this review, and can be seen in Figures 1.2 and 1.3 (accompanied by Tables 1.1 and 1.2, respectively). These data

revealed a decrease in two putative grape PEPC isogenes from veraison (Figure 1.2), which could potentially alter the amount of PEPC activity seen in fruits during ripening. A high level of PEPC activity has been demonstrated during early fruit development, coinciding with malate accumulation and decreasing sharply just prior to the initiation of ripening (Diakou et al., 2000; Guillet et al., 2002; Hawker, 1969; Moing et al., 2000; Ruffner et al., 1976). In grapes, this decline in PEPC activity coincided with observed decreases in CO_2 dark-fixation and glycolytic flux, and may be indicative of PEPC involvement in photosynthesis and sugar breakdown in fruit (Hawker, 1969; Ruffner and Hawker, 1977). At the protein level, grape and tomato PEPC displayed a different pattern to that of gene expression and enzyme activity, whereby protein accumulation was slightly delayed, and retained into the ripening stages (Diakou et al., 2000; Guillet et al., 2002). This may be explained by regulation at the post-translational level, via the phosphorylative activity of PEPC kinase. In the phosphorylated form, PEPC is more active and has reduced sensitivity to malate inhibition (Chollet et al., 1996). Diakou *et al*. (2000) found that the sensitivity of PEPC to sub-optimal pH conditions and inhibition by malate was low in pre-veraison grape berries, but increased during ripening. This was also seen for cherry tomatoes (Guillet et al., 2002). Taken together, one can assume that either the expression of a specific PEPC isoform with these characteristics is favoured during this time, or a change in phosphorylation state of the enzyme has occurred.

Regardless, the enzyme activity may be expected to be higher due to phosphorylation in early fruit development, despite continued high levels of PEPC protein later on. The reason for retaining PEPC protein in the cell may be to ensure that carbohydrate metabolism can still occur when necessary. In particular, PEPC activity can be involved in cytosolic pH balance, and regulation between carbohydrate and nitrogen metabolism (Law and Plaxton, 1995).

1.3.1.2. Malate dehydrogenase (MDH)

MDH catalyses a reversible reaction between OAA and malate, and functions in the maintenance of equilibrium between these two intermediates. However, if cytosolic malate

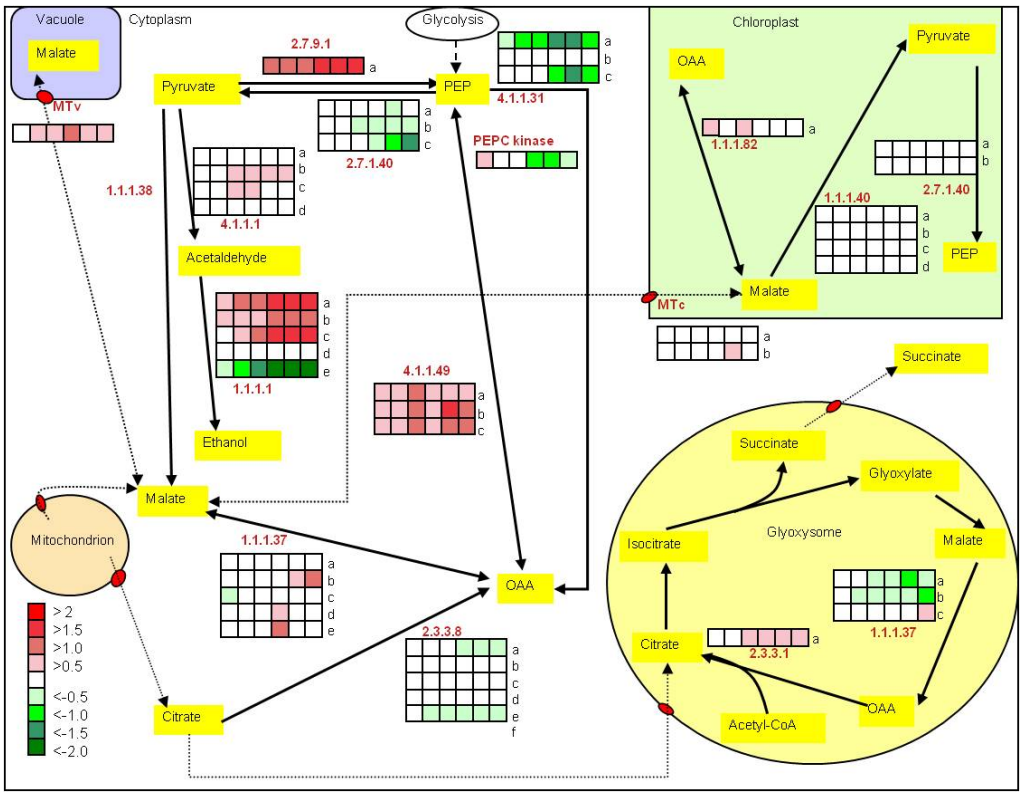

Figure 1.2: Transcript Levels of Extra-mitochondrial Enzymes Involved in Malate Metabolism in *V. vinifera* cv. Cabernet Sauvignon. Changes in gene transcript levels during development of Cabernet Sauvignon grapes were recomputed from the microarray dataset of Deluc *et al*. (2007), at PLEXdb.org. This figure reviews data from enzymes located in the chloroplast, glyoxysome and cytosol. Red text depicts EC numbers of enzymes that were examined for gene expression. Coloured squares illustrate a change in gene transcript level. Rows of squares indicate specific, putative gene isoforms, and columns indicate stage of berry development (increasing development from left to right). The change in expression (log 2 ratio) for each developmental stage is relative to stage 31 (pea-size grapes, as defined by Coombe, 1995). In sequence order (left to right), berries are displayed from stages; 32, bunch closure; 33, still green and hard; 34, softening; 35, veraison; 36, increasing ºBrix and; 38, harvest. Colour from the first column indicates an increase or decrease in fold change of gene transcript, observed between stage 32 and 31. The next column illustrates a change between stage 33 and 31, and so on. Putative gene identities corresponding to EC numbers are given in Table 1.1, along with initial expression values (from stage 31). Enzyme names for each catalytic step can also be seen in Figure 1.1.

Table 1.1: Relative Expression Values for Putative Genes of Malate-metabolising Enzymes from *V. vinifera* cv. Cabernet Sauvignon (External to Mitochondria)

EC Number	Enzyme Prediction[a]	Probe Set ID (PLEXdb)	Genbank Accession	Relative Expression Value[b]
CYTOSOL				
EC 4.1.1.31				
	PEPC (a)	1608100_at	CF404013	2312.889
	PEPC (b)	1611103_at	CF215746	1022.933
	PEPC (c)	1616325_at	CF211815	897.7015
	PEPC kinase	1622074_at	BQ794083	430.1473
EC 1.1.1.37				
	cytMDH (a)	1606799_s_at	CB968910	3535.976
	cytMDH (b)	1609147_at	CB979150	104.3754
	cytMDH (c)	1607043_at	BQ793537	2024.278
	cytMDH (d)	1610035_x_at	CF202356.1	348.0234
	cytMDH (e)	1617497_at	CF202356.1	141.8007
EC 2.7.1.40				
	cytPK (a)	1618753_at	BQ796362	2320.697
	cytPK (b)	1610440_s_at	BQ798062	1509.997
	cytPK (c)	1611154_at	CF204490.1	190.5634
EC 4.1.1.1				
	PDC (a)	1609684_at	CB009168	1715.221
	PDC (b)	1621682_s_at	CF404793	1235.621
	PDC (c)	1611322_at	CF202963.1	1409.543
	PDC (d)	1620983_at	CF207286	11.35753
EC 1.1.1.1				
	ADH (a)	1616500_at	AF194175.1	366.4613
	ADH (b)	1614086_at	AF194173.1	402.5674
	ADH (c)	1608263_a_at	BQ794795	1540.131
	ADH (d)	1606537_at	CD720196	11.02247
	ADH (e)	1619190_at	CD720196	110.9075
EC 2.7.9.1				
	PPDK	1612414_at	CD715284	34.09634
EC 4.1.1.49				
	PEPCK (a)	1621034_at	CF406031	2871.111
	PEPCK (b)	1616630_at	CF603093	1291.988
	PEPCK (c)	1617265_a_at	CF603093	1934.327
EC 2.3.3.8				
	ATP-citrate lyase (a)	1618839_s_at	CF209938	2550.119
	ATP- citrate lyase (b)	1609985_at	CF403736	345.4927
	ATP- citrate lyase (c)	1618362_s_at	CF200684.1	566.3902
	ATP- citrate lyase (d)	1607329_at	CF568978	15.72275
	ATP- citrate lyase (e)	1622766_at	CF209938	1352.295
	ATP- citrate lyase (f)	1622588_s_at	CF207335	1510.961
CHLOROPLAST				
EC 2.7.1.40				
	chlPK (a)	1614016_at	CB008961	278.0493
	chlPK (b)	1617838_at	CB008961	12.07597
EC 1.1.1.40				
	chlNADP-ME (a)	1610626_at	U67426.1	1582.243
	chlNADP-ME (b)	1609345_s_at	L34836.1	2042.676
	chlNADP-ME (c)	1617374_at	CD721072	9.816781
	chlNADP-ME (d)	1621158_at	CD721072	8.857466
EC 1.1.1.82				
	chlNADP-MDH (a)	1611274_at	CF208296	320.0208
Transporters				
	chlMT (a)	1609626_at	CB344866	497.9718
	chlMT (b)	1616229_at	CB344915	439.9644
GLYOXYSOME				
EC 1.1.1.37				
	gMDH (a)	1613332_at	CF606189	172.5623
	gMDH (b)	1620184_at	CB342898	139.4098
	gMDH (c)	1617677_at	BQ792990	461.8955
EC 2.3.3.1				
	gCS	1612913_at	BQ792733	799.5029
VACUOLE				
Transporters				
	vMT	1614820_at	CF404616	463.6996

[a] Enzyme predictions are based on sequence alignments.

[b] Relative expression values are data from stage 31 of grape development. Values are not absolute, but intended for relative comparison of expression level between genes and their isoforms.

is transported and sequestered in the vacuole, then cytosolic MDH activity will be driven toward malate synthesis from OAA, until the equilibrium is re-established. Alternatively, if malate is abundant and OAA is further metabolised to compounds such as PEP or aspartate, then MDH activity will favour the conversion of malate to OAA. It is in such (or similar) situations that MDH is henceforth suggested to be involved in malate "synthesis" or "degradation" respectively.

There are several isoforms of MDH (E.C. 1.1.1.37) in plants, existing in almost every cellular compartment (Figure 1.1), and requiring either NAD or NADP as a cofactor. Taureilles-Saurel *et al*. (1995b) showed that "total MDH activity" (a simultaneous measurement of multiple NAD-dependent MDH isoforms) in grape berries was high in early development, decreasing to a minimum level at veraison, before increasing again throughout ripening. It seems therefore, that MDH may be involved in net malate synthesis and net malate degradation, which could occur through differential regulation of individual MDH isoforms. While the MDH reaction is reversible, affinities of the mitochondrial (mMDH; Figure 1.1, "3") and cytosolic (cytMDH; Figure 1.1, "2") enzymes are higher for NADH and OAA than for NAD^+ and malate, therefore favouring the synthesis of malate *in vitro* (Taureilles-Saurel et al., 1995b). Due to the cytoplasmic existence of PEPC, and hence localisation of OAA production, the current dogma is that cytMDH is responsible for much of the malate synthesis pre-veraison, and mMDH is involved predominantly in malate degradation. However, the existence of dicarboxylate transporters in plant organellar membranes that may enable transport of OAA between cellular compartments (Hanning et al., 1999) makes it possible for other organellar isoforms of MDH to also synthesise malate. Synthesis of malate by mMDH would require the reversal of its catalytic direction relative to the TCA cycle, which was suggested by radiolabelling studies in grape (Beriashvili and Beriashvili, 1996; Ollat and Gaudillere, 2000). This seems unlikely due to the high levels of respiration occurring in grapes at the time of malate accumulation (Ollat and Gaudillere, 2000).

1.3.2. Photosynthesis

In addition to receiving carbon from leaves, fruits are also capable of local photosynthetic assimilation. Work by Beriashvili and Beriashvili (1996) suggested the operation of two photosynthetic routes in the grape berry, whereby CO_2 was assimilated primarily as malate

in early stages of development (C4-type photosynthesis, involving PEPC), and primarily as sugars in berries at the initial stages of ripening (C3-type photosynthesis). The decline in PEPC activity (see Section 1.3.1.1.) at veraison supports this theory of a photosynthetic switch. In cherry a diurnal rhythm of malate accumulation was suggested to be due to CO_2 fixation to malate during the day (Farineau and Laval-Martin, 1977).

Physiologically, fruit contain very few stomata (Palliotti and Cartechini, 2001), and while grapes are incapable of a net rate of photosynthesis at any stage of development, photosynthetic re-assimilation of respired CO_2 in pre-veraison fruit provides some carbon during development (Ollat and Gaudillere, 2000). Young apples and tomato fruit are photosynthetically active, although are less capable of CO_2 fixation than the leaves of the same plants, likely due to the smaller number of stomata, and the structure of tissues and chloroplasts (Blanke and Lenz, 1989; Piechulla et al., 1987). As expected, the photosynthetic capacity of fruits decreases during development, due to the loss of active chloroplasts (or their conversion to chromoplasts), and a decreasing permeability of the skin to gaseous exchange (eg. reduced surface area to volume ratio, sparser stomata and greater wax accumulation) (Blanke and Lenz, 1989; Piechulla et al., 1987).

1.3.2.1. NADP-dependent malic enzyme (NADP-ME)

Malic enzyme (ME) catalyses the reversible conversion between malate and pyruvate, and is potentially involved in both malate synthesis and degradation, depending on the isoform present, cellular conditions and the availability of substrates. In a photosynthetic role, the decarboxylation of malate to pyruvate by NADP-dependent chloroplastic ME (E.C. 1.1.1.40; Figure 1.1, “4”), may release CO_2 to be taken up by RuBisCO and the Calvin Cycle in plant cells (Drincovich et al., 2001), and has been suggested to occur in fruit tissues (Farineau and Laval-Martin, 1977). However NADP-ME protein and activity are principally located close to the vasculature and core tissues (Famiani et al., 2000; Possner et al., 1983). As a result, NADP-ME is likely to be involved in metabolism of translocated assimilates or re-capturing respired CO_2, rather than photosynthetic assimilation of CO_2. These and other studies (Coombe, 1987) demonstrate the influence of tissue heterogeneity on fruit biochemistry, and therefore the importance of partitioning fruits into their constitutive tissues for detailed assessment of particular events.

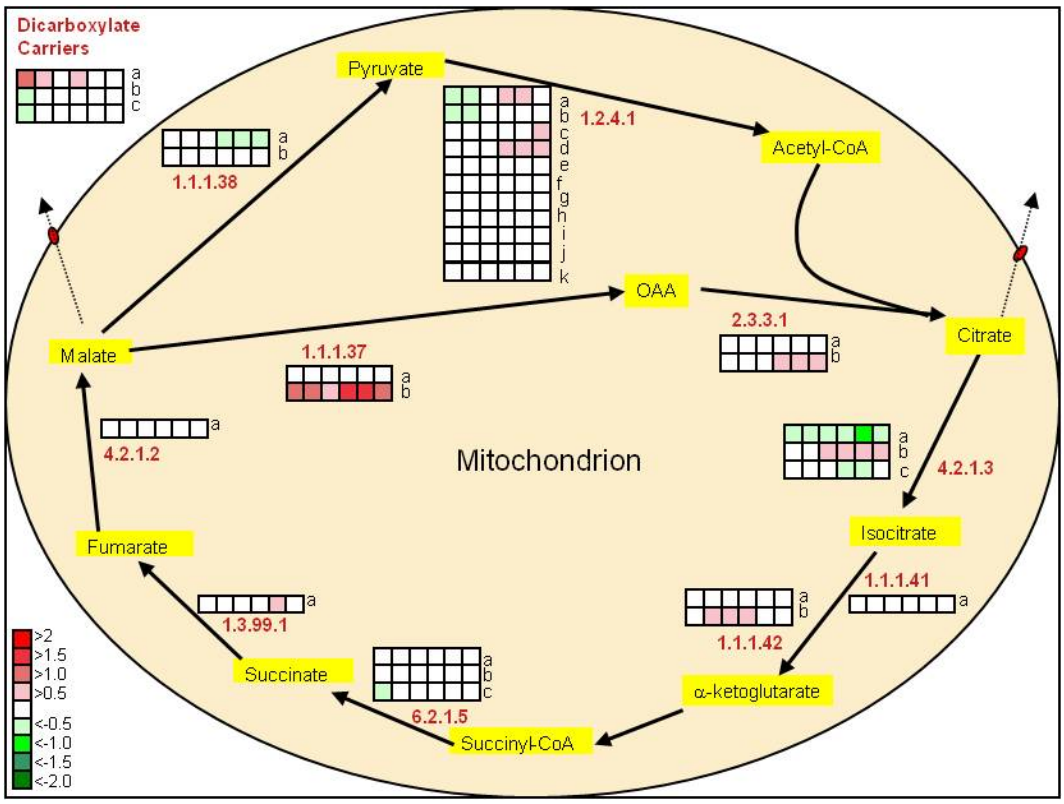

Figure 1.3: Transcript Levels of Mitochondrial Enzymes Involved in Malate Metabolism in *V. vinifera* cv. Cabernet Sauvignon. Changes in gene transcript levels during development of Cabernet Sauvignon grapes were recomputed from the microarray dataset of Deluc *et al*. (2007), at PLEXdb.org. This figure reviews data from enzymes located in the mitochondria. Red text depicts EC numbers of enzymes that were examined for gene expression. Coloured squares illustrate a change in gene transcript level. Rows of squares indicate specific, putative gene isoforms, (eg. EC 1.2.4.1 has 11 putative isogenes, labeled a-k), and columns indicate stage of berry development (increasing development from left to right). The change in expression (log 2 ratio) for each developmental stage is relative to stage 31 (pea-size grapes, as defined by Coombe, 1995). In sequence order (left to right), berries are displayed from stages; 32, bunch closure; 33, still green and hard; 34, softening; 35, veraison; 36, increasing ˚Brix and; 38, harvest. Colour from the first column indicates an increase or decrease in fold change of gene transcript, observed between stage 32 and 31. The next column illustrates a change between stage 33 and 31, and so on. Putative gene identities corresponding to EC numbers are given in Table 1.2, along with initial expression values (from stage 31). Enzyme names for each catalytic step can also be seen in Figure 1.1.

Table 1.2: Relative Expression Values for Putative Genes of Malate-metabolising Enzymes from *V. vinifera* cv. Cabernet Sauvignon (Mitochondrial)

EC Number	Enzyme Prediction[a]	Probe Set ID (PlaxDB)	Genbank Accession	Relative Expression Value[b]
MITOCHONDRIA				
EC 1.1.1.38				
	mNAD-ME (a)	1622775_at	CF415430	458.304
	mNAD-ME (b)	1621510_at	CF405481	16.59513
EC 1.2.4.1				
	PDH (α-a)[c]	1613531_at	CF606004	264.4685
	PDH (α-b)[c]	1613796_at	CB975249	459.4262
	PDH (α-c)[c]	1620802_at	CB976446	599.0287
	PDH (α-d)[c]	1614105_at	CB968800	626.2581
	PDH (β-e)[c]	1615936_at	CD005801	11.87537
	PDH (β-f)[c]	1619705_at	CD005801	6.975943
	PDH (β-g)[c]	1609000_at	CF203971.1	709.3681
	PDH (β-h)[c]	1621359_at	CD802211	243.1342
	PDH (β-i)[c]	1617934_at	BM437104	26.68169
	PDH (β-j)[c]	1619426_s_at	CD003787	476.4753
	PDH (β-k)[c]	1617879_at	CF201094.1	7.690605
EC 2.3.3.1				
	mCS (a)	1619408_at	CB343073	11.59198
	mCS (b)	1618782_at	CF414723	436.5966
EC 4.2.1.3				
	aconitase (a)	1619850_at	CD717468	261.8065
	aconitase (b)	1617606_at	CB978918	1073.845
	aconitase (c)	1621161_at	CF405902	1395.992
EC 1.1.1.41				
	IDH (a)	1618945_at	CF214077	359.25
EC 1.1.1.42				
	IDH (a)	1620526_at	BQ799509	3003.696
	IDH (b)	1612267_at	CF211274	139.8966
EC 6.2.1.5				
	S-CoA synthetase (β-a)[c]	1610222_at	CF514091	7.744316
	S-CoA synthetase (α-b)[c]	1609581_at	CF210434	595.5947
	S-CoA synthetase (α-c)[c]	1612015_at	CF215100	474.833
EC 1.3.99.1				
	SDH	1610760_at	CF211090	1034.845
EC 4.2.1.2				
	fumarase	1617183_at	CB339566	1717.652
EC 1.1.1.37				
	mMDH (a)	1622059_at	CF208844	1645.044
	mMDH (b)	1617448_at	BQ795936	94.32241
Transporters				
	mMT (a)	1615843_at	CD710774	232.845
	mMT (b)	1619168_at	CF405126	1151.788
	mMT (c)	1609917_at	CB917882	634.9385

[a] Enzyme predictions are based on sequence alignments.
[b] Relative expression values are data from stage 31 of grape development. Values are not absolute, but intended for relative comparison of expression level between genes and their isoforms.
[c] Symbols α- and β- are used to identify enzyme subunits.

1.3.3. TCA cycle: fumarase and mitochondrial MDH (mMDH)

1.3.3.1. Fumarase

In fruit mitochondria, malate formed by fumarase (E.C. 4.2.1.2) as part of the TCA cycle (Figure 1.1, "5") may be drawn off for accumulation in the vacuole, rather than procession to OAA by mMDH (E.C. 1.1.1.37). If this is the case, fumarase may be involved in the net synthesis of malate during acid-accumulation phases of fruit. In plant mitochondria fumarase is generally not thought to be rate-limiting, as its activity is controlled by the availability of substrate provided by the preceding step of the TCA cycle (ie. succinate dehydrogenase). However fumarase activity is reversible and, when purified from pea, the enzyme showed inhibition by pyruvate and α-ketoglutarate, as well as AMP, ADP and ATP (Behal and Oliver, 1997), suggesting other forms of enzyme regulation.

A decrease in mMDH activity during the accumulation phase of malate in grapes (Taureilles-Saurel et al., 1995b) suggests that some malate from the TCA cycle may be drawn off and sequestered, although it may need to be converted to pyruvate in order for this to occur. This would require mitochondrial NAD-dependent ME activity (E.C. 1.1.1.38; Figure 1.1, "6"). Malate may also be synthesised from other intermediates of the TCA cycle. For example, citrate can be transported to the cytosol and converted to malate through the activity of ATP-citrate lyase (E.C. 2.3.3.8) in plants (Figure 1.1, "7"). Although the primary function of this reaction is to supply acetyl-CoA for lipid biosynthesis in the cytosol (Fatland et al., 2005), involvement of ATP-citrate lyase in secretion of malate from ion-deficient lupin roots has also been suggested (Langlade et al., 2002). Net synthesis of malate by ATP-citrate lyase is yet to be tested in fruits.

1.3.3.2. Mitochondrial NAD-dependent MDH (mMDH)

A high level of mMDH (E.C. 1.1.1.37; Figure 1.1, "3") expression early in grape berry development (Figure 1.3; Or *et al*., 2000b), supports a role of the enzyme in pre-veraison respiration. Figure 1.3 also shows up-regulation of a putative mMDH gene at veraison, which could therefore be involved in malate degradation. A similar pattern was also seen in strawberry, and transcripts from this fruit were also induced late in ripening (Iannetta et al.,

2004). This indicates independent regulation of expression for particular MDH isoforms, even those that exist within the same organelle.

High levels of mMDH activity observed early in grape berry development (Taureilles-Saurel et al., 1995b) may be due to the high rates of respiration also seen during this phase (Ollat and Gaudillere, 2000). In strawberry, mMDH activity increased early in development, and again at the initiation of ripening before reaching a plateau (Iannetta et al., 2004). This may be indicative of an increasing respiration rate that occurs at ripening (Iannetta et al., 2006), and may be involved in the extended period of malate synthesis that has been observed in this fruit (Moing et al., 2001). In peach, NAD-MDH activity was high in early development and gradually decreased throughout ripening, consistent with patterns of malate accumulation and loss, but not accounting for differences in normal- and low-acid varieties (Moing et al., 1998).

1.3.4. Glyoxylate cycle

In the glyoxysome malate synthase (E.C. 2.3.3.9) converts glyoxylate to malate as part of the glyoxylate cycle (Figure 1.1, “8”), and could therefore be involved in net synthesis of malate. Indeed, microarray analyses revealed high expression of a putative malate synthase gene in young, malate-accumulating grapes (Terrier et al., 2005), and in banana a fruit-specific malate synthase gene was developmentally induced by ethylene (Pua et al., 2003) at a time when malate accumulates in the fruit (Agravante et al., 1991). Radiolabel applied as malate to ripening grapes could be detected in glycolic acid and glycine, which suggested the occurrence of photorespiration at this time, and hence continued operation of the glyoxylate cycle with malate as a source of carbon rather than a product (Beriashvili and Beriashvili, 1996). The detection of malate synthase and isocitrate lyase (E.C. 4.1.3.1; Figure 1.1, “9”) activities suggested a role of the glyoxylate cycle in gluconeogenesis of banana fruit (Surendranathan and Nair, 1976). However, neither isocitrate lyase activity nor expression has been demonstrated in grape or tomato, and protein could not be detected in raspberry, blueberry, strawberry or red currant (Famiani et al., 2005).

1.4. Pathways of malate degradation in fruit

Once grapes reach veraison, sugar metabolism begins to favour hexose accumulation and synthesis rather than catabolism, through regulation of key enzymes of the glycolytic and gluconeogenic pathways (Ruffner and Hawker, 1977). Therefore at this stage sugars relinquish the role of major carbon source for energy metabolism and biosynthesis. Malate released from the vacuole during ripening has the potential to fulfill this function, and can do so through involvement in gluconeogenesis, respiration (aerobic and anaerobic), and biosynthesis of secondary compounds.

Gluconeogenesis

1.4.1.1. Phosphoenolpyruvate carboxykinase (PEPCK)

PEP required for gluconeogenesis in fruit may originate from malate through the activities of MDH (E.C. 1.1.1.37; Figure 1.1, "2") and PEP carboxykinase (E.C. 4.1.1.49; Figure 1.1, "10") or potentially malic enzyme (E.C. 1.1.1.38; Figure 1.1, "11") and pyruvate orthophosphate dikinase (PPDK; E.C. 2.7.9.1; Figure 1.1, "12") (Ruffner, 1982). Evidence from radiolabelling work (Beriashvili and Beriashvili, 1996; Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991) suggests that gluconeogenesis does occur in grape and tomato fruits, particularly during ripening stages, when sugars are accumulating rapidly. Treatment of tomato with acetaldehyde led to a more rapid synthesis of sugars in the fruit, accompanied by a loss of malate, citrate and oxalate, providing further evidence for gluconeogenesis from organic acids (Halinska and Frenkel, 1991).

The expression of two PEP carboxykinase (PEPCK) transcripts (Terrier et al., 2005) coincides with the post-veraison increase in PEPCK activity seen in grapes (Ruffner et al., 1976), indicating that this process may be transcriptionally controlled. Similar expression patterns are shown from grape in Figure 1.2, and furthermore this increase in PEPCK transcript is simultaneous with a decrease in PEPC transcript, and coincides with the malate degradation phase in grape. These data support the hypothesis of a malate-sourced gluconeogenesis, but do not negate a potential role of PEPCK in malate synthesis as suggested earlier (Ruffner and Kliewer, 1975), since gene transcripts and enzyme activity are both present in pre-veraison berries (Figure 1.2; Ruffner *et al*., 1976; Terrier *et al*., 2005). In contrast, a tomato

fruit PEPCK transcript, along with the corresponding protein and activity, was not detectable at all until the breaker stage (initiation of colour change) (Bahrami et al., 2001). Moreover, Famiani *et al*. (2005) uncovered a potential relationship between PEPCK protein level, enzyme activity, and malate loss in raspberry, blueberry and red currant fruits. Meanwhile, neither PEPCK activity nor protein could be detected in strawberries, consistent with the lack of malate degradation seen in this fruit (Famiani et al., 2005).

Plant PEPC and PEPCK activities are differentially regulated by phosphorylation, whereby increased phosphorylation events can generally lead to higher PEPC activity, and lower PEPCK activity (Chollet et al., 1996; Walker and Leegood, 1995). PEPC kinase is the enzyme responsible for phosphorylating PEPC and potentially PEPCK. In blueberries, PEPC and PEPCK proteins are co-located in the cytosol of the same cells (Famiani et al., 2005). Therefore, the phosphorylative regulation of PEPC and PEPCK could occur through the same system. If PEPC and PEPCK enzymes are both phosphorylated, the higher PEPC and lower PEPCK activities will encourage OAA formation from PEP, thus allowing MDH to synthesise malate. Up-regulation of PEPC kinase expression at early stages of grape development (Figure 1.2) suggests that this enzyme could activate PEPC (and potentially inactivate PEPCK) while malate is accumulating within the fruit cells. Subsequently, a decrease in PEPC and PEPC kinase expression from veraison (Figure 1.2), suggests that flux from PEP to OAA may be slowed at this time, simultaneous to a decrease in grape respiration rates (Ollat and Gaudillere, 2000). Expression of PEPCK has been seen to increase in post-veraison grapes (Figure 1.2), potentially supporting a switch from the use of PEP for respiration pre-veraison, to its use in gluconeogenesis post-veraison. This requires a source of OAA, which could occur through the oxidation of malate by MDH.

Continuous turnover of PEPC kinase through the ubiquitin protease pathway, as demonstrated in maize protoplasts (Agetsuma et al., 2005), may be essential for rapid reversal of PEPC (and potentially PEPCK) phosphorylation, and could thereby act as another level of control for malate metabolism. This may also be true for grapevine, although post-translational regulation of PEPCK activity in grape berries is yet to be investigated.

It should be kept in mind that, while a moderately large fraction of grape berry malate could indeed be involved in the synthesis of sugars from veraison (Beriashvili and Beriashvili,

1996), translocation of sugars from leaves still acts as the major supply to the fruit (Ruffner and Hawker, 1977).

1.4.1.2. Pyruvate orthophosphate dikinase (PPDK)

In grape berries, a post-veraison decrease in pyruvate was seen to be followed by a temporary increase in PEP (Ruffner and Hawker, 1977). In plants, the synthesis of PEP directly from pyruvate involves the gluconeogenic enzyme, pyruvate orthophosphate dikinase (PPDK; E.C. 2.7.9.1; Figure 1.1, "12"). This enzyme is yet to be characterised in grape, however a transcript encoding a putative PPDK gene was seen to increase throughout berry development (Figure 1.2). Pyruvate required for PPDK activity may be supplied through the activity of NAD(P)-ME, which could use malate as it is released from the vacuole. No changes in expression of malic enzyme isoforms were detected (Figures 1.2 and 3), however these enzymes are reversible and subject to post-translational regulation (Franke and Adams, 1992; Possner et al., 1981; Ruffner et al., 1984), and activity of NADP-ME has been shown to increase during grape development (Dilley, 1962; Goodenough et al., 1985; Hawker, 1969; Ruffner et al., 1976).

As yet, neither PPDK expression nor activity has been identified in tomato (Bahrami et al., 2001), and while Famiani *et al*. (2005) were unable to detect PPDK protein in raspberry, blueberry, strawberry and red currants, the level of a PPDK protein in peach fruit has been seen to increase during ripening (Borsani et al., 2009). PPDK activity could be linked to a low oxygen stress response (Huang et al., 2005), presumably in providing pyruvate for fermentation pathways. Further work is required to determine the effect of PPDK regulation in relation to fruit development, and whether this could have any bearing on malate metabolism during ripening.

1.4.2. Respiration

The respiratory quotient (RQ; the ratio of CO_2 evolution against O_2 consumption) of numerous grape varieties increases from unity pre-veraison, to as high as 1.5 post-veraison (Harris et al., 1971). This could be due to the use of malate as a fuel for respiration, which releases more CO_2 per molecule of O_2 consumed than the sugars or starch that are used in ripening climacteric fruit. However the production of ethanol, proline or aminobutyrate in

response to stress conditions can also affect the RQ of ripening fruits (Romieu et al., 1992; Taureilles-Saurel et al., 1995b). Nevertheless, addition of endogenous malate to grape berries showed that mature fruit had higher resulting respiratory activities than green fruit (Hardy, 1968). Consistent with other non-climacteric fruit, CO_2 evolution from grapes is highest early in development, decreases rapidly towards the beginning of ripening, and is maintained at a low level thereafter (Ollat and Gaudillere, 2000). Therefore the large release of malate in grapes at veraison does not lead to an increase in respiration rate, but may be necessary to maintain respiration throughout ripening while sugars are redirected to the vacuole.

1.4.2.1. TCA cycle

In plants the majority of ATP synthesis occurs via the mitochondrial Electron Transport Chain (mETC), through the oxidation of electron carriers (NADH and $FADH_2$) that are largely provided from the TCA cycle (Figure 1.1). The withdrawal of cycle intermediates for processes such as amino acid synthesis (Beriashvili and Beriashvili, 1996; Famiani et al., 2000) indicates that carbon must be continuously injected into the cycle. In grapes, large quantities of malate released from the vacuole at veraison may be directly transported to the mitochondria and fed into the TCA cycle, to support ATP synthesis and maintain respiratory flux in the fruit cells. An increase in protein level and activity of mMDH (a TCA cycle enzyme; Figure 1.1, “3”) occurs post-veraison (Peruffo and Pallavicini, 1975; Taureilles-Saurel et al., 1995b), suggesting that malate may be used through this pathway. However the fruit display decreasing rates of respiration throughout ripening (Ollat and Gaudillere, 2000), and the high level of mMDH activity may not be limited to the flux through the TCA cycle.

Pyruvate is also an important respiratory fuel, as its metabolism through pyruvate dehydrogenase (PDH; E.C. 1.2.4.1; Figure 1.1, “13”) provides acetyl-CoA, another intermediate necessary for the operation of the TCA cycle. Pyruvate can also be created through the decarboxylation of malate, through malic enzyme activity. This could occur in the cytosol through NADP-ME (Figure 1.1, “11”), or in the mitochondria through NAD-ME (Figure 1.1, “6”).

1.4.2.2. NADP-dependent malic enzyme (NADP-ME)

In ripening C3 fruits, NADP-ME (E.C. 1.1.1.40; Figure 1.1, "11") may produce the respiratory substrates pyruvate and NADPH from malate and $NADP^+$ (Drincovich et al., 2001; Fernie et al., 2004; Ruffner, 1982) in the cytosol, and may thus partake in malate degradation during ripening (Franke and Adams, 1995; Ruffner et al., 1984). While NADP-ME is reversible, directionality favours malate degradation, and is dependent on post-translational regulators, including $NAD(P)^+$:NAD(P)H ratio, substrate availability and pH (Possner et al., 1981; Ruffner et al., 1984). Franke and Adams (1992) stated that NADP-ME should be able to produce pyruvate from both malate and OAA, and that NADP-ME purified from grapes was capable of doing so. While metal ions (Mn^{2+} and Mg^{2+}) are required as allosteric regulators of activity for the entire reaction, the individual steps require different cofactors, including fumarate, $NAD(P)^+$ and ATP (Chang and Tong, 2003), while other TCA cycle metabolites can act as allosteric regulators (Goodenough et al., 1985).

An increase in NADP-ME activity has been seen during the ripening of grape berries (Hawker, 1969; Ruffner et al., 1976; Ruffner et al., 1984), supporting a role for this enzyme in malate degradation. Years later, using Northern Blotting methods, the transcript of a cytosolic NADP-ME was also seen to increase during grape development (Or et al., 2000b), although this change was not observed for any of the potential NADP-ME isogenes detected by microarray analyses (Figure 1.2; data recomputed from Deluc *et al*., 2007). The loss of NADP-ME protein observed by Famiani *et al*. (2000) during grape ripening implies regulation at the post-translational level. In cross-sections of grape berries, areas furthest from the pedicel and seeds had lower levels of malate (Possner and Kliewer, 1985), and higher levels of NADP-ME protein (Famiani et al., 2000), although these data were taken from ripening and young berries, respectively.

Work with tomato fruit suggested that in early development, the majority of malate oxidation occurred through the TCA cycle, while NADP-ME activity was low. However, NADP-ME activity began to increase simultaneous with a decline in TCA cycle activities, such that the climacteric phase was characterised by a higher level of malate decarboxylation through NADP-ME than through the TCA cycle, at a time when the respiration rate was high (Goodenough et al., 1985). The subsequent observation that the content of NADP-ME

declined with the progress of ripening in tomato (Bahrami et al., 2001) may be indicative of the importance of post-translational modification on the activity state of this enzyme.

Activity of NADP-ME in apple fruit was low early in development, and increased slightly at the pre-climacteric stage, before increasing substantially more in over-ripe fruit (Dilley, 1962). The authors suggested that NADP-ME is involved in CO_2 fixation in young fruit and in malate decarboxylation during ripening. Malate levels throughout apple development support this pattern (Beruter, 2004). Therefore other fruits may also be expected to display increased NADP-ME activity during ripening, particularly where malate is seen to be lost. In addition, some stresses have also been shown to induce activity of NADP-ME in fruit (Tovar et al., 2001).

1.4.2.3. NAD-dependent malic enzyme (NAD-ME)

Activity of the mitochondrial NAD-ME (E.C. 1.1.1.38; Figure 1.1, "6") is yet to be detected in grapes and was thought to be absent from the tissue (Romieu et al., 1992). However, immunohistochemical work revealed the presence and developmental regulation of an NAD-ME protein in grape extracts (Famiani et al., 2000). If this protein is active there may be a role for NAD-ME in malate degradation during grape berry ripening. In tomato, NAD-ME protein was shown to be present at all stages of development and intensity of the band only declined once the fruit had ripened (Bahrami et al., 2001).

In the leaf of the C4 plant *Panicum miliaceum*, a lower yield of ATP resulted from pyruvate production (via NAD-ME) than from OAA production (mMDH) in the mitochondria (Gardestrom and Edwards, 1983). Furthermore, unlike mMDH the activity of NAD-ME was not affected when the phosphorylating pathway of respiration was blocked by cyanide or rotenone in mitochondrial preparations of cauliflower buds (Rustin et al., 1980). This suggests a link between NAD-ME and the "energy-wasting" non-phosphorylating pathway of respiration, which may be explained by the low affinity of non-phosphorylating NADH dehydrogenases for NADH, at least 5-fold lower than that of the phosphorylating pathway Complex I in beetroot (Soole et al., 1990). Specifically, NAD-ME is able to supply high levels of NADH to the mitochondrial matrix, particularly when malate is abundant, and can lead to sufficient NADH accumulation for the activation of the non-phosphorylating dehydrogenases. The reaction carried out by mMDH instead favours NADH oxidation,

unless OAA is actively removed from the system. Therefore the activity of NAD-ME (but not mMDH) can be expected to cause an accumulation of sufficient NADH for activation the non-phosphorylating pathway of respiration, particularly at times of high malate abundance.

1.4.2.4. Non-phosphorylating pathway of respiration

Although grapes are classified as non-climacteric fruit, Pilati *et al*. (2007) observed a release of H_2O_2 from Pinot Noir berries at veraison, simultaneous to an increase in gene expression of the alternative oxidase (AOX) of the non-phosphorylating respiration pathway. AOX activity assists in the reduction of ROS (Reactive Oxygen Species) production (Zhang *et al*., 2003). The increased expression of other ROS scavenging genes (Pilati et al., 2007) suggested that the berry cells were becoming over-reduced. It may be that the release of sequestered malate at veraison can alter the reductive state of the cell, whereupon the induction of AOX by high malate, pyruvate and ROS levels (Gray et al., 2004; Zhang et al., 2003), can function in the removal of ROS. Interestingly, drought and salinity stressed grape berries and leaves also showed increased AOX expression (Cramer et al., 2007; Grimplet et al., 2007).

This information suggests an oxidative burst of sufficient magnitude to trigger an antioxidant response, and cause an increase in flux through non-phosphorylating respiration pathways. As a result, higher TCA cycle flux is required to provide extra reducing equivalents (NADH and $FADH_2$) to the non-phosphorylating pathway and AOX. This in turn may affect malate catabolism, which would be expected to intensify due to increased requirements of the TCA cycle. While grapes do not display an increase in respiration during ripening, the conversion of malate to OAA by MDH may produce NADH in sufficient quantities to induce non-phosphorylating respiration.

In climacteric tomato fruit NADP-ME activity has been suggested to facilitate a Complex I bypass by producing NADPH, which can donate electrons to an external NADPH dehydrogenase instead (Goodenough et al., 1985). Pyruvate also produced in this reaction may be expected to activate the AOX enzyme (Millar et al., 1993), encouraging further avoidance of oxidative phosphorylation (ATP synthesis). The switch from malate use in the TCA cycle to the non-phosphorylating pathway was suggested to be independent of an

ethylene response seen in tomato (Goodenough et al., 1985), and can enable continued catabolism of malate when the oxidative phosphorylation pathway is limited (eg. by high ATP:ADP). In addition, NAD-ME may also facilitate the use of malate when the phosphorylating pathway of respiration is limited (see Section 1.4.2.3). Nevertheless, activities of NAD-ME and non-phosphorylating respiration pathways have not been measured directly in grape, and as such, it is yet to be determined whether gene induction of AOX seen in Pinot Noir (Pilati et al., 2007) is reflected in activity during berry development.

1.4.3. Fermentation

1.4.3.1. *Ethanol fermentation*

The production of ethanol by pyruvate decarboxylase (PDC; E.C. 4.1.1.1; Figure 1.1, "14") and alcohol dehydrogenase (ADH; E.C. 1.1.1.1; Figure 1.1, "15") may occur in ripening fruit under normal atmospheric conditions if conditions inside the cells become too acidic. Such a situation may result when glycolysis exceeds oxidative phosphorylation and an accumulation of pyruvate ensues (Zabalza et al., 2009), or if respiratory activity is limited due to a highly reductive energy status, or high NADH:NAD^+ (Ponce-Valadez and Watkins, 2008).

The concept of aerobic fermentation is gaining extensive credibility, and has been demonstrated in numerous tissues including tomato fruit (Longhurst et al., 1990), tobacco pollen (Bucher et al., 1995; Tadege and Kuhlemeier, 1997) and more recently in pea roots (Zabalza et al., 2009). The induction is likely due to an excess of sugars that increases the rate of glycolysis and the levels of pyruvate (Bucher et al., 1995; Livio et al., 2007). Acidification of the cytosol was thought to be a trigger for increased ADH activity in tomato fruit (Longhurst et al., 1990), and this increase was only slightly reduced when fruit were exposed to higher concentrations of O_2. Production of volatiles as a result of ADH activity was suggested to contribute to the development of taste and aroma within the fruit (Longhurst et al., 1990).

The up-regulation of some ADH genes as a function of fruit ripening (Figure 1.2; Chen and Chase, 1993; Sarni-Manchado *et al*., 1997; Terrier *et al*., 2005), and the increases in ADH

specific activity during tomato ripening (Longhurst et al., 1990) imply that ethanol fermentation is a normal developmental response in grapes and tomatoes. This is further evidenced by the increased expression of two mango fruit ADH genes in response to ethylene, and the decreased expression observed after application of an ethylene inhibitor (Manriquez et al., 2006). These genes were also shown to be up-regulated in the normal ripening mango.

While aerobic ethanol fermentation may occur in some fruits, the same may be true for anaerobic fermentation if the internal environment becomes hypoxic. To give an example, mandarins are less permeable to gas exchange than grapefruit. Consequently, mandarins display a higher basal level of ADH activity and accumulate more pyruvate, acetaldehyde and ethanol (Shi et al., 2007). Transcript levels of PDC, ADH and the glycolytic enzyme pyruvate kinase (Figure 1.1, "16") were also higher in mandarin than in grapefruit. Alternatively, PDC gene expression remained unchanged during the ripening stages of tomato and grape fruits (Chen and Chase, 1993; Or et al., 2000a), which is consistent with the activity pattern observed in grapes (Hawker, 1969). However, the expression of genes for both PDC and ADH were was responsive to anaerobic stress in tomato (Chen and Chase, 1993). Taken together, is seems that ethanol fermentation as a result of hypoxic conditions may involve stimulation of both PDC and ADH activities, whereas developmental cues involving aerobic ethanol fermentation may be expected to affect ADH activity only. According to Imahori *et al*. (2003), ethanol production in response to low oxygen stress in tomato is limited by the rate of PDC activity, which in turn can be limited by the availability of pyruvate. It may be that PDC is present at basal levels throughout development and only induced as a necessary pathway for continued ATP synthesis at times when oxygen becomes limiting, whereas ADH is induced during normal fruit ripening as a necessary pathway to remove acetaldehyde (produced by basal levels of PDC activity and high pyruvate concentrations) and combat changes in cytosolic pH due to acid release from the vacuole. If such is true, then increased PDC activity during ripening suggests that the internal oxygen content of mandarin, pineapple and peach fruits may become sufficiently low to induce anaerobic fermentation (Borsani et al., 2009; Livio et al., 2007; Selvaraj, 1993; Shi et al., 2007), however this needs further clarification.

In maize roots, loss of malate through NADP-ME activity has been implicated as an early response to hypoxia (Edwards et al., 1998). More recently an increase in NADP-ME activity has also been observed at the initiation of pineapple ripening, accompanied by an increased rate of PDC activity, further suggesting a link between malate degradation and fermentative metabolism (Selvaraj, 1993). Ponce-Valadez and Watkins (2008) proposed malate as a source of carbon for aerobic ethanol fermentation with the purpose of NAD^+ regeneration, such that glycolysis can continue to provide ATP during high rates of fruit metabolism. Large amounts of malate that are released from the vacuoles of grape berry cells at veraison may be converted to pyruvate via NAD(P)-ME, and the resulting pyruvate could activate a "PDH bypass" (Terrier et al., 2001). That is, pyruvate may be diverted from the TCA cycle for metabolism through PDC and ADH instead. Indeed, at this stage of development the TCA cycle flux becomes limiting in grapes (Ollat and Gaudillere, 2000), and ethanol fermentation may be required to de-acidify the cytosol of fruit cells (Longhurst et al., 1990).

1.4.3.2. Lactate fermentation

Few studies have explored the fermentation of pyruvate to lactate via lactate dehydrogenase (LDH; E.C. 1.1.1.27; Figure 1.1, "17") in fruit, although litchi flesh and rind have been shown to contain lactate, particularly at the initiation of ripening (Jha et al., 1990). The authors suggested that lactate accumulation occurred due to a rapid glycolytic flux to pyruvate and a simultaneously slowed aerobic respiration rate as indicated by a decrease in succinate dehydrogenase activity. In addition, it has been suggested that fruit may induce fermentative metabolism as a response to high rates of oxygen consumption or limited oxygen diffusion, as these situations can lead to hypoxia (Borsani et al., 2009). These authors detected an increase of PDC and ADH activities but not LDH activity in post-harvest ripening of peach. In tomato and avocado fruits, LDH activity remained at a basal level during ripening, and in response to low oxygen stress (Imahori et al., 2003; Ke et al., 1995). In both cases, pyruvate flux through alcohol fermentation was favoured over lactate fermentation. Of the two LDH genes identified in tomato, neither was highly expressed during ripening, although one did increase in response to low oxygen stress (Germain and Ricard, 1997).

1.5. Intracellular transport of malate in the grape berry

In a recent analysis using introgression lines of tomato, QTL mapping of metabolomic profiles against morphological traits of the fruit and whole plant were used to uncover relationships between genes, metabolites and plant phenotype (Schauer et al., 2006). In this study, lines containing altered levels of malate and other organic acids showed little or no co-localisation with metabolic enzymes, suggesting that membrane transport and other regulatory influences are important contributors to malate metabolism in fruit.

Vacuolar transporters play a critical role in the switch from malate accumulation to degradation in grape berries, as the acid must be released from the vacuole before it can be metabolised. This involves activities of anion transporters that allow passage of malate through the tonoplast, as well as proton pumps that use the hydrolysis of high energy molecules (ATP and PP_i) to drive the import of protons into the vacuole. The latter create an electrochemical gradient that enables malate to be transported into the vacuole against its own electrochemical gradient (Luttge and Ratajczak, 1997).

An increase in activities of the vacuolar proton pumps vATPase and vPPase was seen throughout grape berry ripening and may be necessary for the maintenance of the electrochemical gradient, as the permeability (or "leakiness") of the tonoplast to protons also increases throughout ripening (Terrier et al., 2001). An increase in the proportion of the "less efficient" vPPase activity relative to that of the higher proton-pumping capability of vATPase during ripening (Terrier et al., 2001) could enable a controlled decrease in tonoplastic energisation, allowing malate to be released at a rate that does not cause over-acidification of the cytosol during ripening. Another hypothesis discussed by Terrier *et al*. (2001) states that if supply of ATP and PPi to the proton pumps become limiting (as can occur in ripening fruit due to a higher energetic demand for biosynthetic reactions, a reduction in respiration rate or oxygen limitation in ripening fruit), the ability of the proton pumps to energise the tonoplast will be reduced, thus liberating malate from the vacuole. Inhibition of proton-pumping activity of the vPPase was shown to occur in the presence of low concentrations of the fermentative product ethanol (Terrier and Romieu, 1998).

The plant cell tonoplast also contains gated anion channels through which malate and other dicarboxylates can pass, including the "3-ps" channel identified in the CAM plant *Kalanchoe*

daigremontiana (Hafke et al., 2003), and an AttDt transporter in Arabidopsis leaves (Emmerlich et al., 2003). AttDt gene knockouts in Arabidopsis plants showed no phenotypic differences to the wildtype under normal growth conditions (Hurth et al., 2005). The protein enabled both malate import and export from the vacuole, and leaves of transformed plants could still accumulate some malate and fumarate, although at significantly lower levels than in wild-type plants. An increase in CO_2 evolution and RQ value suggested that acids that were unable to be accumulated in the vacuole were instead respired (Hurth et al., 2005). The authors also suggested that the AttDt protein is likely involved in pH homeostasis. A biochemical pH stat model that uses the transport and retention of malate in the vacuole as a counteractive measure against decreased cellular pH, may involve such enzymes as MDH, malic enzyme, PEPC and PEPCK (Davies, 1986; Sakano et al., 1998),. Notably in peach, the accumulation of malate has been seen to decrease in response to high cytosolic pH (Lobit et al., 2006).

Another study in Arabidopsis demonstrated that one of the members of the AtALMT family (containing malate transporters involved in heavy metal tolerance), AtALMT9, is located in the tonoplast rather than the plasma membrane (Kovermann et al., 2007). Expression of this gene was detected in numerous tissues and organs, including the flower, cotyledon, roots and leaf mesophyll. Gene interruption led to lower inward malate currents, without display of a phenotypic difference to wildtype Arabidopsis, while overexpression in tobacco leaves resulted in increased malate currents, and insertion of the gene into *Xenopus* oocytes showed functional capability of the protein in anion-selective import of malate, which was regulated by the presence of the acid. The authors propose that an additional tonoplastic malate transporter may exist, and along with AtALMT9 and AttDt, can regulate the transport of malate between the cytosol and vacuole in Arabidopsis leaves, but may be confined to the epidermis (Kovermann et al., 2007).

Orthologs to the transporters mentioned here may be responsible for malate passage across the tonoplast of fruit vacuoles, and could play a role in the regulation of fruit acidity. Indeed, expression of a grape berry sequence with homology to the AttDt protein showed moderate (21-fold) up-regulation in Cabernet Sauvignon and Chardonnay berries at veraison (Figure 1.2), coinciding with the malate degradation phase of fruit development (Ruffner and Hawker, 1977).

Mitochondrial malate transporters may also play a role in the regulation of malate degradation, by policing the entry and exit of malate and other carboxylic acids through the inner mitochondrial membrane, to and from the site of the TCA cycle and oxidative phosphorylation. In Arabidopsis there are 58 potential members of the mitochondrial carrier family (MCF) (Picault et al., 2004). Often transport of one acid requires either symport or antiport of another, as in the case of the dicarboxylate-tricarboxylate carrier (DTC) protein family (Picault et al., 2002) and the dicarboxylate carrier (DIC) family (Palmieri et al., 2008).

The level of ion channel proteins are generally low, due to the high rate of turnover (10^6-10^8/sec) (Allen and Sanders, 1997). Therefore these proteins are difficult to study, and much more work is required in this area to identify and characterise transporters that are responsible for the developmentally-regulated loss of malate from grape berry cell vacuoles.

1.6. Approaches to identify genes linked to high and low malate fruit

Patterns of malate and citrate accumulation in a low-acid peach cultivar have been used to investigate causes of variation in fruit acidity, by exploring differences in gene expression and enzyme activity patterns relative to a normal-acid cultivar. These studies have indicated that PEPC activity, while linked to the synthesis of malate in normal acid varieties, cannot explain the variation in malate and citrate levels seen in the low-acid fruit (Moing et al., 2000), nor can NAD-MDH and NADP-ME activities (Moing et al., 1998). A more promising result seems to lie in the ability of the fruit to store organic acids. Low acid fruit showed some divergence in expression of vacuolar proton pumps that could signify a reduced capability of the cells to sequester organic acids, thereby making them accessible to catabolic enzymes (Etienne et al., 2002). Higher levels of PEPC activity and reduced accumulation of sugars in the low-acid fruits during ripening (Moing et al., 1999) may suggest that the acids are constantly recycled.

Another group has exploited high- and low- acid varieties of apple in their discovery of a gene that may control malate levels in fruit (Yao et al., 2007). This was achieved using a cDNA-AFLP (cDNA-amplified fragment length polymorphism) approach, which uncovered a

novel gene “Mal-DDNA” that was found in the cDNA of the low-acid variety only. Expression studies showed that the gene was negatively associated with malate accumulation, and was largely absent in high-acid fruit development (although some transcript was detected late in development of high-acid fruit – at a time when malate was depleting) (Yao et al., 2007). Therefore the “Mal-DDNA” gene must direct either an increase in degradation or a decrease in the synthesis or storage of malate. As yet no function has been allocated, and homologous sequences could not be found in the genome of any other species represented in the National Centre for Biotechnical Information (NCBI) database at the time of this publication, although an EST from peach displays weak similarity (Acc. GB. AM291487, with 64% positive matches using tBalstn).

In tomato, the use of back-crossing and marker-mediated selection has resulted in numerous plant lines that each contains a single chromosome fragment from a wild species (*Lycopersicon pennellii*) within the genome of the cultivated species (*L. esculentum*) (Eshed and Zamir, 1994; http://www.sgn.cornell.edu). These lines were used to identify Quantitative Trait Loci (QTLs) that may be important for regulating tomato growth and composition (Causse et al., 2004). In one line, the *L. escluentum* genome had incorporated a QTL from *L. pennellii* that contained a PEP carboxykinase gene. This line had lower malate, citrate and titratable acidity, as well as a higher pH and increased level of reducing sugars, suggesting increased flux through gluconeogenesis in this plant line. This supports the involvement of PEP carboxykinase in the regulation of organic acids and sugar levels during fruit development. Other QTLs that were associated with altered malate levels using this method, contained sequences for PEP carboxylase, numerous membrane transporters and a fructokinase-like protein. Citrate content was also affected by QTLs containing genes for an alcohol dehydrogenase and an NADP-linked malic enzyme. Therefore it is likely that all of these genes are involved in regulating levels of organic acids in tomato and other fruits.

A systems biology approach, whereby data are collated from transcriptomic, proteomic and metabolomic analyses to investigate flux through a metabolic pathway could also be applied to the question of how malate is metabolised throughout fruit development. Such technologies are currently being applied to grape, including studies by the group of Deluc *et al.* (2007), to which transcriptomic data has been referred throughout this text.

1.7. Temperature regulation of fruit malate metabolism

In some fruits, particularly grape, it is well established that exposure of the ripening fruit to warmer climatic conditions leads to lower levels of malate at harvest (Lakso and Kliewer, 1978; Ruffner et al., 1976). Malate levels were also seen to be reduced upon high exposure to sunlight (Pereira et al., 2006), although this is most likely due to the effect of exposure on berry temperature (Spayd et al., 2002). Synthesis of malate involves an exothermic reaction that may occur more favourably at lower temperatures, although developmental cues are thought to impose a stronger influence on PEPC activity (a major malate synthetic enzyme) than temperature in grapes (Lakso and Kliewer, 1975; Ruffner et al., 1976). Kiwifruit exposed to a higher temperature in early development had double the amount of malate at the peak, but when placed back under normal temperature conditions this decreased to meet the control level (Richardson et al., 2004). From the same experiment, kiwifruit exposed to a higher temperature in later development contained malate levels that were reduced to almost half that of control fruit, even after temperature was returned to normal. Ruffner *et al*. (1976) found that the temperature effect on malate levels in grapes was only evident after veraison, and was due to an extended period of malate degradation at this time. This implicitly suggests up-regulation of respiration as a temperature-sensitive malate metabolic pathway in grapes, due to the involvement of malate in this pathway during ripening. This is consistent with respiration rates in ripe pears, which increased with increasing temperature (Hansen, 1942). Biale (1960) stated that the effect of temperature on fruit respiration is more to do with specific chemical responses, rather than a general speed-up of reactions in the fruit cells. To explore the causes of increased malate degradation in response to temperature, it would be logical to begin investigating those enzymes that serve as regulatory points of the TCA cycle, as well as those that enable entry of metabolites into the cycle.

Taureilles-Saurel *et al*. (1995a) suggested that the expression of developmentally induced MDH isoforms is likely to be also environmentally regulated. At the onset of veraison, both mMDH protein level and enzyme activity increased (Peruffo and Pallavicini, 1975). Furthermore, mMDH isoforms were shown to be more temperature-sensitive than cytMDH (Taureilles-Saurel et al., 1995b), and it may be expected that post-veraison MDH activities will reflect this temperature-sensitivity. According to results from Taureilles-Saurel *et al*.

(1995b), a rise in temperature from 25ºC to 30ºC may result in a switch from predominantly cytMDH activity to mMDH activity. This would support an increase in flux through the TCA cycle in response to higher temperatures in grape.

Additionally, malate could be more rapidly catabolised through the activity of NADP-ME in the cytosol, in response to high temperature. *In vitro*, NADP-ME from Carignane berries showed a specific activity optimum at 50ºC (Lakso and Kliewer, 1975), and was more thermally recoverable than the malate-synthetic enzyme PEPC after heat-treatment of berries (Lakso and Kliewer, 1975). However, according to Ruffner *et al*. (1976), as long as the ratio of NADP:NADPH remains steady in the berry cell, the activity of NADP-ME is relatively independent of temperature. Furthermore, if respiration is increased then the reductive power of the cell is likely to prevent an increase in NADP-ME conversion of malate to pyruvate. Indeed, NADP-ME activity measured in vines exposed to two different temperature regimes (20/15ºC, and 30/25ºC day/night temperatures), was lower in the warmer treatment (Ruffner et al., 1976). In the same experiment, PEPCK activity (representing gluconeogenesis) changed little in response to the increased temperature. In tomato, PEPCK expression was induced by salinity stress (Saito et al., 2008), which suggests that it could also be induced by other stressors. The effect of increased temperature on PEPCK expression and activity should be re-investigated, using higher temperatures that may impose more of a stress on the fruit.

A recent study on the effect of microclimate on the metabolic profile of grapes (Pereira et al., 2006), revealed a temperature effect on some flavonols present in the berry pulp, whereby increased temperature (due to increased sun exposure) led to higher levels of kaempferol-3-glucoside and quercetin-3-gluoside, and reduced levels of myricetin-3-glucoside. The latter compound simultaneously showed an increase in the skin, and may therefore be regulated differently by light and temperature. The formation of such secondary compounds requires a source of carbon that could be provided, at least in part, by the increased usage of malate also observed in the pulp of the treated fruit.

Romieu *et al*. (1992) stated that in whole grape berries the mitochondria reach their phosphorylating capacity at approximately 30ºC, and temperatures exceeding this may lead to ethanol production, as respiratory activity is above that required to meet the energetic

needs of the cell, and possibly above the capacity of the oxidative phosphorylation pathway (mETC). These authors also stated that ethanol production is likely to be very common in grapes grown in the vineyard, as bunches can be subjected to temperatures as high as 50ºC. In the bulky, climacteric apple fruit, increased temperature and respiration, as well as decreased surface area to volume ratio and permeability of skin, led to an accumulation of CO_2 in the fruit (Blanke and Lenz, 1989). In addition, increasing external temperature led to a decrease in the internal O_2 concentration of pear fruit (Hansen, 1942). Under these conditions, the increase in respiration may be greater than the increase of oxygen diffusion to the cells, which also has a lower solubility at warmer temperatures. Taken together, bulky fruit such as apples and pears subjected to high temperatures will have lower internal O_2 and higher internal CO_2 concentrations, which could lead to an induction of anaerobic respiration. Therefore enzymes involved in the ethanol fermentation pathway (PDC and ADH) may play a role in the temperature response of malate degradation. This may not be such an issue in smaller fruits with thinner skins, such as grapes, although Hawker (1969) found that high activities of Sultana PDC corresponded with high temperatures in post-veraison sultana berries. Increased flux through this pathway requires a source of pyruvate, which could occur through an increase in pyruvate kinase (of glycolysis), or the decarboxylation of malate via malic enzyme. Experiments dedicated to controlled temperature conditions on the regulation of enzymes involved in malate metabolism in field-grown fruits are yet to confirm any of these hypotheses.

1.8. Transgenic approaches to modifying malate metabolism

Few attempts at genetic modification aimed at altering malate metabolism have been attempted in fruits, due to the long period required for transformation of plants and subsequent production of fruits. Tomato is perhaps the fastest model fruit-bearing plant to transform, and has been used to over-express and under-express an ADH gene (*Adh2*) (Speirs et al., 1998). ADH-overexpressing fruit had higher levels of some alcohols, in particular hexanol and z-3-hexenol, which gave the fruit a more ripe taste. ADH under-expressors had lower levels of these compounds. The effect on organic acids was not determined in this study. Transgenic grapevines modified to overexpress or repress

expression of an ADH gene (*Adh2*) have also been reported (Tesniere et al., 2006). In the *Adh2* overexpressing lines, transcription of the transgene was under the control of a constitutive promoter, and ADH activity was shown to increase in both vegetative and reproductive tissues. Biochemical analyses revealed lower levels of sucrose in leaves (Tesniere et al., 2006), but not berries (Torregrosa et al., 2008) of transgenic plants over-expressing *Adh2*. This supports a link between ethanol fermentation and sucrose metabolism in grapevine leaves, while translocation of sucrose may have been increased to support the increased rates of fermentation in the berries. Malate was not measured; however there was no effect on total acidity measured in berries either of overexpressing, nor knock-down plants (Torregrosa et al., 2008). The leaves of *Adh2* overexpressors showed increased levels of monoterpenes, carotenoids, proanthocyanidin polymerisation and benzyl alcohol (Tesniere et al., 2006). Changes in such volatile compounds may be expected to alter the aromatic composition of grapevine leaves. However many metabolic changes observed in the leaves could not be detected in the berries, except benzyl alcohol, which was lower in berries of *Adh2* overexpressors (Torregrosa et al., 2008).

Other enzymes involved in carbon metabolism have not yet been modified in grapevine, although in other higher plant species, enzymes such as PEPC, MDH, fumarase, PEPCK, NAD(P)-ME and vATPase have all been investigated through transgenic approaches, often in an attempt to increase the photosynthetic capability. Hausler *et al*. (2001) overexpressed PEPC in potato tubers, and found increased activities of mitochondrial (NAD-) and cytosolic (NADP-) malic enzyme, as well as numerous other enzymes involved in primary carbon metabolism. Such an increase in malic enzyme activities may suggest a rise in the conversion of malate to pyruvate in these plants. In another study, overexpression of active PEPC in potato led to decreased starch accumulation, due to the redirection of carbon flow from sugars to amino acids and malate (Rademacher et al., 2002). The authors suggested that the resultant acidification of the cytosol brought about by increased malate accumulation may have induced nitrate reductases that could increase the availability of nitrogen for amino acid synthesis.

Nunes-Nesi *et al*. (2005) produced tomato plants with reduced mMDH activity, in an attempt to reduce photorespiration through the reduction of malate/aspartate shuttle activity. Plants showed slightly larger fruits, roots, and total dry weight, likely due to the

observed increase in photosynthesis and carbon assimilation rates in the leaves, which also led to increased accumulation of starch and sugars, as well as some organic acids (succinate, ascorbate and dehydroascorbate). As both fumarase and succinate dehydrogenase enzymes (of the TCA cycle) are reversible, the impairment of MDH activity could initiate a back-flow from malate to succinate within the cycle.

Modification of fumarase activity has also been investigated in tomato plants, whereby knock-down of the enzyme lead to lower fruit yield and total dry weight (Nunes-Nesi et al., 2007). Leaves of transformed plants contained lower levels of starch, glucose and fructose, and showed inferior photosynthetic capability and reduced flux through the TCA cycle. This was characteristically opposite to plants that were impaired for mMDH activity. It can be clearly seen that the reduction of flux through the TCA cycle impacts on photosynthetic capabilities, and that these effects are dependent on (i) loss of activity from the enzyme that has been inhibited, (ii) accumulation of the intermediate that is no longer catabolised, or (iii) absence of the intermediate that is no longer produced. Nunes-Nesi *et al*. (2007) concluded however that the decrease in photosynthesis observed in fumarase knock-down plants could be linked to impaired stomatal functioning, rather than a direct metabolic effect. A similar, yet smaller effect on stomatal conductivity was also noticed in mMDH knock-out plants (Nunes-Nesi et al., 2005).

Knock-down of the vacuolar proton pump vATPase from tomato fruit was shown to produce plants with smaller fruit, containing fewer seeds and up to 8-fold higher sucrose levels (Amemiya et al., 2006). Vacuolar pH increased due to lower accumulation of acids, and an observed decrease in the activity of acid invertases likely contributed to the higher sucrose levels, although levels of glucose and fructose remained unchanged in the fruit. The severity of these effects may have been masked by continued activity of another vacuolar proton pump, vPPase (Amemiya et al., 2006).

Enzymes that are not directly involved in malate metabolism may also influence malate levels by controlling product inhibition (eg. pyruvate and ATP) and cofactor availability (eg. CoA and NAD(P)H), and may influence activities of other enzymes directly involved in malate metabolism (ie. MDHs and malic enzymes). Genetic modification of such enzymes in grapevine could be used to alter malate metabolism in the fruit during development and in

response to varied temperature conditions. However, due to the intricate involvement of all above-mentioned enzymes in fundamental cellular processes, including photosynthesis, respiration, energy metabolism, assimilate storage, nitrogen metabolism, carbon cycling, pH balance, and even physiological roles such as stomatal structure, the effects of genetic manipulation on biochemistry of the entire plant should be thoroughly explored. Particular care should be taken with perennial plants, whereby altered metabolism in one season can affect the metabolism and growth in the following seasons.

1.9. Concluding remarks

The information described in this review serves to summarise advances made in fruit malate metabolism research, particularly over the last 20-30 years, as an update to the comprehensive review based in grapes by Ruffner (1982). Levels of malate in harvested fruit may be largely determined by the rate of degradation during ripening (Beruter, 2004; Kortstee et al., 2007; Ruffner and Hawker, 1977; Walton and De Jong, 1990). Results presented from publicly available microarray data (Deluc et al., 2007), suggest the involvement of pyruvate in the degradation of malate in grape, as the expression of enzymes involved in pyruvate metabolism was generally favoured during ripening. These included PPDK (conversion of pyruvate to PEP), PDC and ADH (conversion of pyruvate to ethanol) and PDH (conversion of pyruvate to acetyl-CoA) (Figures 1.2 and 1.3), and although no changes in expression of malic enzyme isoforms were detected, activity of NADP-ME has been shown to increase during fruit development (Dilley, 1962; Goodenough et al., 1985; Hawker, 1969; Ruffner et al., 1976). An increase in expression of the non-phosphorylating respiratory enzyme AOX, which can be regulated by pyruvate levels in plants (Oliver et al., 2008), was seen in grapes at veraison (Pilati et al., 2007) and suggests that this “energy-wasting” pathway of respiration could also be involved in malate degradation during ripening.

Other very important players seem to be vacuolar dicarboxylate transporters and tonoplastic-energizing proton pumps, which facilitate accumulation and release of malate. In particular, the regulation vATPase and vPPase activities may dictate the rate at which malate is accumulated and released from the fruit vacuole, and therefore the rate at which

the acid can be degraded (Amemiya et al., 2006; Terrier et al., 2001). In addition, several vacuolar dicarboxylate channels have been identified in plants (Emmerlich et al., 2003; Hafke et al., 2003; Kovermann et al., 2007), which should be investigated further for their influence on organic acid content of fruit throughout development.

The temperature-sensitivity of fruit malate degradation (Richardson et al., 2004; Ruffner et al., 1976) may be influenced by activities of enzymes involved in pathways such as the TCA cycle and respiration, ethanol fermentation and gluconeogenesis (Hawker, 1969; Lakso and Kliewer, 1975; Romieu et al., 1992; Taureilles-Saurel et al., 1995b). Understanding the biochemical regulation of malate metabolism in field-grown fruits exposed to elevated atmospheric temperature (and CO_2 levels) may assist in the development of methods that can reduce such environmental control. Techniques such as those described in this review (in particular Sections 1.6 and 1.8) will assist in this area, which could become more important with current and looming changes in the global climate. Certainly for grapevines, relocation to more appropriate climatic regions will be regarded with disfavour by the owners of wineries that are reliant on terroir for their reputation of high-quality wines.

1.10. Thesis aims

1.10.1. Developmental regulation of grape berry malate

Fruit of *V.vinifera* show a specific pattern of malate accumulation during development and loss during ripening, suggesting that this acid has specialised roles in developmental metabolism. The switch between malate accumulation and loss in grape berries at véraison must be due to a transition from net malate synthesis to net malate degradation, likely regulated by a combination of synthetic and degradative enzymes, compartmentation, and other cellular conditions that could impact the regulation of general metabolism (eg. hormones, pH, carbon supply). This transition is still largely uncharacterised.

1.10.2. Temperature regulation of grape berry malate

The effect of temperature on acid metabolism is of great interest to viticulturalists and oenologists, as grape and wine characteristics are very reliant on fruit acidity and pH. Changes in seasonal climate could have a large impact on fruit and wine quality through the alteration of malate levels. Previous studies have uncovered a decline in berry malate that is related to heat treatments during ripening. The loss of malate may have resulted from down-regulation of malate synthetic pathways, or up-regulation of malate catabolic pathways. As the fruit undergo a developmental decline in malate at this time, the temperature effect may simply be an acceleration of this loss, and therefore implicates elevated flux through malate catabolic pathways. However, this is yet to be confirmed.

1.10.3. Specific aims

The aims of this study were two-fold. First, to examine metabolic pathways that are key to the regulation of malate during fruit development. Second, to determine the effect of temperature on enzymes of these pathways in relation to changes in berry malate. The aims will be addressed largely through the investigation of specific enzymatic activities, transcript levels and malate contents of grape berries in response to developmental changes and heated vine treatments, as well as mitochondrial isolation for exploration of respiratory machinery and organelle-specific activities. Findings will contribute to the understanding of malate regulation in grape berries, and identify candidate genes for potential improvement of acid control in winegrapes through molecular and breeding practices.

1.11. References

Agetsuma, M., Furumoto, T., Yanagisawa, S., Izui, K., 2005. The ubiquitin-proteasome pathway is involved in rapid degradation of phosphoenolpyruvate carboxylase kinase for C4 photosynthesis. Plant Cell Physiol. 46, 389-398.

Agravante, J. U., Matsui, T., Kitagawa, H., 1991. Sugars and organic acids in ethanol-treated and ethylene-treated banana fruits. J. Jap. Soc. Food Sci. Technol. 38, 441-444.

Albertini, M. V., Carcouet, E., Pailly, O., Gambotti, C., Luro, F., Berti, L., 2006. Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. J. Agric. Food Chem. 54, 8335-8339.

Allen, G. J., Sanders, D., 1997. Vacuolar ion channels of higher plants. Adv. Bot. Res. 25, 217-252.

Amemiya, T., Kanayama, Y., Yamaki, S., Yamada, K., Shiratake, K., 2006. Fruit-specific V-ATPase suppression in antisense-transgenic tomato reduces fruit growth and seed formation. Planta 223, 1272-1280.

Bahrami, A. R., Chen, Z. H., Walker, R. P., Leegood, R. C., Gray, J. E., 2001. Ripening-related occurrence of phosphoenolpyruvate carboxykinase in tomato fruit. Plant Mol. Biol. 47, 499-506.

Behal, R. H., Oliver, D. J., 1997. Biochemical and molecular characterization of fumarase from plants: purification and characterization of the enzyme - cloning, sequencing, and expression of the gene. Arch. Biochem. Biophys. 348, 65-74.

Beriashvili, T. V., Beriashvili, L. T., 1996. Metabolism of malic and tartaric acids in grape berries. Biochem. (Moscow) 61, 1316-1321.

Beruter, J., 2004. Carbohydrate metabolism in two apple genotypes that differ in malate accumulation. J. Plant Physiol. 161, 1011-1029.

Blanke, M. M., Lenz, F., 1989. Fruit Photosynthesis. Plant Cell. Environ. 12, 31-46.

Borsani, J., Budde, C. O., Porrini, L., Lauxmann, M. A., Lombardo, V. A., Murray, R., Andreo, C. S., Drincovich, M. F., Lara, M. V., 2009. Carbon metabolism of peach fruit after harvest: changes in enzymes involved in organic acid and sugar level modifications. J. Exp. Bot. 60, 1823-1837.

Bucher, M., Brander, K. A., Sbicego, S., Mandel, T., Kuhlemeier, C., 1995. Aerobic fermentation in tobacco pollen. Plant Mol. Biol. 28, 739-750.

Causse, M., Duffe, P., Gomez, M. C., Buret, M., Damidaux, R., Zamir, D., Gur, A., Chevalier, C., Lemaire-Chamley, M., Rothan, C., 2004. A genetic map of candidate genes and QTLs involved in tomato fruit size and composition. J. Exp. Bot. 55, 1671-1685.

Chang, G. G., Tong, L., 2003. Structure and function of malic enzymes, a new class of oxidative decarboxylases. Biochem. 42, 12721-12733.

Chen, A. R. S., Chase, T., 1993. Alcohol dehydrogenase 2 and pyruvate decarboxylase induction in ripening and hypoxic tomato fruit. Plant Physiol. Biochem. 31, 875-885.

Chen, J. L., Wang, Z. F., Wu, J. H., Wang, Q., Hu, X. S., 2007. Chemical compositional characterization of eight pear cultivars grown in China. Food Chem. 104, 268-275.

Chollet, R., Vidal, J., Oleary, M. H., 1996. Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol, 47, 273-298.

Coombe, B. G., 1987. Distribution of solutes within the developing grape berry in relation to its morphology. Am. J. Enol. & Vitic. 38, 120-127.

Cramer, G. R., Ergul, A., Grimplet, J., Tillet, R. L., Tattersall, A. R., Bohlman, M. C., Vincent, D., Sonderegger, J., Evans, J., Osborne, C., Quilici, D., Schlauch, K. A., Schooley, D. A., Cushman, J. C., 2007. Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. Funct. Integr. Genomics 7, 111-134.

Davies, D. D., 1986. The fine control of cytosolic pH. Physiol. Plant. 67, 702-706.

Deluc, L. G., Grimplet, J., Wheatley, M. D., Tillett, R. L., Quilici, D. R., Osborne, C., Schooley, D. A., Schlauch, K. A., Cushman, J. C., Cramer, G. R., 2007. Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC Genomics 8.

Diakou, P., Svanella, L., Raymond, P., Gaudillere, J. P., Moing, A., 2000. Phosphoenolpyruvate carboxylase during grape berry development: protein level, enzyme activity and regulation. Aust. J. Plant Physiol. 27, 221-229.

Dilley, D. R., 1962. Malic enzyme activity in apple fruit. Nature 196, 387-388.

Drincovich, M. F., Casati, P., Andreo, C. S., 2001. NADP-malic enzyme from plants: a ubiquitous enzyme involved in different metabolic pathways. FEBS Lett. 490, 1-6.

Edwards, S., Nguyen, B. T., Do, B., Roberts, J. K. M., 1998. Contribution of malic enzyme, pyruvate kinase, phosphoenolpyruvate carboxylase, and the Krebs cycle to respiration and biosynthesis and to intracellular pH regulation during hypoxia in maize root tips observed by nuclear magnetic resonance imaging and gas chromatography mass spectrometry. Plant Physiol. 116, 1073-1081.

Emmerlich, V., Linka, N., Reinhold, T., Hurth, M. A., Traub, M., Martinoia, E., Neuhaus, H. E., 2003. The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. Proc. Natl. Acad. Sci. U.S.A. 100, 11122-11126.

Etienne, C., Moing, A., Dirlewanger, E., Raymond, P., Monet, R., Rothan, C., 2002. Isolation and characterization of six peach cDNAs encoding key proteins in organic acid metabolism and solute accumulation: involvement in regulating peach fruit acidity. Physiol. Plant. 114, 259-270.

Famiani, F., Cultrera, N. G. M., Battistelli, A., Casulli, V., Proietti, P., Standardi, A., Chen, Z. H., Leegood, R. C., Walker, R. P., 2005. Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. J. Exp. Bot. 56, 2959-2969.

Famiani, F., Walker, R. P., Tecsi, L., Chen, Z. H., Proietti, P., Leegood, R. C., 2000. An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries. J. Exp. Bot. 51, 675-683.

Farineau, J., Laval-Martin, D., 1977. Light *versus* dark carbon metabolism in cherry tomato fruits. II. Relationship between malate metabolism and photsynthetic activity. Plant Physiol. 60, 877-880.

Fatland, B. L., Nikolau, B. J., Wurtele, E. S., 2005. Reverse genetic characterization of cytosolic acetyl-CoA generation by ATP-citrate lyase in Arabidopsis. Plant Cell 17, 182-203.

Fernie, A. R., Carrari, F., Sweetlove, L. J., 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Curr. Opin. Plant. Biol. 7, 254-261.

Fernie, A. R., Martinoia, E., 2009. Malate. Jack of all trades or master of a few? Phytochem. 70, 828-832.

Franke, K. E., Adams, D. O., 1992. Inhibition of malic enzyme from grape berries by sulfhydryl-reagents and oxalic-acid. Am. J. Enol. & Vitic. 43, 153-158.

Franke, K. E., Adams, D. O., 1995. Cloning of a full-length cDNA for malic enzyme (EC.1.1.1.40) from grape berries. Plant Physiol. 107, 1009-1010.

Gardestrom, P., Edwards, G. E., 1983. Isolation of mitochondria from leaf tissue of *Panicum miliaceum*, a NAD-malic enzyme type C_4 plant. Plant Physiol. 71, 24-29.

Germain, V., Ricard, B., 1997. Two *ldh* genes from tomato and their expression in different organs, during fruit ripening and in response to stress. Plant Mol. Biol. 35, 949-954.

Givan, C. V., 1999. Evolving concepts in plant glycolysis: two centuries of progress. Biol. Rev. Cambridge Philos. Soc. 74, 277-309.

Goodenough, P. W., Prosser, I. M., Young, K., 1985. NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. Phytochem. 24, 1157-1162.

Gray, G. R., Maxwell, D. P., Villarimo, A. R., McIntosh, L., 2004. Mitochondria/nuclear signaling of alternative oxidase gene expression occurs through distinct pathways involving organic acids and reactive oxygen species. Plant Cell Reports 23, 497-503.

Grimplet, J., Deluc, L. G., Tillett, R. L., Wheatley, M. D., Schlauch, K. A., Cramer, G. R., Cushman, J. C., 2007. Tissue-specific mRNA expression profiling in grape berry tissues. BMC Genomics 8, 187.

Guillet, C., Just, D., Benard, N., Destrac-Irvine, A., Baldet, P., Hernould, M., Causse, M., Raymond, P., Rothan, C., 2002. A fruit-specific phospho*enol*pyruvate carboxylase is related to rapid growth of tomato fruit. Planta 214, 717-726.

Hafke, J. B., Hafke, Y., Smith, J. A. C., Luttge, U., Thiel, G., 2003. Vacuolar malate uptake is mediated by an anion-selective inward rectifier. Plant J. 35, 116-128.

Hale, C. R., 1962. Synthesis of organic acids in the fruit of the grape. Nature 195, 917-918.

Halinska, A., Frenkel, C., 1991. Acetaldehyde stimulation of net gluconeogenic carbon movement from applied malic acid in tomato fruit pericarp tissue. Plant Physiol. 95, 954-960.

Han, S. H., Kawabata, S., 2002. Changes in carbohydrate and water contents of kiwifruit (*Actinidia deliciosa*) during growth. J. Jap. Soc. Hort. Sci. 71, 322-327.

Hanning, I., Baumgarten, K., Schott, K., Heldt, H. W., 1999. Oxaloacetate transport into plant mitochondria. Plant Physiol. 119, 1025-1031.

Hansen, E., 1942. Quantitative study of ethylene production in relation to respiration of pears. Bot. Gazz. 103.

Hardy, P. J., 1968. Metabolism of sugars and organic acids in immature grape berries. Plant Physiol. 43, 224-228.

Hardy, P. J., 1969. Selctive diffusion of basic and acidic products of CO_2 fixation into the transpiration stream in grapevine. J. Exp. Bot. 20, 856-862.

Harris, J. M., Kriedemann, P. E., Possingham, J. V., 1971. Grape berry respiration: Effects of metabolic inhibitors. Vitis 9, 291-298.

Hawker, J. S., 1969. Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochem. 8, 19-23.

Huang, S. B., Greenway, H., Colmer, T. D., Millar, A. H., 2005. Protein synthesis by rice coleoptiles during prolonged anoxia: Implications for glycolysis, growth and energy utilization. Ann. Bot. 96, 703-715.

Hunter, J. J., Ruffner, H. P., 2001. Assimilate transport in grapevines - effect of phloem disruption. Aust. J. Grape & Wine Res. 7, 118-126.

Hurth, M. A., Suh, S. J., Kretzschmar, T., Geis, T., Bregante, M., Gambale, F., Martinoia, E., Neuhaus, H. E., 2005. Impaired pH homeostasis in Arabidopsis lacking the vacuolar dicarboxylate transporter and analysis of carboxylic acid transport across the tonoplast. Plant Physiol. 137, 901-910.

Iannetta, P. P. M., Escobar, N. M., Ross, H. A., Souleyre, E. J. F., Hancock, R. D., Witte, C. P., Davies, H. V., 2004. Identification, cloning and expression analysis of strawberry (*Fragaria x*

ananassa) mitochondrial citrate synthase and mitochondrial malate dehydrogenase. Physiol. Plant. 121, 15-26.

Iannetta, P. P. M., Laarhoven, L. J., Medina-Escobar, N., James, E. K., McManus, M. T., Davies, H. V., Harren, F. J. M., 2006. Ethylene and carbon dioxide production by developing strawberries show a correlative pattern that is indicative of ripening climacteric fruit. Physiol. Plant. 127, 247-259.

Imahori, Y., Matushita, K., Kota, M., Ueda, Y., Ishimaru, M., Chachin, K., 2003. Regulation of fermentative metabolism in tomato fruit under low oxygen stress. J. Hort. Sci. Biotech. 78, 386-393.

Jenner, H. L., Winning, B. M., Millar, A. H., Tomlinson, K. L., Leaver, C. J., Hill, S. A., 2001. NAD malic enzyme and the control of carbohydrate metabolism in potato tubers. Plant Physiol. 126, 1139-1149.

Jha, A. K., Singh, A. K., Prasad, U. S., 1990. Respiratory metabolites in *Litchi chinesis* Sonn. during fruit ripening and senescence. Indian J. Exp. Biol. 28, 537-541.

Ke, D. Y., Yahia, E., Hess, B., Zhou, L., Kader, A. A., 1995. Regulation of fermentative metabolism in avocado fruit under oxygen and carbon dioxide stresses. J. Am. Soc. Hort. Sci. 120, 481-490.

Kliewer, W. M., 1964. Influence of environment on metabolism of organic acids and carbohydrates in *Vitis vinifera*. I. Temperature. Plant Physiol. 39, 869-880.

Kliewer, W. M., Howarth, L., Omori, M., 1967. Concentrations of tartaric acid and malic acids and their salts in *Vitis vinifera* grapes. Am. J. Enol. & Vitic. 18, 42-54.

Kortstee, A. J., Appeldoorn, N. J. G., Oortwijn, M. E. P., Visser, R. G. F., 2007. Differences in regulation of carbohydrate metabolism during early fruit development between domesticated tomato and two wild relatives. Planta 226, 929-939.

Kovermann, P., Meyer, S., Hortensteiner, S., Picco, C., Scholz-Starke, J., Ravera, S., Lee, Y., Martinoia, E., 2007. The Arabidopsis vacuolar malate channel is a member of the ALMT family. The Plant Journal 52, 1169-1180.

Kunkee, R. E., 1991. Some roles of malic acid in the malolactic fermentation in wine-making. FEMS Microbiol. Rev. 88, 55-72.

Lakso, A. N., Kliewer, W. M., 1975. The influence of temperature on malic acid metabolism in grape berries. Plant Physiol. 56, 370-372.

Lakso, A. N., Kliewer, W. M., 1978. The influence of temperature on malic acid metabolism in grape berries. II. Temperature responses of net dark CO_2 fixation and malic acid pools. Am. J. Enol. & Vitic. 29, 145-149.

Langlade, N. B., Messerli, G., Weisskopf, L., Plaza, S., Tomasi, N., Smutny, J., Neumann, G., Martinoia, E., Massonneau, A., 2002. ATP citrate lyase: cloning, heterologous expression and

possible implication in root organic acid metabolism and excretion. Plant Cell. Environ. 25, 1561-1569.

Law, R. D., Plaxton, W. C., 1995. Purification and characterization of a novel phosphoenolpyruvate carboxylase fomr banana fruit. Biochem. J. 307, 807-816.

Livio, T., Tadiello, A., Casadoro, G., 2007. Variations of the peach fruit transcriptome during ripening and in response to hormone treatments. Caryologia 60, 156-159.

Lobit, P., Genard, M., Soing, P., Habib, R., 2006. Modelling malic acid accumulation in fruits: relationships with organic acids, potassium, and temperature. J. Exp. Bot. 57, 1471-1483.

Longhurst, T. J., Tung, H. F., Brady, C. J., 1990. Developmental regulation of the expression of alcohol dehydrogenase in ripening tomato fruits. J. Food Biochem. 14, 421-433.

Luttge, U., Ratajczak, R., 1997. The physiology, biochemistry and molecular biology of the plant vacuolar ATPase. Advances in Botanical Research Incorporating Advances in Plant Pathology, vol. 25, pp. 253-296.

Manriquez, D., El-Sharkawy, I., Flores, F. B., El-Yahyaoui, F., Regad, F., Bouzayen, M., Latche, A., Pech, J. C., 2006. Two highly divergent alcohol dehydrogenases of melon exhibit fruit ripening-specific expression and distinct biochemical characteristics. Plant Mol. Biol. 61, 675-685.

Millar, A. H., Wiskich, J. T., Whelan, J., 1993. Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett. 329, 259-262.

Moing, A., Renaud, C., Gaudillere, M., Raymond, P., Roudeillac, P., Denoyes-Rothan, B., 2001. Biochemical changes during fruit development of four strawberry cultivars. J. Am. Soc. Hort. Sci. 126, 394-403.

Moing, A., Rothan, C., Svanella, L., Just, D., Diakou, P., Raymond, P., Gaudillere, J. P., Monet, R., 2000. Role of phosphoenolpyruvate carboxylase in organic acid accumulation during peach fruit development. Physiol. Plant. 108, 1-10.

Moing, A., Rothan, C., Svanella, L., Just, D., Diakou, P., Rolin, D., Gaudillere, J. P., Monet, R., 1998. Organic acid metabolism during the development of two peach cultivars. Acta Hort. 465, 425-432.

Moing, A., Svanella, L., Gaudillere, M., Gaudillere, J. P., Monet, R., 1999. Organic acid concentration is little controlled by phosphoenolpyruvate carboxylase activity in peach fruit. Aust. J. Plant Physiol. 26, 579-585.

Nunes-Nesi, A., Carrari, F., Gibon, Y., Sulpice, R., Lytovchenko, A., Fisahn, J., Graham, J., Ratcliffe, R. G., Sweetlove, L. J., Fernie, A. R., 2007. Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J. 50, 1093-1106.

Nunes-Nesi, A., Carrari, F., Lytovchenko, A., Smith, A. M. O., Loureiro, M. E., Ratcliffe, R. G., Sweetlove, L. J., Fernie, A. R., 2005. Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol. 137, 611-622.

Oliver, S. N., Lunn, J. E., Urbanczyk-Wochniak, E., Lytovchenko, A., van Dongen, J. T., Faix, B., Schmalzlin, E., Fernie, A. R., Geigenberger, P., 2008. Decreased expression of cytosolic pyruvate kinase in potato tubers leads to a decline in pyruvate resulting in an *in vivo* repression of the alternative oxidase. Plant Physiol. 148, 1640-1654.

Ollat, N., Gaudillere, J. P., 2000. Carbon balance in developing grapevine berries. In: Bravdo, B. A. (Ed.), Proceedings of the V International Symposium on Grapevine Physiology.

Or, E., Baybik, J., Sadka, A., Ogrodovitch, A., 2000a. Fermentative metabolism in grape berries: isolation and characterization of pyruvate decarboxylase cDNA and analysis of its expression throughout berry development. Plant Sci. 156, 151-158.

Or, E., Baybik, J., Sadka, A., Saks, Y., 2000b. Isolation of mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxylase cDNA clones from grape berries and analysis of their expression pattern throughout berry development. J. Plant Physiol. 157, 527-534.

Palliotti, A., Cartechini, A., 2001. Developmental changes in gas exchange activity in flowers, berries, and tendrils of field-grown Cabernet Sauvignon. Am. J. Enol. & Vitic. 52, 317-323.

Palmieri, L., Picault, N., Arrigoni, R., Besin, E., Palmieri, F., Hodges, M., 2008. Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization. Biochem. J. 410, 621-629.

Pandey, R. M., Farmahan, H. L., 1977. Changes in the rate of photosynthesis and respiration in leaves and berries of *Vitis vinifera* grapevines at various stages of berry development. Vitis 16, 106-111.

Pereira, G. E., Gaudillere, J. P., Pieri, P., Hilbert, G., Maucourt, M., Deborde, C., Moing, A., Rolin, D., 2006. Microclimate influence on mineral and metabolic profiles of grape berries. J. Agric. Food Chem. 54, 6765-6775.

Peruffo, A. D. B., Pallavicini, C., 1975. Enzymatic changes associated with ripening of grape berries. J. Sci. Food Agric. 26, 559-566.

Picault, N., Hodges, M., Palmieri, L., Palmieri, F., 2004. The growing family of mitochondrial carriers in *Arabidopsis*. Trends Plant Sci. 9, 138-146.

Picault, N., Palmieri, L., Pisano, I., Hodges, M., Palmieri, F., 2002. Identification of a novel transporter for dicarboxylates and tricarboxylates in plant mitochondria. J. Biol. Chem. 277, 24204-24211.

Piechulla, B. C., Glick, R. E., Bahl, H., Melis, A., Gruissem, W., 1987. Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. Plant Physiol. 84, 911-917.

Pilati, S., Perazzolli, M., Malossini, A., Cestaro, A., Dematte, L., Fontana, P., Dal Ri, A., Viola, R., Velasco, R., Moser, C., 2007. Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at veraison. BMC Genomics 8.

Plaxton, W. C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol, 47, 185-214.

Ponce-Valadez, M., Watkins, C. B., 2008. Fermentation and malate metabolism in response to elevated CO_2 concentrations in two strawberry cultivars. Physiol. Plant. 134, 121-133.

Possner, D., Ruffner, H. P., Rast, D. M., 1981. Isolation and biochemical characterization of grape malic enzyme. Planta 151, 549-554.

Possner, D. R. E., Kliewer, W. M., 1985. The localization of acids, sugars, potassium and calcium in developing grape berries. Vitis 24, 229-240.

Possner, D. R. E., Ruffner, H. P., Rast, D. M., 1983. Regulation of malic acid metabolism in berries of *Vitis vinifera*. Acta Hort. 139, 117-122.

Pua, E. C., Chandramouli, S., Han, P., Liu, P., 2003. Malate synthase gene expression during fruit ripening of Cavendish banana (*Musa acuminata* cv. Williams). J. Exp. Bot. 54, 309-316.

Rademacher, T., Hausler, R. E., Hirsch, H. J., Zhang, L., Lipka, V., Weier, D., Kreuzaler, F., Peterhansel, C., 2002. An engineered phosphoenolpyruvate carboxylase redirects carbon and nitrogen flow in transgenic potato plants. Plant J. 32, 25-39.

Richardson, A. C., Marsh, K. B., Boldingh, H. L., Pickering, A. H., Bulley, S. M., Frearson, N. J., Ferguson, A. R., Thornber, S. E., Bolitho, K. M., Marcrae, E. A., 2004. High growing temperatures reduce fruit carbohydrate and vitamin C in kiwifruit. Plant Cell Environ. 27, 423-435.

Romieu, C., Tesniere, C., Thanham, L., Flanzy, C., Robin, J. P., 1992. An examination of the importance of anaerobiosis and ethanol in causing injury to grape mitochondria. Am. J. Enol. & Vitic. 43, 129-133.

Ruffner, H. P., 1982. Metabolism of tartaric and malic acids in *Vitis*: A review - Part B. Vitis 21, 346-358.

Ruffner, H. P., Hawker, J. S., 1977. Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochem. 16, 1171-1175.

Ruffner, H. P., Hawker, J. S., Hale, C. R., 1976. Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochem. 15, 1877-1880.

Ruffner, H. P., Kliewer, W. M., 1975. Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiol. 56, 67-71.

Ruffner, H. P., Possner, D., Brem, S., Rast, D. M., 1984. The physiological role of malic enzyme in grape ripening. Planta 160, 444-448.

Rustin, P., Moreau, F., Lance, C., 1980. Malate oxidation in plant mitochondria via malic enzyme and the cyanide-insensitive electron transport pathway. Plant Physiol. 66, 457-462.

Saito, T., Matsukura, C., Ban, Y., Shoji, K., Sugiyama, M., Fukuda, N., Nishimura, S., 2008. Salinity stress affects assimilate metabolism at the gene-expression level during fruit development and improves fruit quality in tomato. J. Jap. Soc. Hort. Sci. 77, 61-68.

Sakano, K., Kiyota, S., Yazaki, Y., 1998. Degradation of endogenous organic acids induced by P-i uptake in *Catharanthus roseus* cells: involvement of the biochemical pH-Stat. Plant Cell Physiol. 39, 615-619.

Saradhuldhat, P., Paull, R. E., 2007. Pineapple organic acid metabolism and accumulation during fruit development. Sci. Hort. 112, 297-303.

Schauer, N., Semel, Y., Roessner, U., Gur, A., Balbo, I., Carrari, F., Pleban, T., Perez-Melis, A., Breudigam, C., Kopka, J., Willmitzer, L., Zamir, D., Fernie, A. R., 2006. Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature Biotech. 24, 447-454.

Schulze, J., Tesfaye, M., Litjens, R., Bucciarelli, B., Trepp, G., Miller, S., Samac, D., Allan, D., Vance, C. P., 2002. Malate plays a central role in plant nutrition. Plant Soil 247, 133-139.

Selvaraj, Y., 1993. Changes in enzyme activity in ripening pineapple fruit. Indian J. Hort. 50, 310-317.

Selvaraj, Y., Kumar, R., 1989. Pyruvate kinase activity in ripening mango (*Mangifera indica* L.) fruit. J. Food Sci. Tech. Mysore 26, 228-229.

Shi, J. X., Goldschmidt, E. E., Goren, R., Porat, R., 2007. Molecular, biochemical and anatomical factors governing ethanol fermentation metabolism and accumulation of off-flavors in mandarins and grapefruit. Postharvest Biol. Tehcnol. 46, 242-251.

Shiraishi, S., Kawakami, K., Widodo, S. E., Shiraishi, M., Kitazaki, M., 1996. Organic acid profiles in the juice of fig fruits. J. Faculty Agric. Kyushu Univ. 40, 29-33.

Singh, S. P., Singh, Z., 2008. Major flavor components in some commercial cultivars of Japanese plum. J. Am. Pomol. Soc. 62, 185-190.

Soole, K. L., Dry, I. B., Wiskich, J. T., 1990. Oxidation of NADH by plant mitochondria - kinetics and effects of calcium-ions. Physiol. Plant. 78, 205-210.

Spayd, S. E., Tarara, J. M., Mee, D. L., Ferguson, J. C., 2002. Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. Am. J. Enol. & Vitic. 53, 171-182.

Speirs, J., Lee, E., Holt, K., Yong-Duk, K., Scott, N. S., Loveys, B. R., Schuch, W., 1998. Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit affects the balance of some flavor aldehydes and alcohols. Plant Physiol. 117, 1047-1058.

Surendranathan, K. K., Nair, P. M., 1976. Stimulation of the glyoxylate shunt in gamma-irradiated banana. Phytochem. 15, 371-373.

Tadege, M., Kuhlemeier, C., 1997. Aerobic fermentation during tobacco pollen development. Plant Mol. Biol. 35, 343-354.

Taureilles-Saurel, C., Romieu, C. G., Robin, J. P., Flanzy, C., 1995a. Grape (*Vitis vinifera* L.) malate dehydrogenase .I. Intracellular compartmentation of the isoforms. Am. J. Enol. & Vitic. 46, 22-28.

Taureilles-Saurel, C., Romieu, C. G., Robin, J. P., Flanzy, C., 1995b. Grape (*Vitis vinifera* L.) malate dehydrogenase .II. Characterization of the major mitochondrial and cytosolic isoforms and their role in ripening. Am. J. Enol. & Vitic. 46, 29-36.

Terrier, N., Glissant, D., Grimplet, J., Barrieu, F., Abbal, P., Couture, C., Ageorges, A., Atanassova, R., C., L., Renaudin, J. P., Dedaldechamp, F., Romieu, C., Delrot, S., Hanmdi, S., 2005. Isogene specific oligo arrays reveal multifaceted changes in gene expression during grape berry (*Vitis vinifera* L.) development. Planta 222, 832-847.

Terrier, N., Romieu, C., 1998. Inhibition of vacuolar proton pumps by ethanol impairs grape berry compartmentation. Aust. J. Grape & Wine Res. 4, 39-45.

Terrier, N., Sauvage, F. X., Ageorges, A., Romieu, C., 2001. Changes in acidity and in proton transport at the tonoplast of grape berries during development. Planta 213, 20-28.

Tesniere, C., Torregrosa, L., Pradal, M., Souquet, J. M., Gilles, C., Santos, K. D., Chatelet, P., Gunata, Z., 2006. Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. J. Exp. Bot. 2006, 1.

Torregrosa, L., Pradal, M., Souquet, J. M., Rambert, M., Gunata, Z., Tesniere, C., 2008. Manipulation of *VvAdh* to investigate its function in grape berry development. Plant Sci. 174, 149-155.

Tovar, B., Garcia, H. S., Mata, M., 2001. Physiology of pre-cut mango II. Evolution of organic acids. Food Res. Internat. 34, 705-714.

Usenik, V., Fabcic, J., Stampar, F., 2008. Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (*Prunus avium* L.). Food Chem. 107, 185-192.

Wada, H., Shackel, K., Matthews, M. A., 2008. Fruit ripening in *Vitis vinifera*: apoplastic solute accumulation accounts for pre-veraison turgor loss in berries. Planta 227, 1351-1361.

Walker, R. P., Leegood, R. C., 1995. Purification, and Phosphorylation in-Vivo and in-Vitro, of Phosphoenolpyruvate Carboxykinase from Cucumber Cotyledons. FEBS Lett. 362, 70-74.

Walton, E. F., De Jong, T. M., 1990. Growth and compositional changes in kiwifruit berries from three Californian locations. Ann. Bot. 66, 285-298.

Wu, B. H., Quilot, B., Genard, M., Kervella, J., Li, S. H., 2005. Changes in sugar and organic acid concentrations during fruit maturation in peaches, *P. davidiana* and hybrids as analyzed by principal component analysis. Sci. Hort. 103, 429-439.

Yao, Y., Li, M., Liu, Z., Hao, Y., Zhai, H., 2007. A novel gene, screened by cDNA-AFLP approach, contributes to lowering the acidity of fruit in apple. Plant Physiol. Biochem. 45, 139-145.

Zabalza, A., Van Dongen, J. T., Froehlich, A., Oliver, S. N., Faix, B., Gupta, K. J., Schmalzlin, E., Igal, M., Orcaray, L., Royuela, M., Geigenberger, P., 2009. Regulation of respiration and fermentation to control the plant internal oxygen concentration. Plant Physiol. 149, 1087-1098.

Zhang, Q., Moore, C. S., Soole, K. L., Wiskich, J. T., 2003. Over-reduction of cultured tombacco cells mediates changes in respiratory activities. Physiol. Plant. 119, 183-191.

Zhang, X. Y., Wang, X. L., Wang, X. F., Xia, G. H., Pan, Q. H., Fan, R. C., Wu, F. Q., Yu, X. C., Zhang, D. P., 2006. A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. Plant Physiol. 142, 220-232.

Chapter 2

Developmental regulation of malate and its involvement in primary and secondary metabolism of *Vitis vinifera* fruit

Developmental regulation of malate and its involvement in primary and secondary metabolism of *Vitis vinifera* fruit

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2.1. Abstract

Malate, one of the predominant acids of *Vitis vinifera* fruit, is woven into a complex network of plant metabolic pathways and, as such, plays vital roles in plant biochemistry. In grape, the acid displays two distinct phases of malate metabolism; a net accumulation phase during early development, followed by a period of acid loss during ripening. The metabolism of malate has been investigated during development of the grape berry, to assist with the identification of pathways important in the regulation of plant malate metabolism. The activities and transcripts of numerous enzymes potentially involved in changes in malate metabolism during development were measured from whole grape berry cellular extracts and, in some instances, from purified grape berry mitochondria. A general metabolic analysis was also conducted, to pinpoint changes in other facets of metabolism that may be linked to changes in malate metabolism during berry development. Results support previous findings regarding developmental changes in primary metabolic pathways, and present new information regarding the control of PEP metabolism, with the use of C4-like photosynthetic enzymes in the metabolic pathways of this C3-type plant. These data highlight changes in sugar metabolism including glycolysis and gluconeogenesis, through changes in sugar and sugar-phosphate levels, as well as activities and transcripts of PEP carboxylase, PEP carboxykinase, pyruvate kinase and pyruvate,orthophosphate dikinase. There was a general decrease in numerous TCA cycle intermediates and amino acids, suggesting a use for these acids in other pathways during the ripening of the fruit. Activities of malate dehydrogenase and malic enzyme isoforms demonstrated varying trends throughout development, suggesting involvement of these enzymes in both malate synthesis and degradation, with possible regulation through post-translational modification and substrate availability, according to requirements of other metabolic pathways. Alcohol fermentation and the shikimate pathway may have also indirectly utilised malate during ripening to support the synthesis of secondary metabolites important for fruit ripening.

2.2. Introduction

Grapes commonly accumulate large quantities of malate and tartrate, in the realm of 10-80 µmoles per berry depending on variety and developmental stage (Melino et al. 2009). These

acids are significant in both the biochemical and sensory properties of the fruit. As the predominant acids in the berry, malate and tartrate are also very influential in winemaking due to their contribution to flavour and aroma effects in finished wines. Malate has long been known to play a significant role in grape berry metabolism (Ruffner et al., 1983), and recent evidence suggests that active metabolism of tartrate may occur in the ripening berry (Melino et al., 2009), although the latter is largely thought to be inert and sequestered in the vacuole throughout development (Ruffner, 1982a). Malate is a functional organic acid; it is an intermediate of the TCA cycle and a source of carbon for gluconeogenesis, amino acid biosynthesis and respiration. In plants it also plays roles in photosynthesis (Meister et al., 1996), control of stomatal aperture (Dietrich et al., 2001), pH regulation (Mathieu et al., 1986), and heavy metal tolerance (Delhaize et al., 2007), among others (for a review, see Fernie & Martinoia, 2009). In *V. vinifera* a clear pattern emerges during development of the fruit, whereby malate undergoes net accumulation to a high concentration during early growth and development, and then switches to net degradation during ripening (Harris et al., 1968). This switch occurs at, or just before the initiation of fruit ripening (veraison), where the berries begin to soften and accumulate sugars and complex compounds, including pigments in coloured cultivars. The veraison phenomenon has been of keen interest to plant scientists for decades (Coombe, 1984), and yet the regulation of the switch between net malate synthesis and net degradation is still unclear.

Within the cell of a grape berry there exist two pools of malate, such that the acid can be accumulated by sequestration in the vacuole (storage pool), whilst still available for cellular processes (metabolic pool) (Ruffner, 1982b). While sequestration, and hence availability of the acid to cellular metabolism is undoubtedly important in the regulation of malate catabolism, the rapid loss is likely to also be regulated by the activities of metabolic enzymes in the cell (Figure 1.1; Sweetman et al. 2009). Such enzymes as NADP-linked malic enzyme, malate dehydrogenase and PEP carboxylase have been investigated in previous studies with grapes (Hawker, 1969; Diakou et al., 2000), and may be likely candidates. Glycolytic enzymes such as hexokinase and glucose 6-phosphate dehydrogenase also appear to have no correlation with malate levels (Hawker, 1968), while the gluconeogenic enzyme PEP carboxykinase has been suggested to play a role in the synthesis of sugars from malate (Walker et al., 1999; Famiani et al., 2000). Other enzymes such as alcohol dehydrogenase

have also been investigated (Tesniere and Verries, 2000; Tesniere et al., 2006), and while these reports were not applied to the study of organic acid metabolism, the patterns of transcript and activity levels demonstrated throughout development suggest that malate may be an upstream substrate for these pathways. The regulation of malate metabolism in grapes, in addition to its release from the vacuole, may occur through the presence and post-translational regulation of enzymes from the pathways mentioned above, the synthesis of new enzyme units, or by a combination of these events. The current study aims to deliver a thorough investigation of activities and transcript levels of enzymes thought to be involved in the regulation of malate metabolism during grape berry development, as well as their potential influence on some metabolic intermediates.

2.3. Materials and methods

2.3.1. Materials

Berries were collected from the Coombe Vineyard, University of Adelaide Waite Campus, Urrbrae, South Australia (123m elevation, -34.964˚ latitude, 138.648˚ longitude) in the 2007-08 and 2009-2010 seasons. Vines were spur-pruned to 30-40 nodes per vine, and drip irrigated weekly. Five East-West facing rows of Shiraz clone BVRC12 vines, established in 1993 on Schwarzmann rootstocks (3 m row spacing and 1.8 m vine spacing), were used. Each row was divided into panels of four vines. A panel was selected for each of the five rows (i.e. 20 vines in total). Each of the four vines in a panel was designated to a separate replicate such that four replicate samples would contain fruit from vines across five different rows. Approximately six bunches were selected from each vine in each of the five panels and tagged at 50% cap-fall, or “anthesis”. Therefore each replicate consisted of berries from 30 synchronous bunches. Samples were taken from fruit set until maturity. At each sampling date the fruit was harvested between 7:30-9:30 am, with 1-4 berries per bunch taken from various positions within the bunches. The fruit was immediately frozen in liquid nitrogen before transfer to the laboratory and storage at -80˚C until further use.

Molecular grade reagents were purchased from Sigma-Aldrich (NSW, Australia), unless stated otherwise.

2.3.2. Malate quantification

Organic acids were extracted from 500 mg frozen berry powder, in 5 ml of 3% (w/v) metaphosphoric acid (MPA; with 1 mM EDTA), with shaking for 60 mins at 4˚C. The homogenate was centrifuged at 2,500 *x g* for 5 mins and the supernatant was passed through a Millipex 0.45 µm hydrophilic PVDF syringe-driven filter (Millipore, NSW, Australia). The filtrate was used directly for Reversed-Phase HPLC quantification according to Melino et al. (2009b).

2.3.3. Enzyme extraction

Active enzymes were extracted using a method adapted from Ruffner and Kliewer (1975). Twelve volumes of extraction buffer (0.5 M Tris-Cl, pH 8.5 with 200 M KCl, 20 mM $MgCl_2$, 10 mM EDTA, 8% (w/v) PEG-4000, 8 mM cysteine-HCl, 7 mM diethyldithiocarbamate, 5 mM DTT, 2% (w/v) PVPP, 0.25% (w/v) BSA, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine) were added to 1g of frozen grape berry powder and mixed gently at 4˚C for 15 mins. After centrifugation at 2,750 *x g* for 5 mins to remove cell debris, PEG-4000 was added to a final concentration of 65% (w/v) in the supernatant and mixed by gentle shaking at room temperature until dissolved. After centrifugation at 30,000 *x g*, the precipitated protein was carefully resuspended in 2mL final volume (5 mM Tris-Cl, pH 7.0, with 20 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, 3% (v/v) Triton X-100, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine), re-centrifuged at 3,000 *x g* and the supernatant used in enzyme activity assays.

2.3.4. Mitochondrial isolation

All steps were carried out at 4˚C using a method adapted from Romieu and Flanzy (1988). Fresh grape berry tissue (50 g), was gently homogenised in 150 ml of Isolation Medium (200 mM MOPS pH 8.0, 15 mM Na_2HPO_4, 5 mM EDTA, 0.3 M sucrose, 0.35 M mannitol, 1% (w/v) PVP-40, 0.25% (w/v) casein, 4 mM cysteine-HCl, 5 mM iso-ascorbate, 0.5% (w/v) BSA), using a Polytron at minimum speed to ensure seeds remained intact. The homogenate was filtered through 2 layers of Miracloth to remove skin and seeds, then centrifuged at 1,500 *x g* for 15 mins. The supernatant was centrifuged at 10,000 *x g* for 30 mins, and the pellet resuspended in approximately 30 ml of Wash Medium (15 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 0.3 M sucrose, 0.5 M mannitol, 1% (w/v) PVP-40, 0.1% (w/v) BSA). After repeating the

centrifugations, the pellet was carefully resuspended in 2 ml of Wash Medium (without BSA). This crude mitochondrial preparation was purified on a 4-step discontinuous density gradient (10%, 15%, 25% and 45% (v/v) Percoll in Wash Medium), in a swing-out rotor at 18,000 rpm for 60 mins. Mitochondria were collected at the 15:25 and 25:45 Percoll interfaces, pooled and washed in 30 ml of Wash Medium (containing 0.4 M sucrose) and pelleted at 10,000 *x g* for 15 mins. The final pellet was resuspended in 1 ml of Wash Medium (without BSA) and used in spectrophotometric and polarographic enzyme assays.

2.3.5. Enzyme activity assays

Unless specified otherwise, all assays were carried out at 25˚C using a FLUOstar UV/vis plate reader (BMG Labtech, Victoria, Australia), in a final volume of 200 µl. The rate of change in absorbance at 340nm was used to measure the reduction of 2 mM $NAD(P)^+$ or oxidation of 0.5 mM NAD(P)H, as outlined below. In each case the assay was initiated by the addition of the reagent listed last.

NAD-dependent malate dehydrogenase (NAD-MDH) activity was quantified as the rate of NADH oxidation in 50 mM MES (pH 6.0), in the presence of 5 mM oxaloacetate, as described previously (Ruffner et al., 1976).

NADP-dependent malate dehydrogenase (NADP-MDH) activity was quantified as the rate of NADPH oxidation in 50 mM TES (pH 8.0), in the presence of 5 mM DTT and 5mM oxaloacetate, using a method modified from Jacquot et al. (1981).

PEP carboxylase (PEPC) activity was quantified as the rate of NADH oxidation in 50 mM TES (pH 8.0), in the presence of 10 mM $MgCl_2$, 5 mM DTT, 5 mM $KHCO_3$, 6 U malate dehydrogenase (Sigma) and 2.5 mM PEP, a modification from Ruffner et al. (1976).

PEP carboxykinase (PEPCK) activity was quantified as the rate of NADH oxidation in 50 mM MES (pH 6.7), in the presence of 0.1 M KCl, 6 mM $MnCl_2$, 25 mM DTT, 90 mM $KHCO_3$, 6 U malate dehydrogenase (Sigma), 6 mM PEP and 1 mM ADP, as described previously (Walker et al., 1999).

NADP-dependent malic enzyme (NADP-ME) activity was quantified as the rate of NADP reduction in 50 mM MES (pH 6.0) in the presence of 8 mM $MnCl_2$, 2.5 mM potassium cyanide, 0.3 µM octyl gallate and 5 mM malate, a modification from Ruffner et al. (1976).

Alcohol dehydrogenase (ADH) activity was quantified as the rate of NADH oxidation in 50 mM MES (pH 6.0) in the presence of 50 µM acetaldehyde, as described previously (Molina et al., 1987).

Pyruvate kinase (PK) activity was quantified as the rate of NADH oxidation in 50 mM TES (t pH 7.2) in the presence of 70 mM KCl, 30 mM $MgCl_2$, 6 U lactate dehydrogenase, 5 mM PEP and 8 mM ADP, as described previously (Turner and Plaxton, 2000).

NAD-dependent malic enzyme (NAD-ME) activity was measured using an oxygen electrode (Rank Brothers) in a Standard Reaction Medium (10 mM TES pH 6.8, 10 mM KH_2PO_4, 0.4 M sucrose, 0.5 M mannitol, 2 mM $MgCl_2$). Activity was defined as the rate of oxygen consumption of purified mitochondria in the presence of 10 mM malate, 2 mM ADP, 4 mM $MnCl_2$, 1 mM NAD^+ and 75 µM Coenzyme A (CoA). With malate as the sole respiratory substrate, the activity of malic enzyme can define the amount of acetyl-CoA made available to the TCA cycle, and thus the rate of oxygen consumption through oxidative phosphorylation. An initial rate in the absence of CoA was ascribed to NAD-MDH activity, and was left to plateau before the addition of CoA.

2.3.6. Bioinformatics

Gene sequences for enzymes potentially involved in malate metabolism were detected with keyword searches and BLAST searches using sequences from other fruits (eg. tomato and mango), from grape EST databases such as
DFCI (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape) and
NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=29760). *V. vinifera* protein accessions for each gene are given in Table 2.1. Where necessary, ESTs were assembled into contiguous sequences. Corresponding genomic sequences were identified using the MegaBLAST function on NCBI (http://blast.ncbi.nlm.nih.gov) in the *V. vinifera* Whole Genome Shotgun (WGS) database. Grape EST and genomic contigs were aligned using Spidey (NCBI; http://www.ncbi.nlm.nih.gov/spidey), to identify potential intron/exon

Table 2.1: List of accession numbers, primer sets and probes used for qRT-PCR. Probe numbers are from the Human Universal Probe Library (Roche, Australia).

Gene	Accession #	Protein	Primer Sequences (5'-3')	Probe
ADH1	AF194173 (CDS)	AAG01381	F: tttagtgaatacaccgtcgttca R: ggttttgccacattgagagtg	76
ADH2	AF194174 (CDS)	AAG01382	F: tgtgttcttagctgtggaatctct R: cgattgatgaacccttcgat	145
ADH3	AF194175 (CDS)	AAG01383	F: aaccccaaagatcatgacaaa R: gcaacgcttccagtacactct	25
PEPC1	AF236126 (mRNA)	AAL83719	F: cgccagttattatcaagtggaa R: caagcacagagtgatctatagcaga	34
PEPC2	XM_002280806	CAN62388	F: atgcccgacttgctgttagt R: aatagaggaaccacccgtagc	113
PEPC3	XM_002285406	CBI20195	F: aatagaggaaccacccgtagc R: caagaaggtccttgtgtccag	25
PEPCK	XM_002277794.1	CBI35761	F: cattgatctctcaagggagaaag R: tcgaacaccacattttccaac	147
NAD-ME	XM_002266661	XP_002266697	F: gactgactttgtcaaccaaaagatag R: gccatattaagcacaccaatcc	4
PPDK	CA808125 (EST)	CBI26150.3	F: tgggaaccatgattgagattc R: gaagaattctgcctccttcg	164
Ankyrin	XM_002283462	XP_002283498	F: ggttatggcaggaaggagtg R: ggtgtcttgccatccatgtt	120
Ubiquitin	BN000705 (mRNA)	CAI56329	F: gtggccacagcaaccagt R: gcaacctccaatccagtcat	143

splice sites. Primer and probe sets for qRT-PCR assays were developed using the Roche Universal ProbeLibrary Assay Design Center, such that primers flanked intron splice sites, with expected products of 70-110 bp. Primer and probe details are shown in Table 2.1.

2.3.7. Quantitative real-time reverse transcriptional PCR

Total grape berry RNA was extracted and purified as previously described (Melino et al., 2009). First-strand cDNA synthesis was carried out with oligo$(dT)_{20}$ primer and Superscript III reverse transcriptase (Invitrogen, Victoria, Australia), as per the manufacturer's protocol. The concentration of resultant cDNA was estimated using a Nanodrop spectrometer, then diluted to 12.5 ng/µl in nuclease-free water. Products from each primer set were amplified from cDNA template using GoTaq Flexi polymerase (Promega, NSW, Australia), then purified using a Wizard PCR Purification Kit (Promega, NSW, Australia) and quantified using a Nanodrop spectrometer. The molecular weight of each PCR product was calculated using the product sequence and an online molecular weight calculator (Chang Bioscience DNA/RNA/Protein/Chemical Molecular Weight Calculator, http://www.changbioscience.com/genetics/mw.html). Purified PCR products were then diluted to 100 fmol/µl stocks in nuclease-free water. These stocks were used to create

standards at 1, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} fmol/µl, for use in qRT-PCR reactions. Reactions were set up in Faststart Universal Probe Master (Rox) master mix (Roche, NSW, Australia) with a final concentration of 0.9 µM gene-specific primers (Geneworks, SA, Australia) and 0.1 µM Universal ProbeLibrary probes (Roche, NSW, Australia), and initiated with 50 ng of cDNA in a final volume of 16 µl. Thermal cycling conditions for all qRT-PCR assays involved an initial 95˚C activation step (10 min), followed by 45 cycles of 95˚C (15 sec) melting and 57˚C (1 min) annealing/extension steps, using an ABI HT-700 thermocycler, and analysed with SDS 2.3 software (Applied Biosystems, Victoria, Australia).

2.3.8. Metabolic profiling

Metabolite extraction and analysis were carried out by the Melbourne node of Metabolomics Australia (University of Melbourne, Parkville, Victoria). Metabolites were extracted from 30 mg of frozen grape berry powder in 100% methanol, using internal standards ribitol and norleucine, then diluted in an equal volume of water and centrifuged

at 13,000 rpm for 15 mins. The supernatant (containing 'TBS' metabolites) was dried and stored. Further purification was carried out by centrifuging at 13,000 rpm with an equal volume of $CHCl_3$. The polar phase (containing 'TMS' metabolites) was collected, dried and stored. Dried residues were re-dissolved in 10 µl methoxyamine hydrochloride (30 mg/ml, in pyridine), derivatised for 2 h at 37˚C, then treated for 45 mins with N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (with 1% tert-butyldimethylchlorosilane) at 65˚C. Samples (1 µl) were injected into a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole mass spectrometer with a Gerstel 2.5.2 autosampler (Agilent, Santa Clara, USA) through a 30 m VF-5MS gas chromatography column (0.2 µm film thickness) with a 10 m Integra guard column (Varian, Inc, Victoria, Australia). The injection temperature was 250˚C, the MS transfer line was 280˚C, the ion source was at 250˚C and the quadrupole was set to 150˚C. Helium was used as the carrier gas at a flow rate of 0.8 ml min^{-1}. The analysis of TBS samples was performed under the following temperature program; injection at 100˚C (1min), 12.5˚C min^{-1} oven temperature ramp to 325˚C, then held for 6 mins. The analysis of TMS samples differed only by injection temperature; 70˚C. The system was temperature-equilibrated for 1 min at 100°C prior to injection of the next sample.

Mass spectra were recorded at 2 scan/sec with an *m/z* 50-600 scanning range. Both chromatograms and mass spectra were evaluated using the Chemstation program (Agilent, Santa Clara, USA). Mass spectra of eluting compounds were identified by Metabolomics Australia using the commercial mass spectra library NIST (http://www.nist.gov), the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html) and the in-house Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios normalised to extracted fresh weight for each analysed metabolite were prepared as described by Roessner et al. (2001).

2.3.9. Statistical analyses

Linear regression of gene transcript abundance and temperature was conducted for all genes. Those producing strong correlations have been displayed inset within gene transcript figures. For metabolic profiling, boxes indicate the fold-change difference in metabolite level

for each stage as compared to the previous stage of development. Statistically significant differences ($p < 0.05$) were defined between at least two adjacent stages, using non-parametric t-tests.

2.4. Results

2.4.1. Fruit growth and developmental changes in malate levels

Berry growth (measured as fresh weight), occurred in two phases: early development, which occurred from fruit set until approximately 50 days after flowering (D.A.F.), and ripening, which occurred from 60-110 D.A.F. (Figure 2.1). Véraison occurred around 60 D.A.F., initiating the second phase of growth. Fruit harvest occurred at approximately 100 D.A.F., at approximately 22 ˚Brix, with berries weighing approximately 0.9 grams each, although some shrinking may have begun around this time (Figure 2.1). Malate content reached a peak of around 50 µmoles.berry^{-1} just prior to véraison, upon which time malate content declined dramatically throughout ripening (Figure 2.1). The malate content of berries at harvest was less than 10 µmoles.berry^{-1}. The average rate of malate loss during the initial degradation phase (59-66 D.A.F) was 3.12 nmoles.min^{-1}.berry^{-1}.

2.4.2. Developmental patterns of gene transcript levels and enzyme activities

PEPC activity was observed at all stages of development and increased during ripening to a maximum rate of approximately 200 nmolNADH.min^{-1}.berry^{-1} (Figure 2.2). *VvPepc1* transcript levels were highest during pre-véraison berry development, with two peaks during early development and pre-véraison, before a decrease at véraison. *VvPepc2* transcript levels were steady during early development and fell at veraison, remaining low during ripening. *VvPepc3* transcript levels were much lower than *VvPepc1* and *VvPepc2* transcript levels, and changed very little throughout development.

NADP-MDH and NAD-MDH (NAD(P)-MDH) activities (Figure 2.3a,b) were present at each stage of development tested here. Both activities increased throughout development to a maximum of almost 200 and 4,000 and nmolNADH.min^{-1}.berry^{-1}, respectively. The activity of NAD-MDH was higher than that of any other enzyme measured in this study. However,

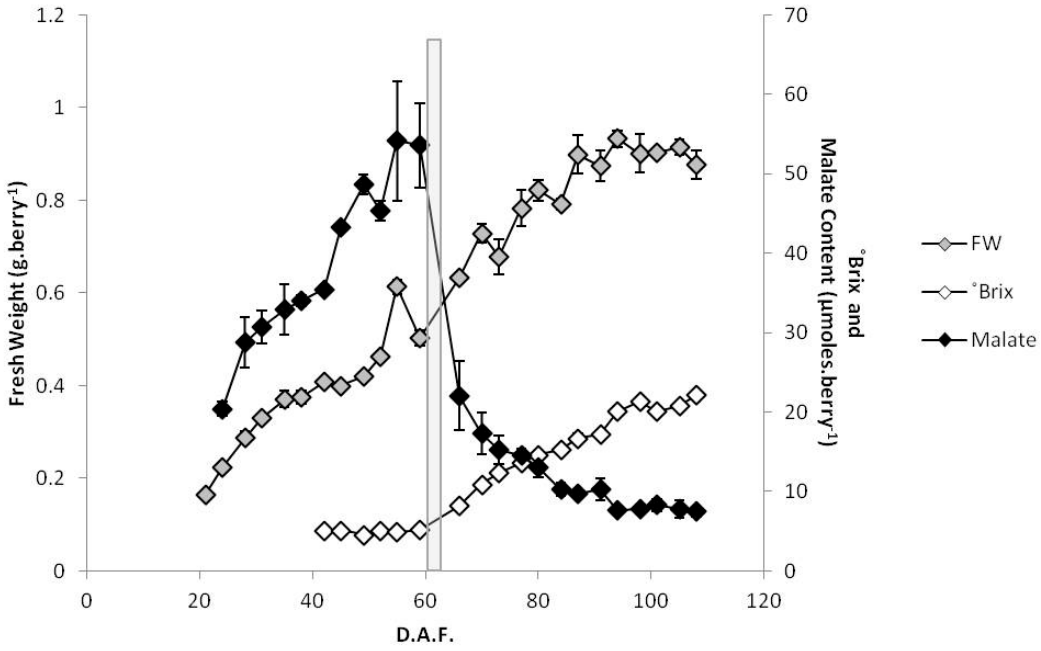

Figure 2.1: Attributes of berry development throughout ripening. Seasonal changes in Shiraz berry fresh weight (FW), ˚Brix and malate content (amount of malate per berry). Presented as the mean of four biological replicates (± SEM). Shaded region indicates veraison.

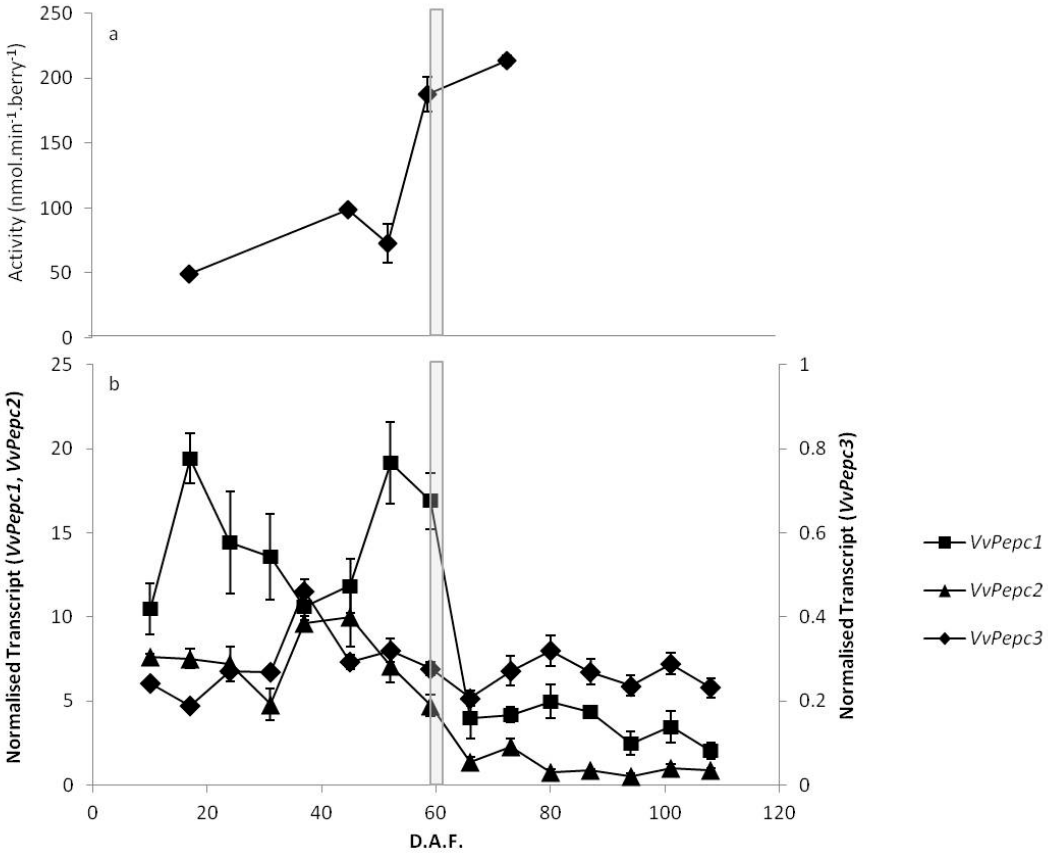

Figure 2.2: Developmental changes in PEPC activity and transcripts. PEPC activity (a) and *VvPepc* transcripts (b) across developmental time series of Shiraz berries. Activity is expressed as nmolNADH.min^{-1}.berry^{-1}. Transcript levels were normalised to the geometric mean of two reference gene transcripts: ankyrin and ubiquitin. Data are presented as means of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison.

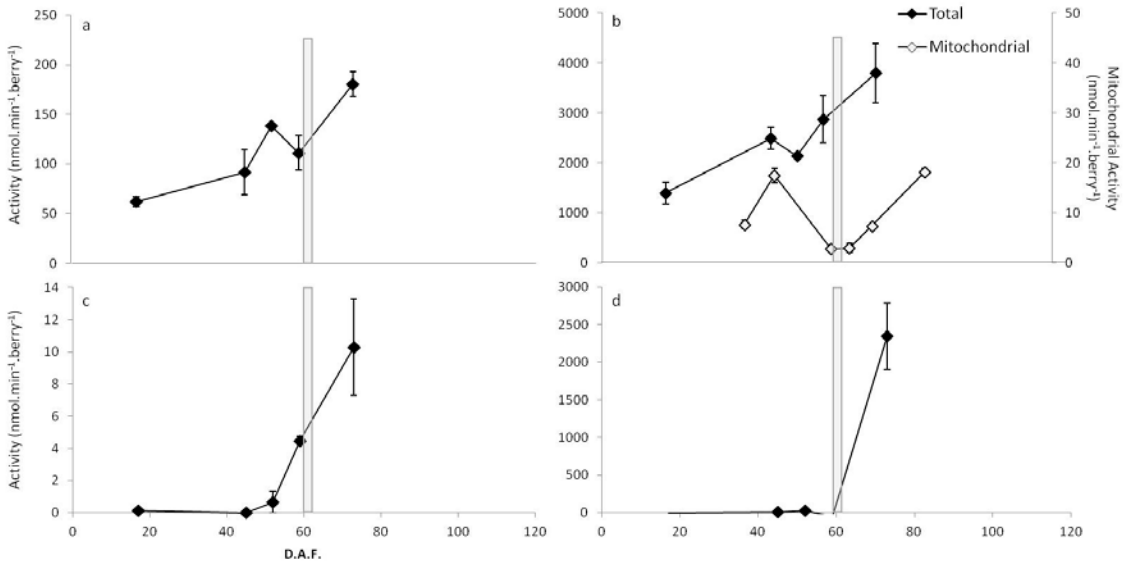

Figure 2.3: Developmental changes in NAD(P)-MDH, NADP-ME and PK activities. NADP-MDH (a), NAD-MDH (b), NADP-ME (c) and PK (d) activities across developmental time series of Shiraz berries. Activities are expressed as nmolNADH.min^{-1}.berry^{-1}. All activities were measured in whole cell extracts, except for mitochondrial NAD-MDH activity (d), which was from purified, lysed mitochondria. Data are presented as mean of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison.

mitochondrial-specific NAD-MDH (Figure 2.3b) occurred at much lower levels, with a maximum observed rate of 18 nmolNADH.min^{-1}.berry^{-1}, and showed a different pattern throughout development, peaking early during development and dropping suddenly with véraison, before climbing again throughout the ripening phase. NADP-ME and PK activities (Figure 2.3c,d) were barely detectable until véraison, whereby each increased throughout ripening to approximately 10 and 2,000 nmolNADH.min^{-1}.berry^{-1}, respectively.

NAD-ME activity from isolated grape berry mitochondria declined to almost undetectable levels during development, then increased suddenly to a peak of approximately 1 nmolNADH.min^{-1}.berry^{-1} around veraison, before declining throughout ripening (Figure 2.4a). A *VvNad-me* transcript was highest in very young fruit, with a sharp decline during early berry development (Figure 2.4b).

PPDK transcript levels were very low in young fruit and increased pre-veraison, reaching a peak just after veraison before declining to a steady level throughout ripening (Figure 2.5). As well as showing developmental induction, the *VvPpdk* transcript level was also positively correlated (R^2 = 0.4437) with the minimum ambient temperature taken the night before sampling (Figure 2.5, inset).

PEPCK activity increased approximately 10-fold throughout development, particularly upon reaching veraison (Figure 2.6a). *VvPepck* transcript levels demonstrated a 6-fold increase throughout early development, and then fluctuated during berry ripening (Figure 2.6b). When plotted alongside the ambient minimum temperature for this area (i.e. the temperature from the night before sampling, taken from the nearest weather station and recorded by the Bureau of Meteorology; ABM, 2008), post-veraison transcript appeared to mirror the pattern of temperature, and when plotted against minimum ambient temperature (Figure 2.6b, inset), displayed a negative correlation (R^2 = 0.7482).

ADH activity increased throughout development (> 7-fold) to a maximum of approximately 80 nmolNADH.min^{-1}.berry^{-1}, particularly once veraison had occurred (Figure 2.7a). *VvAdh1* transcript showed a general decrease across development, while *VvAdh2* and *VvAdh3* transcripts increased before veraison to reach a plateau throughout ripening (Figure 2.7b). *VvAdh1* transcript was also plotted alongside minimum ambient temperature (Figure 2.7c),

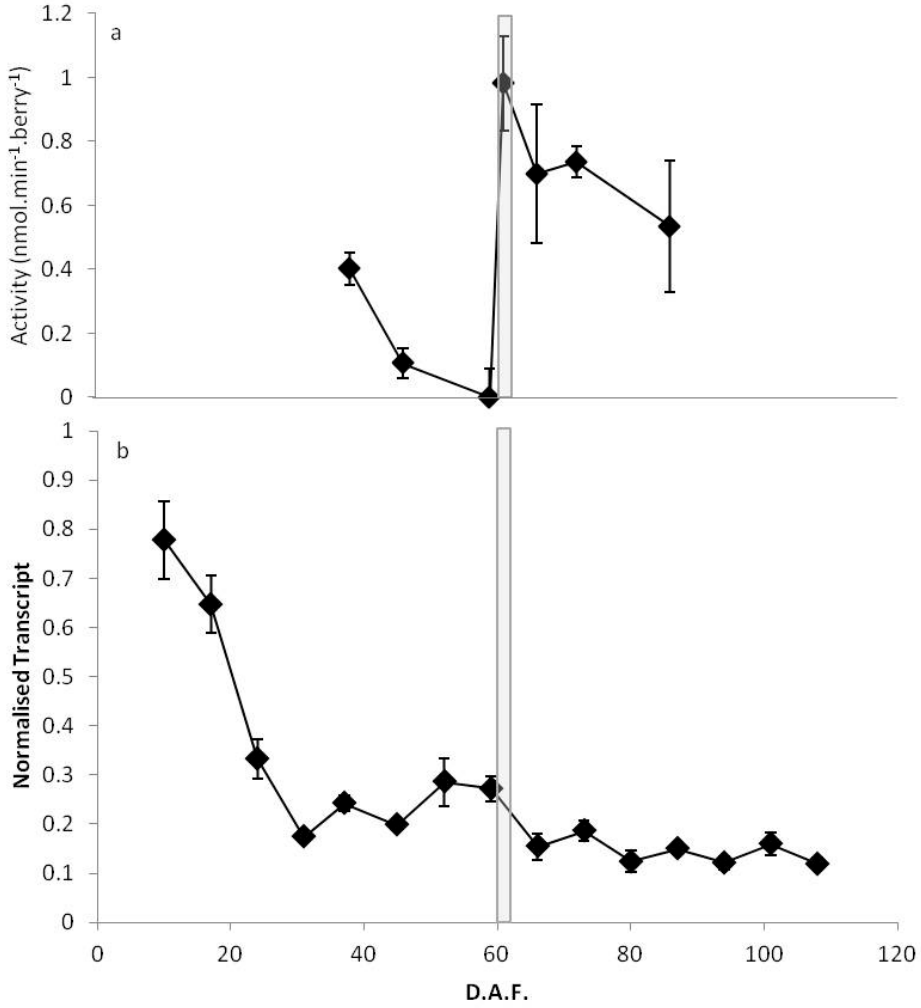

Figure 2.4: Developmental changes in NAD-ME activity and transcript. Mitochondrial NAD-ME activity (a) and *VvNadme* transcript (a) across developmental time series of Shiraz berries. Activity is expressed as $nmolO_2.min^{-1}.berry^{-1}$ consumed by purified mitochondria in the presence of malate, NAD^+, $MnCl_2$ and Coenzyme A. Transcript levels were normalised to the geometric mean of two reference gene transcripts: ankyrin and ubiquitin. Data are presented as means of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison.

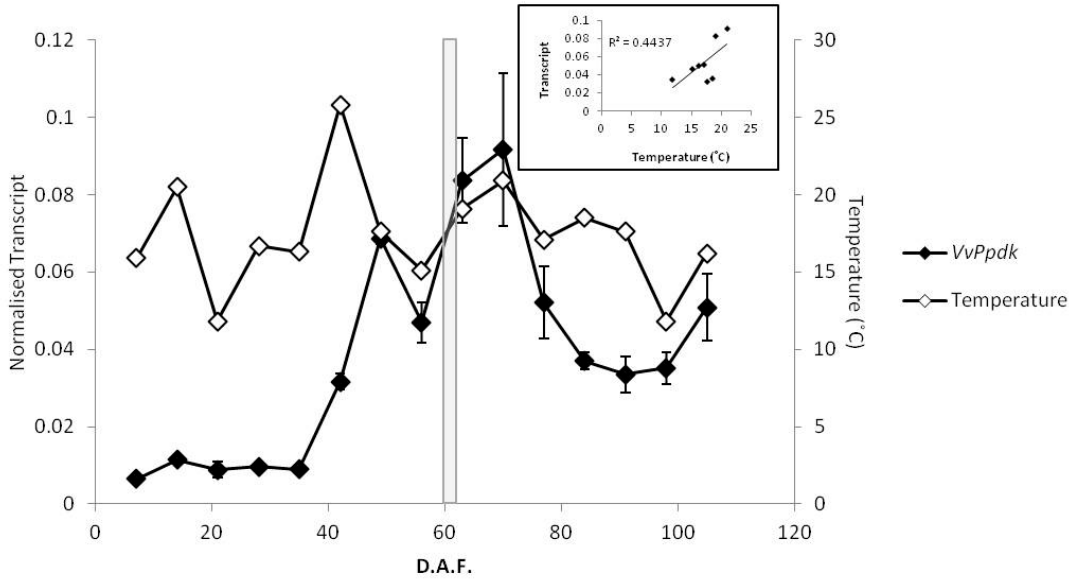

Figure 2.5: Developmental changes in *VvPpdk* transcript. Transcript levels were normalised to the geometric mean of two reference gene transcripts: ankyrin and ubiquitin and plotted alongside minimum temperature data from the preceeding night of each sampling timepoint. Data are presented as mean of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison. Inset: linear regression of temperature and transcript level data from from post-veraison fruit.

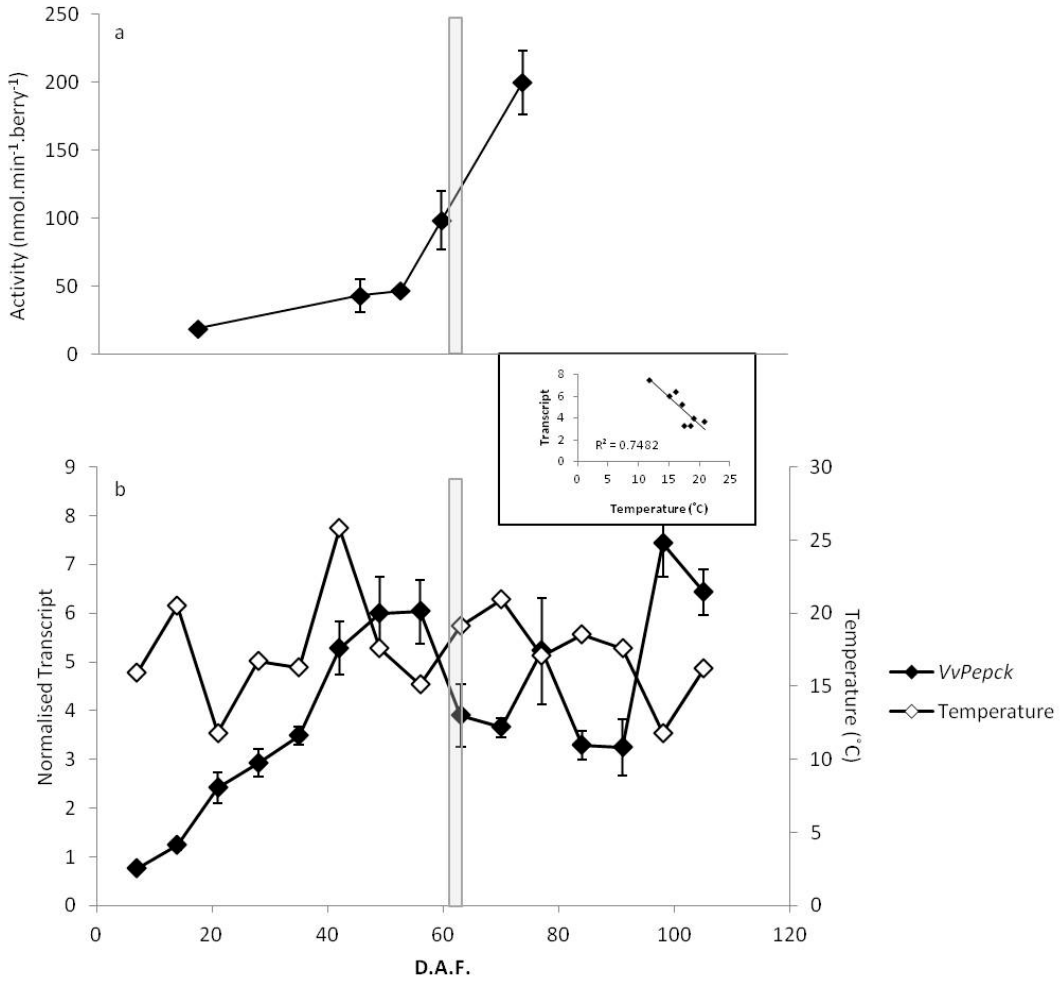

Figure 2.6: Developmental changes in PEPCK activity and transcript. PEPCK activity (a) and *VvPepck* transcript (b) across developmental time series of Shiraz berries. Activity is expressed as nmolNADH.min^{-1}.berry^{-1}. Transcript levels were normalised to the geometric mean of two reference gene transcripts: ankyrin and ubiquitin and plotted alongside minimum temperature data from the preceeding night of each sampling timepoint. Data are presented as means of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison. Inset: linear regression of temperature and transcript level data from post-veraison fruit.

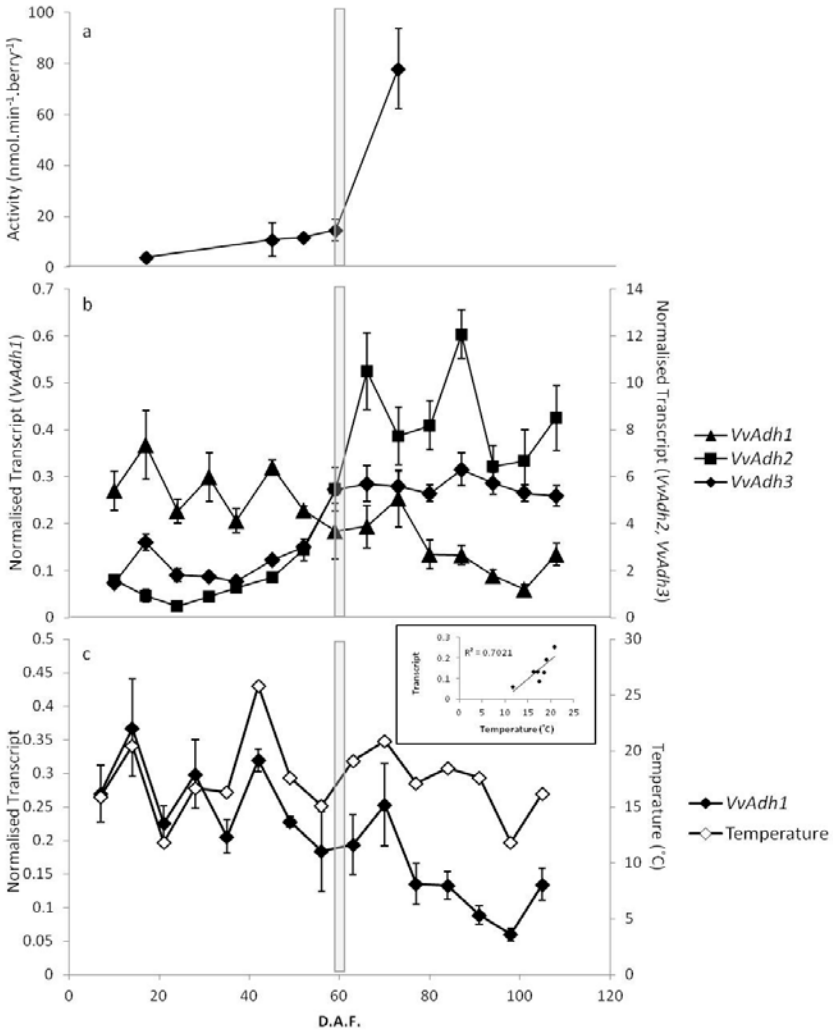

Figure 2.7: Developmental changes in ADH activity and transcripts. ADH activity (a), *VvAdh* transcripts (b) and *VvAdh1* transcript plotted with temperature (c) across developmental time series of Shiraz berries. Activity is expressed as nmolNADH.min^{-1}.berry^{-1}. Transcript levels were normalised to the geometric mean of two reference gene transcripts: ankyrin and ubiquitin, and plotted alongside minimum temperature data from the preceeding night of each sampling timepoint. Data presented as mean of four biological replicates with error bars representing standard error of the mean. The shaded region indicates veraison. Inset of (c): linear regression of temperature and transcript level from samples taken from veraison and throughout ripening.

showing some trend similarity and a positive correlation with linear regression (R^2 = 0.7021), with samples from veraison onwards (Figure 2.7c, inset).

Activities of enzymes at the switch from malate accumulation to degradation (59 D.A.F.) are summarised in Table 2.2, and used for calculation of potential capacity for malate flux.

2.4.3. Metabolic profile of berry development

Data obtained from the metabolic profiling GC-MS technique demonstrated the differences in developmental patterns of individual compounds (Figure 2.8). Of the organic acids, glycerate, 2-keto-L-gulonate and citrate each increased from week 2 (early development) to week 6 (pre-véraison), before declining throughout véraison and ripening. Benzoate increased throughout development, particularly during early development and later stages of ripening. Fumarate and malate levels did not change significantly, while tartrate showed a significant increase during early development, and increased again slightly with ripening. Threonate, gluconate, succinate and shikimate all showed a significant decrease during grape development. Oxalate and quinate each showed a decline at the earlier stages of berry development or ripening, before increasing significantly during the later stage of ripening.

Of the amino acids measured here (Figure 2.8), alanine and asparagine were the only two that showed dramatic or significant increases during grape berry development, although both showed a decline later in ripening. Glycine showed a slight, but insignificant increase at maturity, while threonine and norleucine did show a temporary increase before switching to a decrease with véraison. Proline showed a slight, but significant decrease around véraison in Shiraz berries, while aspartate, glutamate, glutamine, pyroglutamate and serine each displayed strong and consistent decreasing trends throughout the various stages of development.

The majority of measured sugars increased with the ripening of the fruit, except raffinose, which declined with ripening. Cellular phosphate and phosphorylated glucose compounds also declined with véraison and early ripening.

Table 2.2: Capacities of enzymes for metabolism of malate at véraison. Data taken from mean enzyme activities at véraison time-point (59 D.A.F.), from cellular extracts unless indicated otherwise. * Data estimated from Ollat & Gaudillere (2000). ** Ratio may depend on pathway of malate respiration (e.g. direct uptake into TCA cycle, or decarboxylation to pyruvate).

Enzyme	Measured Rate ($nmol.min^{-1}.berry^{-1}$)	Stoichiometry (molar ratio with malate)	Normalised Rate ($nmolMalate.min^{-1}.berry^{-1}$)
PEPC	187.76	1:1	187.6
PEPCK	98.69	1:1	98.69
NADP-MDH	110.99	1:1	110.99
NAD-MDH	2874.80	1:1	2874.80
Mitochondrial NAD-MDH	2.76	1:1	2.76
NADP-ME	4.49	1:1	4.49
Mitochondrial NAD-ME	0.98	1:1	0.98
ADH	14.66	1:1	14.66
Mitochondrial State III Respiration	0.36	2:3**	0.24
Dark CO_2 Evolution*	12.5*	2:1**	6.25

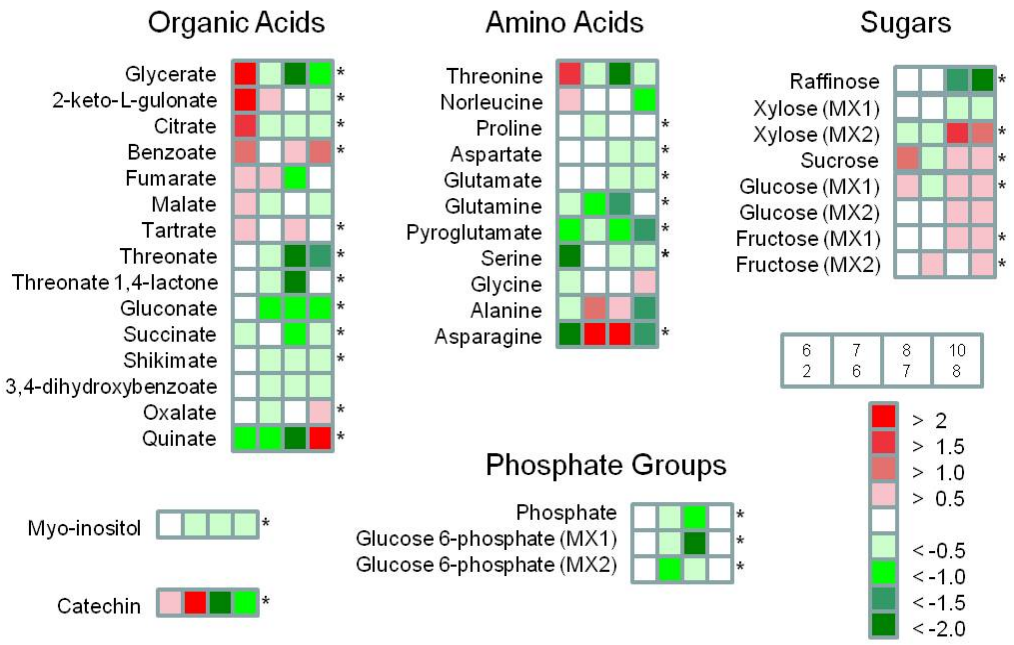

Figure 2.8: Changes in metabolite content across stages of berry development. Boxes indicate the fold-change difference in metabolite level for each stage as compared to the previous stage of development, quantified in weeks (Wk). From left to right, Wk6/2 (pre-veraison relative to early development), Wk7/6 (veraison relative to pre-veraison), Wk8/7 (post-veraison relative to veraison) and Wk10/8 (late ripening relative to post-veraison), calculated as $\log_2$(Wk6/Wk2), $\log_2$(Wk7/Wk6), etc. MX1 and MX2 indicate cis- and trans-isomers of methoximated sugar derivatives (Roessner et al., 2000).*statistically significant differences ($p < 0.05$) between at least 2 adjacent stages.

2.5. Discussion

To investigate the importance of particular metabolic pathways in the metabolism of grape berry malate, the levels of enzyme transcripts and activities, as well as relative changes in some metabolites were measured from Shiraz fruit collected across the developmental season. Changes reflect metabolism of the whole berry, and are not specific to particular cell types or tissue regions. Grapes displayed the typical double sigmoid pattern of growth, with veraison occurring at the lag between the two growth phases at approximately 60 days after flowering. This also coincided with the initiation of sugar accumulation (total soluble solids), and a switch from malate accumulation to degradation. According to the GC-MS metabolic analysis, malate levels did not change significantly at any stage of development. However, more detailed HPLC analysis with samples from additional time-points displayed a clear developmental pattern of malate content. This suggests that the limited number of time-point samples used for the GC-MS analysis of relative changes in compounds may have been too broad to detect specific changes in some compounds throughout development. On the other hand, it may be that some organic acid anions were not fully recovered with the use of this extraction process, which has been shown in the past to be maximal with a sequential ethanol and water extraction method (Iland and Coombe, 1988). Nevertheless, tartrate, an end product of ascorbate catabolism, was seen early during berry development and showed a slight increase during ripening, similar to patterns seen with detailed HPLC analyses of fruit from the same sample set (Melino et al., 2009). Oxalate, another product of ascorbate catabolism, showed a slight increase during ripening, while 2-keto-L-gulonate, an intermediate of the biosynthetic pathway of tartrate from ascorbate (Saito and Kasai, 1982) under recent scrutiny in Shiraz grapes (Burbidge, 2011), showed a dramatic increase in early development, followed by a slight decline during late ripening. Together, these patterns support the findings of Melino et al (2009a), whereby flux of ascorbate towards tartrate biosynthesis occurs early in grape berry development (accumulation of 2-keto-gulonate and tartrate), while an increase in the ascorbate recycling system likely occurs during ripening (accumulation of oxalate).

2.5.1. Malate synthesis during berry development

Net accumulation of malate during early grape berry development is due to a higher rate of malate synthesis and sequestration relative to the rate of malate degradation. However, as malate is a fundamental intermediate of cellular metabolism, its synthesis must continue throughout the entirety of grape berry development and ripening. Malate synthesis can occur through the reduction of oxaloacetate (OAA), catalysed by MDH. OAA for this step can be derived by the carboxylation of PEP, catalysed by PEPC (Hawker, 1969). Together, these two enzymes have the capacity to divert flux of sugar breakdown (glycolysis) products toward malate synthesis rather than pyruvate synthesis.

PEPC can have numerous roles in the plant, including photosynthesis, carbon partitioning and seed formation (Chollet et al., 1996), which may be important in grape berry metabolism. During the development of Shiraz berries, PEPC activity showed little change relative to fresh weight, but a slight increase when expressed on a per-berry basis. Similar patterns of PEPC activity and protein levels have been reported for Pinot Noir and Cabernet Sauvignon fruits (Ruffner and Kliewer, 1975; Diakou et al., 2000). The Km of PEPC in mature Shiraz berries was 0.45 mM for PEP and 0.2 mM for $KHCO_3$ (see Appendix 2); similar to that of tomato fruit PEPC (Guillet et al., 2002). Such high Km values for PEP are more common with PEPC from C4 plants (Engelmann et al., 2003). In young grapes, the level of PEP was measured at approximately 15 µmoles/kgFW (Ruffner and Hawker, 1977), which seems insufficient for activation of the PEPC activity. However, this metabolite is very unstable and transitory in the cell, and may have been difficult to accurately quantify with previous methods. It should also be noted that the enzyme extraction and assay procedures used in this study did not take into account the phosphorylation state of PEPC, which can affect the affinity of the enzyme for its substrate (Svensson et al., 2003).

The affinity of tomato PEPC for PEP and bicarbonate changed throughout fruit development and in response to pH (Guillet et al., 2002). In addition, PEPC protein and two PEPC genes were regulated during development of tomato fruit, with protein and transcript levels at their highest during the malate accumulation period. In the current study, three isogenes of PEPC were tested for transcript patterns across grape berry development. These have been designated as *VvPepc1*, *VvPepc2* and *VvPepc3*. According to Blastx alignments with the non-

redundant protein database in NCBI, *VvPepc1* was identified as a potential PEPC homolog, while *VvPepc2* and *VvPepc3* aligned primarily with unnamed, hypothetical proteins from *V. vinifera* and with PEPC genes from other plant species. Transcript patterns of *VvPepc1* and *VvPepc2* showed initially high expression that declined particularly towards veraison, as consistent with Northern blots of a PEPC gene in Perlette berries (Or et al., 2000), and with patterns seen using an analysis of microarray data (Sweetman et al., 2009). While these transcript patterns are consistent with a role for PEPC in malate synthesis and accumulation alone, this was not reflected in the enzyme activity data, which remained high and may be involved in other cellular processes during berry ripening. For example, PEPC in grape berries may be involved in re-assimilation of respired CO_2 (Ruffner, 1982b; Ollat and Gaudillere, 2000). Of the three putative PEPC transcripts measured, *VvPepc1* displayed the most similar changes to PEPC activity.

PEPC activity can be controlled through phosphorylation, which is regulated by PEPC kinase activity in plants (Chollet et al., 1996). Cabernet Sauvignon berry microarray data demonstrated a developmental decline in one putative PEPC kinase transcript, which would be expected to decrease the phosphorylation state of PEPC protein in ripening grapes (Sweetman et al., 2009), and thus decrease PEPC activity *in vivo*. Plant PEPC activity is also sensitive to inhibition by oxaloacetate and malate, and activation by glucose 6-phosphate (reviewed by Chollet et al. 1996). Data from GC-MS analyses showed a decrease in glucose 6-phosphate levels around veraison, simultaneous with the release of malate from the vacuole. Together, these changes could lead to an *in vivo* decrease of grape berry PEPC activity from véraison. An investigation of the phosphorylation state of the grape berry PEPC at different stages of development, potentially through the use of an anti-phosphorylation antibody (Tripodi et al., 2005), could be used to complement data concerning sensitivity of grape berry PEPC to malate inhibition (Diakou et al., 2000), and provide valuable information concerning PEPC regulation during fruit development. It would also be practical to investigate activity of grape berry carbonic anhydrase, which supplies PEPC with the bicarbonate substrate, and could provide more insight into the regulation of PEPC activity during the malate accumulation and degradation phases.

NAD-MDH and NADP-MDH activities increased in a manner similar to that of PEPC. This was unexpected for the NADP-MDH, as it is a photosynthetic enzyme and expected to be

present only in the chloroplast (Edwards et al., 1985). While rates of the PEPC and NADP-MDH activities were very similar, they were greatly exceeded (20-fold) by total NAD-MDH activity. NAD-MDH enzymes exist as numerous isoforms in the cell, which may play different roles in grape berry metabolism, and may be influential at different stages of development (Taureilles-Saurel et al., 1995). As the quantification of total NAD-MDH activity may overlook patterns specific to the individual isoforms, NAD-MDH activity was also measured in mitochondria isolated from Shiraz fruit during the '09-10 growing season. Results suggested that the mitochondrial isoform is responsible for only a small fraction of the total NAD-MDH activity. The observed decline in mitochondrial NAD-MDH activity around veraison is consistent with a decrease in the transcript of a putative mitochondrial NAD-MDH gene in Perlette (Or et al., 2000) and Cabernet Sauvignon (Sweetman et al., 2009), and may contribute to the slight dip in total NAD-MDH activity observed with extracts from whole Shiraz berries. The decrease in mitochondrial NAD-MDH may be related to the increase in mitochondrial NAD-ME activity that also coincided with véraison.

Glycolysis is thought to be inhibited in berries once veraison has occurred (Ruffner and Hawker, 1977; Martinez-Esteso et al., 2011). However, contrary to the decrease in glycolytic proteins, including pyruvate kinase (PK), observed during Muscat Hamburg berry development (Martinez-Esteso et al., 2011), PK was present and active in Shiraz ripening grape berries in the current study. In fact, PK activity was almost undetectable until after véraison, suggesting that the fruit are more capable of converting phosphoenolpyruvate to pyruvate during the malate degradation phase. From activity patterns observed here, it seems that PEPC and MDH are recruited to a glycolytic role in place of pyruvate kinase, and may be responsible for malate synthesis at least during early grape development.

2.5.2. Malate degradation during berry development

Net malate loss occurs from veraison throughout ripening, and is due to degradation of the acid upon its release from sequestration. Malate degradation can be driven by many pathways, but occurs directly through decarboxylation to oxaloacetate through MDH, or pyruvate through ME. The changes in MDH activities described in the previous section are conducive to a role in malate catabolism during berry ripening, with an observed increase for both NAD-MDH and NADP-MDH throughout development. While the activities of NAD-

ME and NADP-ME showed differing patterns across Shiraz berry development, both also tended towards an increase during ripening. NAD-ME was previously thought to be absent from grapes (Romieu et al., 1992). Data summarised in Table 2.2 indicated that most of the activities measured in this study had the capacity to account for the dramatic loss of malate that occurred at this time (approximately 3.12 nmoles.min^{-1}.berry^{-1}). Therefore the degradation of malate is likely to be regulated by other drivers, such as the TCA cycle, gluconeogenesis or secondary metabolic pathways (Sweetman et al., 2009).

As mentioned previously, some steps of glycolysis are thought to be inhibited once veraison occurs (Ruffner and Hawker, 1977). The accumulation of glucose and fructose and decline in glucose 6-phosphate does suggest a decline in glycolytic flux during ripening. These data also suggest that a point of control for glycolysis in grapes may be at the hexokinase reaction. This is not unheard of for plant cells (Kubota and Ashihara, 1990), and hexokinase activity as well as glucose 6-phosphate dehydrogenase has been seen to decline in post-véraison Sultana berries when expressed as a function of fresh weight (Hawker, 1968). Due to the inhibition of glycolysis, the post-veraison supply of carbon to the TCA cycle is expected to occur through malate released from the vacuole. The TCA cycle also requires acetyl-CoA, from the catabolism of pyruvate. In the absence of glycolysis, malate must act as a source of pyruvate through the activities of the malic enzymes, which were indeed elevated at, or after, veraison. However, it was also noted that pyruvate kinase was largely absent until the ripening stage. Upon induction, PK activity reached rates comparable to NAD-MDH and much higher than those of PEPC, suggesting that a proportion of PEP metabolism may be directed towards pyruvate rather than OAA metabolism. It is clear that the metabolism of PEP is very important at this time, and that this may be supplied by some mechanism other than glycolysis. The measurement of activity and substrate levels from previous steps in glycolysis (ie. conversion of 2-phosphoglycerate to PEP via enolase) may assist in determining whether other late steps of glycolysis are up-regulated during fruit ripening, or whether PK has a specialised function outside of glycolysis..

In addition to PK, ME can supply pyruvate to cellular pathways of metabolism. As the enzyme exists as different isoforms in specific compartments of the cell, it can be involved in numerous pathways. NADP-ME activity, which could be representative of cytosolic and/or chloroplastic isoforms of this enzyme, was present at basal levels in young fruit before

induction at véraison. In Sultanina berries, NADP-ME activity was detectable at all stages of development, but showed irregular patterns with respect to fresh weight and per-berry measures (Hawker, 1969), while in Chardonnay, the level of NADP-ME protein from grape flesh did not change significantly (Famiani et al., 2007). In other fruits, such as apple and loquat, NADP-ME protein and activity levels also increased at the later stages of ripening (Yoshioka et al., 1991; Chen et al., 2009; Yao et al., 2009; Yang et al., 2011). Meanwhile, transcript of a malic enzyme cDNA clone measured using Northern blotting method was detectable in young Perlette berries, but also increased throughout ripening (Or et al., 2000). Chloroplastic NADP-ME is involved in photosynthetic carbon assimilation in numerous tissues (Edwards et al., 1985; Edwards and Andreo, 1992), and may have such a function in young green fruits. However, increased transcript level and enzyme activity of the NADP-ME seems to be a common occurrence in ripening fruits, suggesting that a non-photosynthetic, cytosolic isoform may be important in the metabolism of malate later in development.

The transcript of a gene likely to encode a grape PPDK, another photosynthetic enzyme, also increased at veraison. The enzyme converts pyruvate to PEP and functions in the release of carbon dioxide to RuBisCo in C4 photosynthesis (Edwards et al., 1985). The gene identified in *V. vinifera* aligns with a hypothetical *V. vinifera* protein in the NCBI database, and with PPDK genes from other plants. Inconsistent with a role in photosynthesis, the relative quantity of this transcript peaked at veraison. Transcript levels also correlated positively with ambient minimum temperature, although this effect was strongest in young fruit and lost during the later stages of ripening, suggesting that a stimulation of *VvPpdk* transcription by elevated temperature may be dependent on developmental stage. Photosynthetic PPDK is well known to be light responsive and further controlled through pH changes in the chloroplast (Hatch, 1987). The enzyme is also sensitive to cold, causing irreversible deactivation *in vitro* (Hatch and Slack, 1968). Therefore elevated temperature may also be an important regulatory factor of *VvPpdk* expression. PPDK activity was not measured here, due to the difficulty of its extraction from grape berries. PPDK protein could not be detected in Pinot Noir, Chardonnay, Zibbibo, nor Cabernet Sauvignon grapes (Famiani et al., 2007), nor in other fleshy fruits (Famiani et al., 2005), although has recently been detected in cactus pear (Walker et al., 2011). These studies suggest that either the PPDK protein of *V.*

vinifera cannot be picked up using the antibody from maize, or that the protein is not present at all, or at least not at detectable levels in grape berries. While it is unlikely that such a tightly regulated transcript has no function in grape berry metabolism, the activity must be assayed to confirm its presence and role in the fruit. If the pattern of PPDK protein and activity levels increase throughout fruit development as seen for the transcript, then the enzyme could potentially play a gluconeogenic role, and may draw on carbon from malate for the synthesis of sugars in the berry. Activity of the gluconeogenic pathway has been suggested in grape berries previously (Ruffner and Kliewer, 1975; Ruffner, 1982b).

The gluconeogenic enzyme, PEPCK, which catalyses the reversible conversion between OAA and PEP, was also investigated in this study. A putative PEPCK EST contig was identified in *V.vinifera*, although in conceptual translation it did not align with any annotated hypothetical proteins from *V.vinifera* in NCBI. The top alignment to this sequence was the *Flaveria trinvervia* PEPCK gene. Transcript of the *VvPepck* sequence showed strong developmental regulation, with a large increase (approximately 6-fold) in transcript level during green berry development towards veraison, consistent with patterns from microarray data (Sweetman et al., 2009). From veraison, *VvPepck* transcript showed irregular changes that appeared to be negatively correlated with temperature.

PEPCK activity from extracts of whole Pinot Noir berries have been seen to peak at around 4 weeks after anthesis, followed by a gradual decline in activity during ripening (Ruffner and Kliewer, 1975). Consistent with this, PEPCK activity was detected at a relatively high rate in seeds of developing Pinot Noir grapes (Walker et al., 1999), with a peak in protein level and activity in seeds at 40 days after bloom (pre-veraison). This is thought to be due to the requirement of PEPCK for gluconeogenesis and the provision of sugars in the seed (Graham, 2008), or with a role in storage of nitrogen assimilates (Walker et al., 1999). On the contrary, from extracts of numerous *V. vinifera* varieties, Famiani et al (2007) observed only a small amount of PEPCK protein in pre-veraison grapes, but a large increase at veraison, which remained steady thereafter. This latter study is consistent with results seen here, whereby PEPCK activity was very low in young berries and increased 4-fold from veraison, despite the use of whole berry extracts (including seeds). When adjusted to the same units, maximum rates of PEPCK activity from Pinot Noir seeds (approx. 0.8 $\mu mol.gFW^{-1}.min^{-1}$; Walker et al. 1999) and Pinot Noir whole berry extracts (approx. 0.5 $\mu mol.gFW^{-1}.min^{-1}$; Ruffner & Kliewer

1975) were very similar to those seen here in whole Shiraz berry extracts (approx. 1 $\mu mol.gFW^{-1}.min^{-1}$).

These data support suggestions that PEPCK may be involved in the catabolism of malate during grape berry ripening (Famiani et al., 2007), as the enzyme became highly active around veraison, coinciding with the dramatic phase of malate degradation. Furthermore, the enzyme is at least partially regulated at the transcriptional level, although the abrupt increase in PEPCK activity does not completely reflect the gradual increase in *VvPepck* transcript, suggesting that another level of regulation is imposed upon the PEPCK protein. The *VvPepck* transcript displayed some sensitivity to temperature during the ripening phase. However, the negative correlation suggests that the enzyme activity may be decreased when temperatures are elevated.

As observed from GC-MS analyses, a decline in the levels of organic acids involved in the TCA cycle, namely citrate, succinate, fumarate and malate, as well as numerous amino acids including glutamate and aspartate, could be due to the utilisation of key TCA cycle intermediates in gluconeogenesis. Nevertheless, a decrease in the rate of glycolysis, along with continued imported of sucrose from the leaves may be more likely to account for the increase in sugar levels seen with the GC-MS analysis, rather than an increase in gluconeogenesis, which is estimated to contribute less than 5% of total grape berry sugar accumulation (Ruffner and Kliewer, 1975; Ruffner and Hawker, 1977).

Of the sugars detected in this study, only raffinose exhibited a significant and dramatic decline after véraison. This pattern does not coincide with data from a previous, in-depth investigation of trace sugars during grape berry development (Kliewer, 1965). However, Kliewer (1965) did illustrate varietal differences in raffinose accumulation. Consistent with the decrease in raffinose, a decline in the level of *myo*-inositol was also observed. *Myo*-inositol is involved in numerous plant metabolic pathways, including ascorbate biosynthesis and synthesis of aminoglycosides (i.e. streptomycin), as well as numerous signalling pathways (Loewus and Murthy, 2000). The compound arises through the metabolism of glucose 6-phosphate. The observed down-regulation of glucose 6-phosphate, tentatively attributed to an inhibition of glycolysis, may therefore be the cause of decreased *myo*-inositol that was observed with ripening. Furthermore, this could cause down-regulation of

phosphate accumulation, nutrient storage, some stress responses, cell wall metabolism and many other processes (Loewus and Murthy, 2000), some of which may have downstream effects on malate metabolism in the grape berry. For example, down-regulation of *myo*-inositol could have led to the decrease in inorganic phosphate accumulation seen during ripening, which may influence cellular respiration rates and therefore malate metabolism through decreased function of the mitochondrial electron transport chain and subsequent feedback inhibition on the TCA cycle and glycolysis pathways.

Together, activity and transcript data presented in this study suggest that combinations of MDH, PEPCK and PPDK, or NADP-ME and PPDK may provide mechanisms for the conversion of malate to PEP during the ripening stage, for use in gluconeogenesis or other pathways such as the shikimate pathway for secondary metabolite biosynthesis. The decline in shikimate and increase in quinate observed during grape berry ripening suggests that shikimate may support the synthesis of quinate at this time, competing with flux through the shikimate pathway and biosynthesis of amino acids tyrosine, phenylalanine and tryptophan (of which none were detected in this study). This is consistent with a decrease in shikimate:NADP oxidoreductase protein seen with Muscat Hamburg berry development (Martinez-Esteso et al., 2011). However, the reverse seems to occur in kiwifruit, whereby a high level of quinate accumulates during early development and is used for the synthesis of shikimate during ripening (Marsh et al., 2009). The synthesis of benzoate, a simple aromatic carboxylic acid, can occur as a branch from the shikimate pathway. Benzoate accumulated during berry ripening and is likely related to the increase in flavour and aroma compounds and the decrease in malate that occurs during fruit ripening. Catechin, a flavonoid subunit of condensed tannins in grape, increased dramatically towards véraison before declining rapidly. Such a pattern has been seen previously in grape skins and seeds, along with similar patterns of proanthocyanidins levels (Downey et al., 2003). Flavonoids are synthesised through complex metabolic pathways that largely stem from the shikimate pathway. The decline in catechin observed in the present study may be due its decreased synthesis or increased polymerisation and decreased extractability (Downey et al., 2003).

The NAD-dependent malic enzyme (NAD-ME) is located in mitochondria, and the activity has been measured for the first time in grapes, as a part of this study. A sequence that potentially encodes NAD-linked malic enzyme was identified in *V. vinifera*, and aligned with

a hypothetical *V. vinifera* protein along with NAD-ME genes from other plants in the NCBI database. Expression of this *VvNadme* gene was high in very young berries and, save for a small peak at veraison, decreased to a basal expression level for the rest of development. NAD-ME activity measured from purified mitochondria during the 2009-10 season was also high at the early stage of berry development, decreasing towards veraison and then peaking again post-veraison. If this pattern is seasonally consistent, then it may be that the NAD-ME activity is involved in malate synthesis early during development, and may play an important role in the post-veraison metabolism of malate that has been released from the vacuole and transported to the mitochondria. The maximum rate of NAD^+ reduction observed during grape development was 1.1 µmoles.min^{-1}.gFW^{-1}. While this rate was much lower than activities of other enzymes measured in the grape berry, it was 10-fold higher than rates observed with pineapple leaves (Hong et al., 2004), similar to leaves of C3 and C4 *Flaveria* spp. (Gowik et al., 2011) and *Arabidopsis thaliana* (Tronconi et al., 2008), although low relative NAD-ME from the leaf mitochondria of CAM species such as *Hoya carnosa* (Hong et al., 2008). Such a presence of NAD-ME activity in the fruit is suggestive of some importance in grape berry metabolism. Goodenough et al. (1985) suggested that increased ME activity during fruit ripening may also assist in the catabolism of malate in the presence of oxaloacetate, (i.e. when malate dehydrogenase cannot utilise malate). In grapes, a large quantity of malate is lost very rapidly during ripening, therefore it is possible that a sudden shift in malate:oxaloacetate may reduce the catabolism of malate through the malate dehydrogenases at a certain point during ripening. Thus, NAD(P)-ME may be required to continue degrading malate, providing pyruvate as a source of carbon for respiration and ripening-related biosynthetic pathways.

Pyruvate that is formed by NAD(P)-ME can be used in several different pathways. In the mitochondria, NAD-ME can supply acetyl-CoA to the TCA cycle, through the metabolism of pyruvate. In the cytosol, NADP-ME could supply pyruvate for anaerobic respiration, with the function of regenerating NAD for the TCA cycle. This can occur when aerobic respiration is inhibited or cannot keep up with the energy requirements of the cell. Alcohol fermentation is one pathway of anaerobic respiration, catalysed by pyruvate decarboxylase and alcohol dehydrogenase, respectively channeling pyruvate to acetaldehyde and ethanol or other alcohols. ADH activity was very low in young berries and increased gradually throughout

development, before an induction in post-veraison fruit that was delayed with respect to the switch from malate accumulation to degradation. The activity of ADH reported for Danuta berries (2 µmol.min^{-1}.berry^{-1}; Tesniere & Verries (2000) was much higher than rates seen in the present work for Shiraz (0.1 µmol.min^{-1}.berry^{-1}), although both varieties showed very similar patterns of activity across development. The Km for acetaldehyde of ADH purified from Carignane berries (0.48 mM; Molina et al. 1987) was similar to that observed with the crude Shiraz extract in this study (0.25 mM; data not shown).

Transcripts of three isogenes, designated *VvAdh1*, *VvAdh2* and *VvAdh3*, as described by Tesniere & Verries (2000), were also quantified in this study. Results from semi-quantitative PCR (Labat et al., 2000; Tesniere and Verries, 2000), and microarray data analyses (Kobayashi et al., 2009; Sweetman et al., 2009) have been supported by quantitative real-time PCR in this study, whereby the expression of both *VvAdh2* and *VvAdh3* were developmentally induced in *V. vinifera* berries. Therefore one or both of these genes is likely to be responsible for the developmental regulation of ADH activity. A putative pyruvate decarboxylase transcript also increased during ripening (Kobayashi et al., 2009), and may facilitate *VvAdh2* and *VvAdh3* in the up-regulation of the alcohol fermentation pathway enzymes. Transcript levels of *VvAdh1* were much lower and decreased slightly throughout development, consistent with reports by Tesniere et al. (2006). This gene is either unimportant with respect to controlling ADH activity, or could play a role in response to other stimuli. Indeed, a positive correlation between *VvAdh1* transcript level and temperature data throughout berry development suggests that this gene may be involved in coordinating changes in ADH activity in response to environmental conditions. Furthermore, a study with field-grown Shiraz grapevines showed some stimulation of *VvAdh1* transcript with exposure to elevated temperature conditions (see Section 4).

Together, these data suggest that NADP-ME activity in the cytosol of ripening grape berries could provide pyruvate to support PEP-driven pathways such as the shikimate pathway (Herrmann and Weaver, 1999), or fermentation reactions that regenerate NAD through anaerobic formation of alcohols or lactate(Lara et al., 2011), while NAD-ME activity could supply pyruvate to the mitochondrial matrix, for maintenance of the TCA cycle (Grover and Wedding, 1982)when glycolytic formation of pyruvate may be limited (Ruffner and Hawker, 1977).

Another set of processes that may use pyruvate provided by NAD(P)-ME, along with various intermediates from the TCA cycle, are the biosynthetic pathways of amino acids. The decline in aspartate observed with GC-MS analyses during grape berry development may suggest net flux from aspartate to alanine and asparagine, of which both increased during véraison and early ripening stages and may also affect pyruvate and oxaloacetate levels, respectively. Overall, it could be seen that there was a decreasing trend in the majority of free amino acids detected in this study. This is inconsistent with previous reports, where amino acid level has tended towards an increase during grape berry development and ripening (Kobayashi et al., 2009; Mulas et al., 2011). A dramatic decline in serine levels during early development coincided with a sharp increase in glycerate and threonine, while a slower, steady decline in serine during ripening coincided with a slight increase in glycine. As such, it would seem that serine metabolism may be involved in the synthesis of glycerate, threonine and glycine at these different stages of development. The metabolically linked glutamine, glutamate and proline all showed decreasing trends throughout various stages of development. The significant (albeit slight) decrease in proline around véraison is inconsistent with other studies that have observed an increase of proline during development and ripening of Tokay, Cardinal and Cabernet Sauvignon grape berries (Kliewer, 1973; Deluc et al., 2007).

2.6. Concluding remarks

Grape PEPC had some similar characteristics to C4 PEPC enzymes, including a low affinity for PEP (Bauwe and Chollet, 1986) and the potential for assimilating carbon through malate synthesis. Glycolysis may provide carbon to the PEPC-MDH reaction and malate synthesis in place of the PK-driven pyruvate synthesis during early development, however this pathway may be inhibited at the hexokinase step with the initiation of ripening. PK and other enzymes involving pyruvate and PEP metabolism, such as PPDK, PEPCK, NADP-ME and ADH seem to be more important post-veraison, lending to the possibility of increased flux through gluconeogenesis, fermentative metabolism for NAD^+ regeneration and the shikimate pathway for biosynthesis of sugars and secondary metabolites, including flavonoids and aromatic carboxylic acids. An upregulation of gluconeogenesis simultaneous

to a decline in glycolysis was also indicated by an increase in glucose and fructose and a decrease in glucose 6-phosphate, carboxylic acids and amino acids.

NAD-ME appears to be important during early grape berry development, with rates similar to those seen in leaves of some C4 plants. The enzyme may play a photosynthetic role, despite its compartmentation in the mitochondrial matrix. It also appears to be stimulated from véraison, possibly working with NADP-ME in the degradation of malate when MDH is inhibited by high OAA.

Together, these data suggest that the catabolism of malate may be closely linked with the metabolism of pyruvate and PEP during grape berry ripening. NAD(P)- MDH activities shared an increasing trend that suggests the activities of these enzymes are important throughout the entirety of development, but not likely to confer rate-limiting roles in malate metabolism. Some preliminary work with organellar isolation has indicated that the mitochondrial isoforms may be contributing a very small proportion of MDH activity, and suggests a differential regulation, which may account for changes in malate content during development. Malate metabolism may also be subject to regulation by complex cellular networks, including signalling molecules such as myo-inositol.

It should be noted that these changes are reflective of a heterogeneous mixture of berry skins, seeds and flesh, and therefore may be more important in particular berry tissues. Analyses of separated tissues are useful in determining localised berry metabolism (Coombe, 1987) and may provide further insights into the regulation of organic acid metabolism in fruits.

2.7. References

ABM. (2008). Adelaide, SA - Daily Weather Observations. Australian Bureau of Meteorology, 2007-2008, from http://www.bom.gov.au/climate/dwo/IDCJDW5002.latest.shtml.

Bauwe, H. and R. Chollet (1986). Kinetic properties of phosphoenolpyruvate carboxylase from C3, C4 and C3-C4 intermediate species of *Flaveria* (Asteraceae). Plant Physiology **82**(3): 695-699.

Burbidge, C. A. (2011). Identification and characterisation of the enzymes involved in the biosynthetic pathway of tartaric acid in *Vitis vinifera*. Faculty of Science & Engineering. Adelaide, Flinders University of South Australia. **PhD thesis**.

Chen, F. X., X. H. Liu and L. S. Chen (2009). Developmental changes in pulp organic acid concentration and activities of acid-metabolising enzymes during the fruit development of two loquat (Eriobotrya japonica Lindl.) cultivars differing in fruit acidity. Food Chemistry **114**(2): 657-664.

Chollet, R., J. Vidal and M. H. Oleary (1996). Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**: 273-298.

Coombe, B. G. (1984). The inception of ripening in the grape berry. Quad. Vitic. Enol. Univ. Torino **8**: 87-99.

Coombe, B. G. (1987). Distribution of solutes within the developing grape berry in relation to its morphology. American Journal of Enology and Viticulture **38**(2): 120-127.

Delhaize, E., B. D. Gruber and P. R. Ryan (2007). The roles of organic anion permeases in aluminium resistance and mineral nutrition. FEBS Letters **581**(12): 2255-2262.

Deluc, L. G., J. Grimplet, M. D. Wheatley, R. L. Tillett, D. R. Quilici, C. Osborne, D. A. Schooley, K. A. Schlauch, J. C. Cushman and G. R. Cramer (2007). Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC Genomics **8**(1): 429.

Diakou, P., L. Svanella, P. Raymond, J. P. Gaudillere and A. Moing (2000). Phosphoenolpyruvate carboxylase during grape berry development: protein level, enzyme activity and regulation. Australian Journal of Plant Physiology **27**(3): 221-229.

Dietrich, P., D. Sanders and R. Hedrich (2001). The role of ion channels in light-dependent stomatal opening. Journal of Experimental Botany **52**(363): 1959-1967.

Downey, M. O., J. S. Harvey and S. P. Robinson (2003). Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Australian Journal of Grape and Wine Research **9**(1): 15-27.

Edwards, G. E. and C. S. Andreo (1992). NADP-malic enzyme from plants. Phytochemistry **31**(6): 1845-1857.

Edwards, G. E., H. Nakamoto, J. N. Burnell and M. D. Hatch (1985). Pyruvate,P_i dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: Properties and mechanism of light/dark regulation. Annual Review of Plant Physiology **36**: 255-286.

Engelmann, S., O. E. Blasing, U. Gowik, P. Svensson and P. Westhoff (2003). Molecular evolution of C4 phosphoenolpyruvate carboxylase in the genus *Flaveria* - a gradual increase from C3 to C4 characteristics. Planta **217**(5): 717-725.

Famiani, F., V. Casulli, P. Proietti, R. P. Walker and A. Battistelli (2007). Organic acid metabolism in grape: Role of phosphoenolpyruvate carboxykinase. Proceedings of the International Workshop on Advances in Grapevine and Wine Research. V. Nuzzo, P. Giorio and C. Giulivo**:** 599-602.

Famiani, F., R. P. Walker, L. Tecsi, Z. H. Chen, P. Proietti and R. C. Leegood (2000). An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries. Journal of Experimental Botany **51**(345): 675-683.

Famiani, F., N. G. M. Cultrera, A. Battistelli, V. Casulli, P. Proietti, A. Standardi, Z. H. Chen, R. C. Leegood and R. P. Walker (2005). Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. Journal of Experimental Botany **56**(421): 2959-2969.

Fernie, A. R. and E. Martinoia (2009). Malate. Jack of all trades or master of a few? Phytochemistry **70**: 828-832.

Goodenough, P. W., I. M. Prosser and K. Young (1985). NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. Phytochemistry **24**(6): 1157-1162.

Gowik, U., A. Brautigam, K. L. Weber, A. P. M. Weber and P. Westhoff (2011). Evolution of C(4) Photosynthesis in the Genus Flaveria: How Many and Which Genes Does It Take to Make C(4)? Plant Cell **23**(6): 2087-2105.

Graham, I. A. (2008). Seed storage oil mobilization. Annual Review of Plant Biology **59**: 115-142.

Grover, S. D. and R. T. Wedding (1982). Kinetic ramifications of the association-disassociation behaviour of NAD malic enzyme. Plant Physiology **70**: 1169-1172.

Guillet, C., D. Just, N. Benard, A. Destrac-Irvine, P. Baldet, M. Hernould, M. Causse, P. Raymond and C. Rothan (2002). A fruit-specific phospho*enol*pyruvate carboxylase is related to rapid growth of tomato fruit. Planta **214**(5): 717-726.

Harris, J. M., P. E. Kriedemann and J. V. Possingham (1968). Anatomical aspects of grape berry enlargement. Vitis **7**: 106-119.

Hatch, M. D. (1987). C4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta **895**(2): 81-106.

Hatch, M. D. and C. R. Slack (1968). A new enzyme for interconversion of pyruvate and phosphopyruvate and its role in C4 dicarboxylic acid pathway of photosynthesis. Biochemical Journal **106**(1): 141-146.

Hawker, J. S. (1968). Changes in the activities of enzymes concerned with sugar metabolism during the development of grape berries. Phytochemistry **8**: 9-17.

Hawker, J. S. (1969). Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry **8**: 19-23.

Herrmann, K. M. and L. M. Weaver (1999). The shikimate pathway. Annual Review of Plant Physiology and Plant Molecular Biology **50**: 473-503.

Hong, H. T. K., A. Nose and S. Agarie (2004). Respiratory properties and malate metabolism in Percoll-purified mitochondria isolated from pineapple, Ananas comosus (L.) Merr. cv. smooth cayenne. Journal of Experimental Botany **55**(406): 2201-2211.

Hong, H. T. K., A. Nose, S. Agarie and T. Yoshida (2008). Malate metabolism in *Hoya carnosa* mitochondria and its role in photosynthesis during CAM phase III. Journal of Experimental Botany **59**(7): 1819-1827.

Iland, P. G. and B. G. Coombe (1988). Malate, Tartrate, Potassium, and Sodium in Flesh and Skin of Shiraz Grapes During Ripening - Concentration and Compartmentation. American Journal of Enology and Viticulture **39**(1): 71-76.

Jacquot, J. P. P., B. B. Buchanan, F. Martin and J. Vidal (1981). Enzyme regulation in C4 photosynthesis. Purification and properties of thioredoxin-linked NADP-malate dehydrogenase from corn leaves. Plant Physiology **68**(2): 300-304.

Kliewer, W. M. (1965). The sugars of grapevines. II. Identification and seasonal changes in the concentration of several trace sugars in *Vitis vinifera*. American Journal of Enology and Viticulture **16**(3): 168-178.

Kliewer, W. M. (1973). Berry composition of *Vitis vinifera* cultivars as influenced by photo- and nycto- temperatures during maturation. Journal of the American Society for Horticultural Science **98**: 153-159.

Kobayashi, H., K. Fujita, S. Suzuki and T. Takayanagi (2009). Molecular characterization of Japanese indigenous grape cultivar 'Koshu' (Vitis vinifera) leaf and berry skin during grape development. Plant Biotechnology Reports **3**(3): 225-241.

Kubota, K. and H. Ashihara (1990). Metabolic regulation in plant cell culture. 37. Identification of nonequilibrium glycolytic reactions in suspension cultured plant cells. Biochimica Et Biophysica Acta **1036**(2): 138-142.

Labat, E., M. Calas, P. Sarni-Manchado, C. Verries and C. Tesniere (2000). Grapevine (*Vitis vinifera* L.) alcohol dehydrogenase gene: An important marker of the physiological state

during fruit ripening. Proceedings of the Fifth International Symposium on Grapevine Physiology. B. A. Bravdo**:** 303-310.

Lara, M. V., C. O. Budde, L. Porrini, J. Borsani, R. Murray, C. S. Andreo and M. F. Drincovich (2011). Peach (*Prunus persica*) fruit response to anoxia: reversible ripening delay and biochemical changes. Plant and Cell Physiology **52**(2): 392-403.

Loewus, F. A. and P. P. N. Murthy (2000). Myo-inositol metabolism in plants. Plant Science **150**(1): 1-19.

Marsh, K. B., H. L. Boldingh, R. S. Shilton and W. A. Laing (2009). Changes in quinic acid metabolism during fruit development in three kiwifruit species. Functional Plant Biology **36**(5): 463-470.

Martinez-Esteso, M. J., S. Selles-Marchart, J. C. Vera-Urbina, M. A. Pedreno and R. Bru-Martinez (2011). DIGE analysis of proteome changes accompanying large resveratrol production by grapevine (Vitis vinifera cv. Gamay) cell cultures in response to methyl-beta-cyclodextrin and methyl jasmonate elicitors. Journal of Proteomics **74**(8): 1421-1436.

Mathieu, Y., J. Guern, M. Pean, C. Pasquier, J. C. Beloeil and J. Y. Lallemand (1986). Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. II. Possible mechanisms involved in pH regulation during acid load. Plant Physiology **82**(3): 846-852.

Meister, M., A. Agostino and M. D. Hatch (1996). The roles of malate and aspartate in C-4 photosynthetic metabolism of *Flaveria bidentis* (L). Planta **199**(2): 262-269.

Melino, V. J., K. L. Soole and C. M. Ford (2009a). Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. Bmc Plant Biology **9**.

Melino, V. J., K. L. Soole and C. M. Ford (2009b). A method for determination of fruit-derived ascorbic, tartaric, oxalic and malic acids, and its application to the study of ascorbic acid catabolism in grapevines. Australian Journal of Grape and Wine Research **15**(3): 293-302.

Molina, I., C. Salles, M. Nicolas and J. Crouzet (1987). Grape alcohol dehydrogenase. II. Kinetic studies: mechanism, substrate, and coenzyme specificity. American Journal of Enology and Viticulture **38**(1): 60-64.

Mulas, G., M. G. Galaffu, L. Pretti, G. Nieddu, L. Mercenaro, R. Tonelli and R. Anedda (2011). NMR Analysis of Seven Selections of Vermentino Grape Berry: Metabolites Composition and Development. Journal of Agricultural and Food Chemistry **59**(3): 793-802.

Ollat, N. and J. P. Gaudillere (2000). Carbon balance in developing grapevine berries. Proceedings of the V International Symposium on Grapevine Physiology.

Or, E., J. Baybik, A. Sadka and Y. Saks (2000). Isolation of mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxylase cDNA clones from grape berries and analysis of their expression pattern throughout berry development. Journal of Plant Physiology **157**(5): 527-534.

Roessner, U., C. Wagner, J. Kopka, R. N. Trethewey and L. Willmitzer (2000). Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant Journal **23**(1): 131-142.

Roessner, U., A. Luedemann, D. Brust, O. Fiehn, T. Linke, L. Willmitzer and A. R. Fernie (2001). Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell **13**(1): 11-29.

Romieu, C. and C. Flanzy (1988). Extraction des mitochondries de baies de raisin (*Vitis vinifera*). Plant Physiology and Biochemistry **26**(5): 589-596.

Romieu, C., C. Tesniere, L. Thanham, C. Flanzy and J. P. Robin (1992). An examination of the importance of anaerobiosis and ethanol in causing injury to grape mitochondria. American Journal of Enology and Viticulture **43**(2): 129-133.

Ruffner, H. P. (1982a). Metabolism of tartaric anc malic acids in *Vitis*: A Review - Part A. Vitis **21**(4): 247-259.

Ruffner, H. P. (1982b). Metabolism of tartaric and malic acids in *Vitis*: A review - Part B. Vitis **21**(4): 346-358.

Ruffner, H. P. and W. M. Kliewer (1975). Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiology **56**: 67-71.

Ruffner, H. P. and J. S. Hawker (1977). Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochemistry **16**: 1171-1175.

Ruffner, H. P., J. S. Hawker and C. R. Hale (1976). Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15**: 1877-1880.

Ruffner, H. P., S. Brem and U. Malipiero (1983). The physiology of acid metabolism in grape berry ripening. Acta Horticulturae **139**: 123-128.

Saito, K. and Z. Kasai (1982). Conversion of L-ascorbic acid to L-idonic acid, L-idono-gamma-lactone and 2-keto-L-idonic acid in slices of immature grapes. Plant and Cell Physiology **23**(3): 499-507.

Svensson, P., O. E. Blasing and P. Westhoff (2003). Evolution of C4 phosphoenolpyruvate carboxylase. Archives of Biochemistry and Biophysics **414**(2): 180-188.

Sweetman, C., L. G. Deluc, G. R. Cramer, C. M. Ford and K. L. Soole (2009). Regulation of malate metabolism in grape berry and other developing fruits. Phytochemistry **70**(11-12): 1329-1344.

Taureilles-Saurel, C., C. G. Romieu, J. P. Robin and C. Flanzy (1995). Grape (*Vitis vinifera* L.) malate dehydrogenase .I. Intracellular compartmentation of the isoforms. American Journal of Enology and Viticulture **46**(1): 22-28.

Tesniere, C. and C. Verries (2000). Molecular cloning and expression of cDNAs encoding alcohol dehydrogenases from *Vitis vinifera* L. during berry development. Plant Science **157**: 77-88.

Tesniere, C., C. Davies, L. Sreekantan, J. Bogs, M. Thomas and L. Torregrosa (2006). Analysis of the transport levels of *VvAdh1*, *VvAdh2* and *VvGrip4*, three genes highly expressed during Vitis vinifera L. berry development. Vitis **45**(2): 75-79.

Tripodi, K. E., W. L. Turner, S. Gennidakis and W. C. Plaxton (2005). *In vivo* regulatory phosphorylation of novel phosphoenolpyruvate carboxylase isoforms in endosperm of developing castor oil seeds. Plant Physiology **139**(2): 969-978.

Tronconi, M. A., H. Fahnenstich, M. C. G. Weehler, C. S. Andreo, U. I. Flugge, M. F. Drincovich and V. G. Maurino (2008). *Arabidopsis* NAD-malic enzyme functions as a homodimer and heterodimer and has a major impact on nocturnal metabolism. Plant Physiology **146**(4): 1540-1552.

Turner, W. L. and W. C. Plaxton (2000). Purification and characterization of cytosolic pyruvate kinase from banana fruit. Biochemistry Journal **352**: 875-882.

Walker, R. P., F. Famiani, A. Baldicchi, J. G. Cruz-Castillo and P. Inglese (2011). Changes in enzymes involved in photosynthesis and other metabolic processes in the fruit of Opuntia ficus-indica during growth and ripening. Scientia Horticulturae **128**(3): 213-219.

Walker, R. P., Z. H. Chen, L. I. Tecsi, F. Famiani, P. J. Lea and R. C. Leegood (1999). Phosphoenolpyruvate carboxykinase plays a role in interactions of carbon and nitrogen metabolism during grape seed development. Planta **210**(1): 9-18.

Yang, L. T., C. Y. Xie, H. X. Jiang and L. S. Chen (2011). Expression of six malate-related genes in pulp during the fruit development of two loquat (Eriobotrya japonica) cultivars differing in fruit acidity. African Journal of Biotechnology **10**(13): 2414-2422.

Yao, Y. X., M. Li, Z. Liu, C. X. You, D. M. Wang, H. Zhai and Y. J. Hao (2009). Molecular cloning of three malic acid related genes MdPEPC, MdVHA-A, MdcyME and their expression analysis in apple fruits. Scientia Horticulturae **122**(3): 404-408.

Yoshioka, H., K. Aoba, M. Fukumoto and K. Fujimoto (1991). Purification of NADP-malic enzyme from apple fruit and its change with development and ripening. Journal of the Japanese Society for Horticultural Science **60**(2): 449-455.

Chapter 3

Importance of mitochondrial respiration and acid metabolism in *Vitis vinifera* berry development and ripening

Importance of mitochondrial respiration and acid metabolism in *Vitis vinifera* berry development and ripening

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3.1. Abstract

Respiration of fruits has long been an interest of plant scientists. Studies have often focussed on the climacteric or non-climacteric nature of the fruit, and the optimisation of post-harvest processes and storage conditions for preservation of such fruits. In this study the pre-harvest metabolism of grape berries, which do not ripen if removed from the vine, has been investigated. Although grapes are non-climacteric, dramatic changes occur in respiratory metabolism with the onset of grape berry ripening. Of these changes, a switch to the utilisation of malate as a carbon source for fruit respiration may play one of the most prominent roles in respiratory metabolism associated with grape berry ripening. Here, we seek to determine the impact of malate on fruit respiratory pathways. We present the first evidence for activities of the alternative oxidase and the mitochondrial-specific, NAD-dependent malic enzyme in grapevine, using preparations of purified grape mitochondria. Further, we provide an analysis of three putative *Vitis vinifera* alternative oxidase gene sequences, and explore transcriptional regulation and enzymatic activities of the phosphorylating and non-phosphorylating pathways of respiration in developing grape berries, with a focus on the initiation of ripening. As malate is mobilised from grape berry vacuoles, respiratory patterns change dramatically, with a stimulation of both phosphorylating and non-phosphorylating pathway enzymes, mitochondrial malate dehydrogenase and malic enzyme activities, and alternative oxidase gene transcripts. The coordinated regulation of these pathways may ensure provision of respiratory products and intermediates that are essential for the ripening of the fruit.

3.2. Introduction

Grape (*Vitis vinifera*) berry metabolism is important for the production of healthy, pleasant-tasting fruit that can be harvested and used as table grapes, or for production of dried fruit, juice and wine. As with many fruit, young grapes are hard and green with rapidly dividing cells, functional stomata and chloroplasts (Harris et al., 1968; Blanke and Leyhe, 1987; Palliotti and Cartechini, 2001). Throughout early development, photosynthesis in the fruit and translocation of sugars from other parts of the vine provide fuel for cell growth and division, resulting in rapid fresh weight accumulation. During this early developmental stage, sugars are respired and organic acids (mostly

tartrate and malate) accumulate in the cell vacuole (Hale, 1962; Ruffner and Hawker, 1977). Approximately 10 weeks into development the berry weight reaches a plateau and the fruit undergoes a dramatic metabolic shift, marking the initiation of ripening (véraison). In post-véraison fruit, growth resumes and is mainly driven by cell expansion and accumulation of sugars (along with other solutes) and water (Coombe, 1992). Simultaneous to this increase in sugars, the fruit show a dramatic decline in organic acid content, mostly through the loss of malate. Together, these events generally lead to the production of a sweet-tasting fruit containing complex taste, aroma and texture compounds, which can also provide favourable conditions for wine production and pleasing sensory characters in the finished wine.

The switch from fruit development to fruit ripening is of a molecular and biochemical interest, due to the dramatic, controlled changes in metabolism. At véraison, even the fundamental pathways of cellular respiration are altered, as evidenced from changes in the respiratory quotient (Harris et al., 1971). Young grapes metabolise sugars that are mainly imported from the leaves. Upon véraison, when glycolysis seems to slow (Ruffner and Hawker, 1977), the vacuolar pool of malate may be released as a new respiratory driver (Hardy, 1968; Ruffner, 1982). Such a change in respiratory mechanisms could have a number of downstream effects, including changes in the nature and quantity of metabolic intermediates, as well as redox states in the mitochondria. Furthermore, changes in cellular respiration may alter the synthesis of ATP, which is involved in a plethora of cellular processes including cell maintenance, division and biosynthesis.

Fundamental cellular respiration generally encompasses three pathways: glycolysis in the cytosol, which involves catabolism of glucose monosaccharides to pyruvate (and malate in some cases); the TCA cycle in the mitochondrial matrix, which is fuelled by glycolytic pyruvate or malate and drives the reduction of electron carriers NADH and $FADH_2$, and the mitochondrial electron transport chain (mETC) of the inner mitochondrial membrane, which oxidises electron carriers from the mitochondrial matrix and establishes an electrochemical gradient that can drive ATP production.

The common mETC is comprised of four enzyme complexes (CI, CII, CIII, CIV) and two carrier proteins (ubiquitin and cytochrome c), and is responsible for oxidative phosphorylation. Electron donors from the TCA cycle (ie. NADH and $FADH_2$) provide electrons to the mETC, which, along with the reduction of oxygen to water, form an electrochemical gradient across the inner mitochondrial membrane that enables the

ATP synthase complex to phosphorylate ADP to ATP. Plants (as well as some bacteria, yeast and algae), exploit an additional pathway of cellular respiration, which is "oxidative" but "non-phosphorylating", and is resistant to inhibitors of the phosphorylating pathway (Moore and Siedow, 1991). This non-phosphorylating pathway of cellular respiration encompasses a series of enzymes that transfer electrons without contributing to the electrochemical gradient and thereby ATP synthesis. This pathway consists of a series of non-phosphorylating dehydrogenases (npDHs), which collect electrons from mitochondrial NADH and NADPH (Soole and Menz, 1995), and an alternative oxidase (AOX) reduces oxygen to water and further bypasses complexes of the phosphorylating pathway (Finnegan et al., 2004). Through the activities of various phosphorylating and non-phosphorylating respiratory enzymes, the efficiency of ATP production from cellular respiration can be altered. In a fully operational non-phosphorylating pathway, there is potential to completely uncouple electron transport from ATP synthesis. While the function for this mechanism remains unresolved, a large and ever-growing body of evidence from numerous sources suggest an involvement in cellular protection from reactive oxygen species (Zhang et al., 2003). The non-phosphorylating pathway of respiration has also been identified as a part of plant stress response (Smith et al., 2009; Vijayraghavan and Soole, 2010) and another suggested function is to enable consistent flux through metabolic pathways such as glycolysis and the TCA cycle, without the feedback inhibition imposed by increased synthesis of ATP that would otherwise occur in the fully operational phosphorylating pathway (Day et al., 1995). This may be useful in providing carbon for the synthesis of useful secondary metabolites. In fruiting plants, an increase in respiratory flux through the non-phosphorylating pathway may enable an increase in the development of pigments and complex aroma and flavour compounds during ripening. This may assist the plant by attracting more pollen-distributing insects and seed-dispersers, and may additionally benefit viticulturalists and winemakers by increasing the complexity of harvest-ripe fruit.

Since the discovery of the first AOX gene in *Arabidopsis thaliana* (Kumar and Soll, 1992), a family of five isogenes has been elucidated in this species; *AtAox1a-1d* and *Ataox2* (Saisho et al., 1997; Clifton et al., 2006). AOX genes, which are present in all plants (Siedow and Umbach, 1995), can be divided into two subfamilies; *Aox1* isogenes, present in monocots and dicots, and; *Aox2* isogenes, exclusive to eudicots (Considine et al., 2002). Orthologous AOX gene sequences have also been detected in the genomes of numerous other plant species, including fruits (Considine et al., 2001). In *Vitis vinifera* at

least three putative AOX genes have been identified; denoted *VvAox1a-1b* and *VvAox2* (Costa et al., 2009), although the activities of these gene products are yet to be confirmed.

While AOX genes may not always be directly comparable between species (Clifton et al., 2006), tentative conclusions on genetic similarities can be drawn using a bioinformatics approach. However, to understand roles of AOX in *V. vinifera*, the function must be clarified within the plant system itself. Unfortunately the use of forward or reverse genetics processes are difficult when dealing with perennial plants, due to extended growth and development periods. Therefore, the aim of this study was to investigate functionality of AOX in *V. vinifera*, as facilitated by observed changes in gene transcript levels and enzyme activities measured throughout development of the fruit. In addition, the shift of grape berry primary respiration pathways has been exploited to determine whether malate is a potential driver of fruit respiration, and what effects this switch from predominantly sugar-fuelled, to largely acid-fuelled respiration may have on the phosphorylating and non-phosphorylating pathways of the mETC.

3.3. Materials and methods

3.3.1. Materials

Berries were collected from the Coombe Vineyard, University of Adelaide Waite Campus, Urrbrae, South Australia (123m elevation, -34.964˚ latitude, 138.648˚ longitude). Five East-West facing rows of Shiraz clone BVRC12 vines, established in 1993 on Schwarzmann rootstocks (3m row spacing and 1.8m vine spacing), were selected. Vines were spur-pruned to 30-40 nodes per vine, and drip irrigated weekly. Bunches from one panel (four vines) in each row (ie. a total of 20 vines) were tagged at 50% capfall (denoted “anthesis”), such that each replicate consisted of berries from approximately 30 bunches from five different vines across the five rows. Samples were taken from the initiation of berry development until maturity. At each sampling date the fruit was harvested between 7:30-9:30 am, with 1-4 berries per bunch, taken from the front, back, top, bottom and centre of the bunches. The fruit was immediately frozen in liquid nitrogen before transporting to the laboratory, and stored at -80˚C until use. At particular time-points throughout the season, whole bunches were removed, transported to the laboratory on ice, and used fresh for mitochondrial isolations.

3.3.2. Malate quantification

Organic acids were extracted from 500 mg frozen berry powder, using the method of Melino et al. (2009) in 5 ml of 3% (w/v) metaphosphoric acid (MPA; with 1 mM EDTA) with shaking for 60 mins at 4˚C. The homogenate was centrifuged at 2,500 xg for 5 mins and the supernatant was passed through a Millipex 0.45 µm hydrophilic PVDF syringe-driven filter (Millipore, NSW, Australia). The filtrate was diluted in 0.1M MOPS (pH 8.0) and used for enzyme-linked spectrophotometric assays, as modified from Mollering (1974). This method measured NADH formation (340 nm) in 200 µl of 0.1 M MOPS (pH 8.0) containing 10 mM NAD^+, 50 mM glutamate, alanine aminotransferase (3 U) and malate dehydrogenase (2 U), before and after a 30 minute incubation with extract (20 µl). Malate was quantified according to standards of known concentrations dissolved in MPA and diluted 1/10 with 0.1 M MOPS (pH 8.0).

3.3.3. Mitochondrial isolation

Whole tagged bunches of Shiraz grape berries were harvested at numerous time-points, measured as days after flowering (D.A.F.), throughout the berry developmental season. Fruit were transported to the laboratory on ice, and all further steps were carried out at 4˚C using a method adapted from Romieu & Flanzy (1988). Fresh grape berry tissue (50 g), was gently homogenised in 3 volumes of Isolation Medium (200 mM MOPS pH 8.0, 15 mM Na_2HPO_4, 5 mM EDTA, 0.3 M sucrose, 0.35 M mannitol, 1% PVP-40, 0.25% casein, 4 mM cysteine-HCl, 5 mM iso-ascorbate, 0.5% BSA), using a polytron at minimum speed to ensure seeds remained intact. The homogenate was filtered through 2 layers of Miracloth to remove skin and seeds, then centrifuged at 1,500 xg for 15 mins. The supernatant was centrifuged at 10,000 xg for 30 mins, and the pellet resuspended in approximately 30 ml of Wash Medium (15 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 0.3 M sucrose, 0.5 M mannitol, 1% PVP-40, 0.1% BSA). After repeating the centrifugations, the pellet was carefully resuspended in 2 ml of Wash Medium (without BSA). This crude mitochondrial preparation was purified on a 4-step discontinuous density gradient (10%, 15%, 25% and 45% v/v Percoll), in a swing-out rotor at 18,000 rpm for 60 mins. Mitochondria were collected at the 15:25% and 25:45% Percoll interfaces, pooled and washed in 30 ml of Wash Medium (containing 0.4 M sucrose) and pelleted at 10,000 xg for 15 mins. The final pellet was resuspended in 1 ml of Wash Medium (without BSA) and used in spectrophotometric and polarographic enzyme assays.

3.3.4. Cellular enzyme extraction

Active enzymes were extracted using a method adapted from Ruffner & Kliewer (1975). Twelve volumes of extraction buffer (0.5 M Tris-Cl, pH 8.5 with 200 M KCl, 20 mM $MgCl_2$, 10 mM EDTA, 8% (w/v) PEG-4000, 8 mM cysteine-HCl, 7 mM diethyldithiocarbamate, 5 mM DTT, 2% (w/v) PVPP, 0.25% (w/v) BSA, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine) were added to 1g of frozen grape berry powder such that the extract remained greater than pH 7.0, and mixed gently at 4˚C for 15 mins. After centrifugation at 2,750 xg for 5 mins to remove cell debris, PEG-4000 was added to a final concentration of 65% in the supernatant, then mixed by gentle shaking at room temperature until dissolved. After centrifugation at 30,000 xg, the precipitated protein was carefully resuspended in 2mL final volume (5 mM Tris-Cl, pH 7.0, with 20 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, 3% (v/v) Triton X-100, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine), re-centrifuged at 3,000 x g and the supernatant used in enzyme activity assays.

3.3.5. Spectrophotometric enzyme activity assays

COX activity was quantified as the rate of Cytochrome C oxidation (detectable at 550 nm) from approximately 100 µg mitochondrial protein in 0.1M MES and 10 µM EDTA (modified from Wharton & Tzagoloff, 1967).

NAD-MDH activity was quantified as the rate of NADH oxidation (0.5 mM) in a standard reaction media (SRM; 10 mM TES, pH 6.8, 10 mM KH_2PO_4, 0.4 M sucrose, 0.5 M mannitol, 2 mM $MgCl_2$), containing 10 mM KCl, 5 mM potassium cyanide (KCN), 150 nM octyl gallate (OG) and 5 mM OAA (modified from Ruffner et al. 1976).

Catalase activity was quantified as the azide-sensitive (1mM) rate of H_2O_2 (0.05%) consumption (detectable at 240 nm) in 0.1 M potassium phosphate, pH 7.0 (modified from Beers & Sizer, 1952) for detection of peroxisomal contamination.

NAD-ME activity was quantified as the rate of CoA-dependent (75 µM) NAD^+ (2 mM) reduction in 50 mM TES (pH 7.4) in the presence of 5 mM $MnCl_2$, 0.02% (v/v) Triton X-100, 5 mM DTT, 5 mM KCN, 50 nM OG and 5 mM malate (modified from Hatch et al. 1982).

NADP-ME activity was quantified as the rate of $NADP^+$ (2 mM) reduction in 50 mM MES (pH 6.0) in the presence of 8 mM $MnCl_2$, 0.02% (v/v) Triton, 5 mM KCN, 150 nM OG and

5 mM malate (modified from Ruffner et al. 1976), for detection of cytosolic and chloroplastic contamination.

NADP-MDH activity was quantified as the rate of NADPH (0.5 mM) oxidation in 50 mM TES (pH 8.0) in the presence of 5 mM DTT, 0.02% Triton X-100 and 5 mM OAA (modified from Jacquot et al. 1981), for detection of chloroplastic contamination.

PEPC activity was quantified as the rate of NADH (0.5 mM) oxidation in 50 mM TES(pH 8.0) in the presence of 15% (v/v) glycerol, 5 mM $KHCO_3$, 10 mM $MgCl_2$, 5 mM DTT, 6 U MDH and 3 mM PEP (modified from Ruffner et al. 1976), for detection of cytosolic contamination.

Mitochondrial protein concentration was determined using a BCA Protein Estimation Kit (Pierce) according to the manufacturer's instructions.

3.3.6. Polarographic enzyme activity assays

Mitochondrial enzyme activities were assayed through rates of oxygen consumption, as measured by an oxygen electrode (Rank Brothers) in SRM (pH 7.2), in the presence of exogenous malate (10 mM), succinate (10 mM), ascorbate:TMPD (5mM:1mM), NADH (1 mM), or reduced cytochrome c (30 µM).

The presence of succinate dehydrogenase was confirmed through the measurement of succinate respiration (10 mM), in the presence of 0.1 mM ATP. KCN-sensitive, ascorbate-cytochrome c-dependent O_2 uptake was measured to confirm the presence of cytochrome oxidase activity. Activity was measured in SRM (pH 7.2), as the rate of oxygen consumption in the presence of 8 mM ascorbate and 30 µM cytochrome c, that was sensitive to 2 mM KCN. Assays were run with intact and osmotically lysed mitochondria, to estimate the level of membrane integrity.

NAD-ME activity was quantified as the rate of mitochondrial oxygen consumption in the presence of 10 mM malate, 2 mM ADP, 4 mM $MnCl_2$ and 1 mM NAD^+, in SRM (pH 6.8). With malate as the sole respiratory substrate, the activity of malic enzyme (whereby malate is converted to pyruvate) can define the amount of acetyl-CoA made available to the TCA cycle, and thus the rate of oxygen consumption through oxidative phosphorylation. In this way, oxygen consumption in the presence of 10 mM malate, 2 mM KCN, 0.12 µM OG, 2 mM ADP, 1 mM NAD and 4 mM MnCl2, was used to estimate activity of NAD-ME. This enzyme was also assayed spectrophotometrically, as described.

The capacity for COX activity was determined as the rate of oxygen consumption in SRM (pH 7.2), that was resistant to 0.12 µM OG and sensitive to 2 mM KCN, in the presence of 10 mM succinate, 10 mM pyruvate, 1 mM NADH, 5 mM DTT and 2 mM ADP.

The capacity for AOX activity was determined as the rate of oxygen consumption in SRM (pH 7.2), that was resistant to 2 mM potassium cyanide and sensitive to 0.12 µM octyl gallate, in the presence of 10 mM succinate, 10 mM pyruvate, 1 mM NADH, 5 mM DTT and 2 mM ADP.

3.3.7. Bioinformatics

Grape sequences for AOX and COX subunit 3b were collected through tBLASTn searches in grape EST databases, including TGI
(now DFCI; http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape) and NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=29760), using known protein sequences of other plants (eg. tomato, mango and *Arabidopsis*). ESTs were assembled into contiguous sequences using Sequencher 4.7 (Gene Codes, MI, USA), and three resultant isogenes were predicted. These sequences were later found to be very similar to those published by Costa et al (2009). Corresponding genomic sequences were identified using a MegaBLAST in NCBI (http://blast.ncbi.nlm.nih.gov), from the *Vitis vinifera* (WGS) database. EST and genomic contigs were aligned using Spidey (NCBI; http://www.ncbi.nlm.nih.gov/spidey), to identify potential intron/exon splice sites. Primer and probe sets for qRT-PCR assays were developed using Roche Universal (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) such that primers flanked intron splice sites and targeted small (65-110 bp), unique regions of the gene. Primers were checked for specificity by BLASTn in the DFCI grape database, and outlined in Table 3.1.

3.3.8. Quantitative real-time reverse transcriptional PCR

RNA was extracted from approximately 1g of frozen berry powder, using a sodium perchlorate method modified from Davies and Robinson (1996), and purified using an RNeasy Mini Kit (Qiagen, Australia) as described by Melino et al. (2009). Genomic DNA contamination was removed by an on-column DNase step, using RNase-free DNase I (Qiagen, Australia). Grape RNA quality was assessed by agarose gel electrophoresis, and quantified with a Nanodrop spectrometer (Thermo Scientific, Biolab, Australia). First-strand cDNA synthesis from grape berry RNA was achieved with oligo$(dT)_{20}$ primer and

Table 3.1: List of accession numbers, primer sets and probes used for qRT-PCR. Probe numbers are from the Human Universal Probe Library (Roche, Australia).

Gene	Accession #	Protein	Primer Sequences (5'-3')	Probe
AOX1a	EU165202	ACI28876.1	F: aatggaaatggagctgtttca R: aagctactttctcagtccataccc	60
AOX1b	EU165203	ACI28877.1	F: tcttcaacgcatacttcctaacat R: tccttgaggaattcggtgtgag	25
AOX2	EU523224	ACB45425.1	F: ggaattcagcggcctaaga R: ttgaagtgtctgcatgat	126
COX3	XR_078009	YP_002608380.1	F: agggggtgcaacacttctc R: gccaccatacgaacatggta	105
Ankyrin	XM_002283462	XP_002283498	F: ggttatggcaggaaggagtg R: ggtgtcttgccatccatgtt	120
Ubiquitin	BN000705 (mRNA)	CAI56329	F: gtggccacagcaaccagt R: gcaacctccaatccagtcat	143

Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The resultant cDNA was diluted in DNase free water and 50 ng used for each qRT-PCR assay, in a final volume of 16 µl. Reactions were set up in Faststart Universal Probe Master (Rox) master mix (Roche, Australia) with gene-specific primers and Universal ProbeLibrary probes (Roche, Australia) in a final volume of 16 µl. Thermal cycling conditions involved an initial 95˚C melt step (10 mins), followed by 45 cycles of; 95˚C (15 secs), 57˚C (1 min). Assays were conducted with an ABI HT-700 thermocycler, and analysed using SDS 2.3 software (Applied Biosystems, Australia). Transcript levels were normalised against a reference number, derived from Ankyrin and Ubiquitin genes. These genes were chosen due to their stable transcript levels in multiple microarray experiments and within the cDNA samples used in these experiments.

3.4. Results

3.4.1. Genes of the grapevine mitochondrial electron transport chain

Vitis vinifera AOX genes have been classified as *VvAox1a*, *VvAox1b* and *VvAox2* by Costa et al. (2009). *VvAox* peptides showed high similarity to each other and to Arabidopsis *AtAox* and mango (*Mangifera indica*) *MiAox* peptides (Figure 3.1), particularly at the C-terminal regions.

3.4.1.1. VvCox

The transcript of a putative cytochrome oxidase subunit 3 gene (*VvCox3*) was present at a high level in grape berries at all stages of development analysed here, relative to the normalised transcript levels of the *VvAox* genes (Figure 3.2a). *VvCox3* transcript remained steady throughout berry development, although showed a small peak at véraison.

3.4.1.2. VvAox1a

The *VvAox1a* transcript was present at all stages of grape berry development, albeit at very low levels in young grape berry fruit (Figure 3.2b). From véraison to harvest-ripe fruit, the levels of this transcript increased 10-fold, reaching levels 25-fold greater than those seen in the initial stages of development.

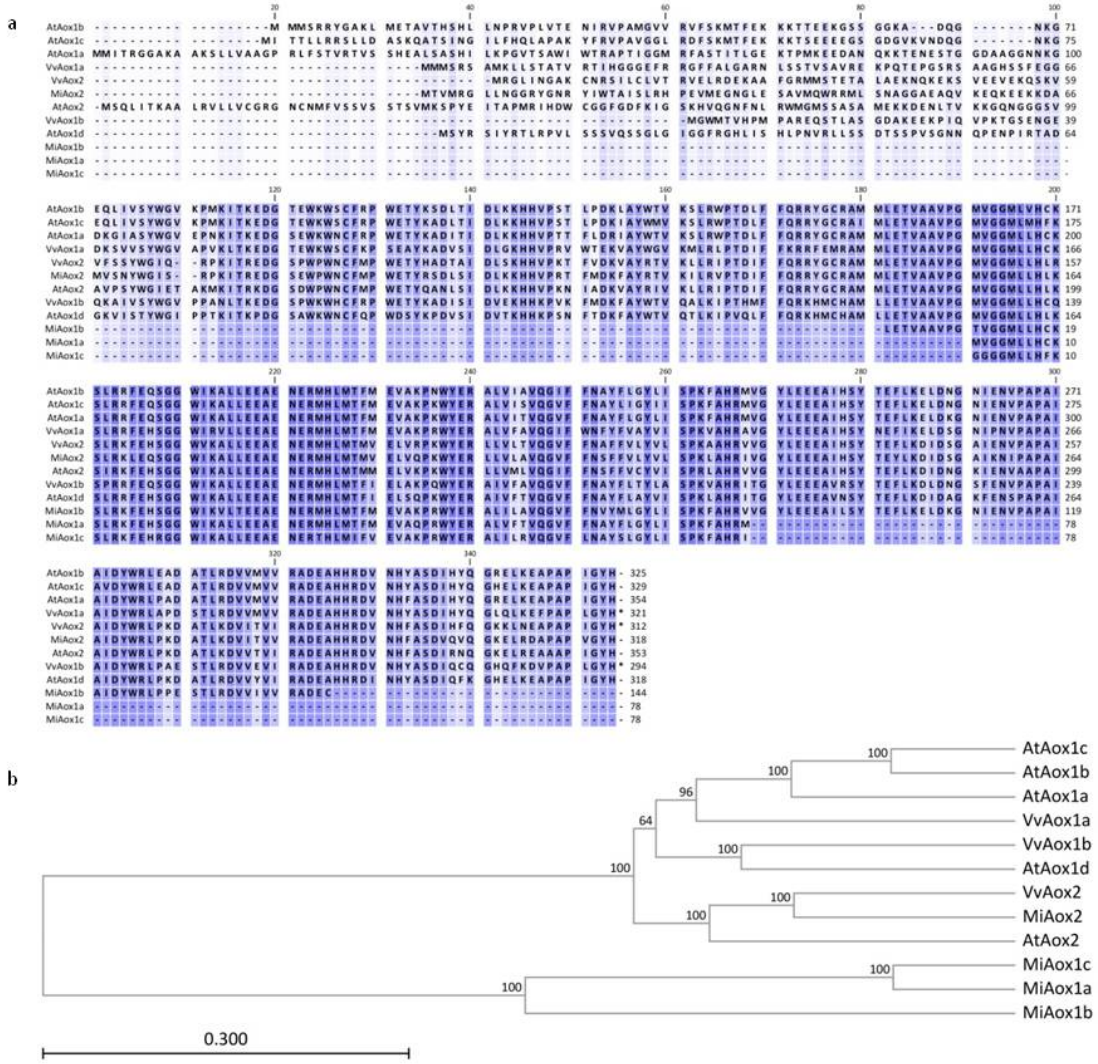

Figure 3.1: Sequence alignment and phylogenetic tree of translated *V. vinifera*, *M. indica* and *A. thaliana* AOX genes. ClustalW alignment (a) and phylogenetic tree (b) of *V. vinifera, M. indica* and *A. thaliana* AOX amino acid sequences as classified by Costa et al. (2009) and Considine et al. (2001; 2002). The phylogenetic tree was obtained with the Neighbour-joining method (Saitou and Nei, 1987), showing bootstrap values (from 100 replicates), using CLC Sequence Viewer 6.4 software.

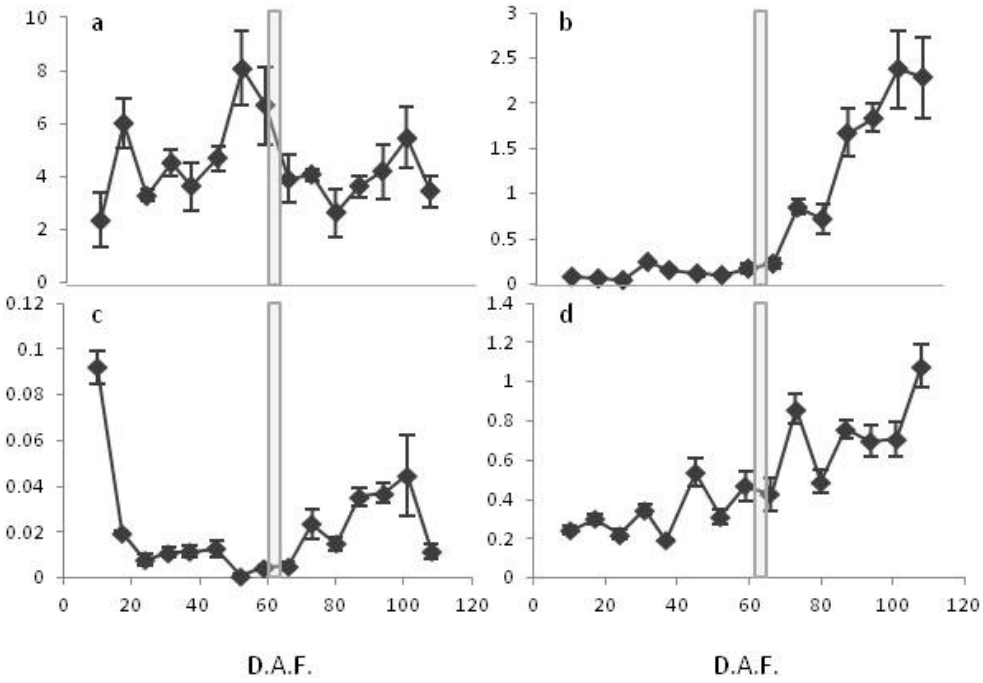

Figure 3.2: Normalised gene transcript level data across development for a) *VvCox3*, b) *VvAox1a*, c) *VvAox1b* and d) *VvAox2.* Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

3.4.1.3. VvAox1b

The maximum transcript level of the *VvAox1b* (0.09 normalised units; Figure 3.2c) was similar to the minimum transcript levels of the VvAox1a and VvAox2 (0.08 and 0.2 normalised units, respectively; Figures 3.2b,d), all of which coincided at 10 D.A.F.. *VvAox1b* transcript decreased 20-fold during early development, but recovered partially (to 0.04 normalised units) through a gradual increase during ripening.

3.4.1.4. VvAox2

VvAox2 transcript was high in young fruit, relative to the other *VvAox* transcripts (Figure 3.2d). Levels of this transcript showed an unsteady increase during development that may have been slightly accelerated from véraison, resulting in levels that were 4-fold higher in ripened berries relative to very young berries.

3.4.2. Purification of grape berry mitochondria

PEPC activity levels in crude and purified mitochondria were approximately 1.5 and 0.2 nmol.min^{-1}.berry^{-1} respectively (Figure 3.3a), whereas in whole fruit this activity was around 50-200 nmol.min^{-1}.berry^{-1} (Section 2.4.2). NADP-MDH activity levels in crude and purified mitochondria were less than 2.5 and 0.6 nmol.min^{-1}.berry^{-1} respectively (Figure 3.3b), whereas in whole fruit this activity was around 50-150 nmol.min^{-1}.berry^{-1} (Section 2.4.2). NADP-ME activity in crude and purified mitochondria were generally less than 1 and 0.2 nmol.min^{-1}.berry^{-1} respectively (except for the first time-point where they were

unusually high; Figure 3.3c), whereas in whole fruit this activity was up to 10 nmol.min^{-1}.berry^{-1} (Section 2.4.2). Catalase activity in crude and purified mitochondria reached no higher than 5 and 2.3 nmol.min^{-1}.berry^{-1} (Figure 3.3d).

COX activity was measured in mitochondrial preparations that were either lysed (diluted in water) or kept intact (diluted in wash medium). Integrity of the mitochondrial membranes was similar in the crude and purified preparations and increased from 75% to 90% after véraison, before decreasing slightly in harvest-ripe fruit (Figure 3.4).

3.4.3. Mitochondrial respiration

The rate of oxygen consumption by grape mitochondria purified from young fruit, measured polarographically, was less than 1 nmol.min^{-1}.mg^{-1} protein regardless of substrate combination (Figure 3.5a). Post-véraison fruit mitochondria had higher rates

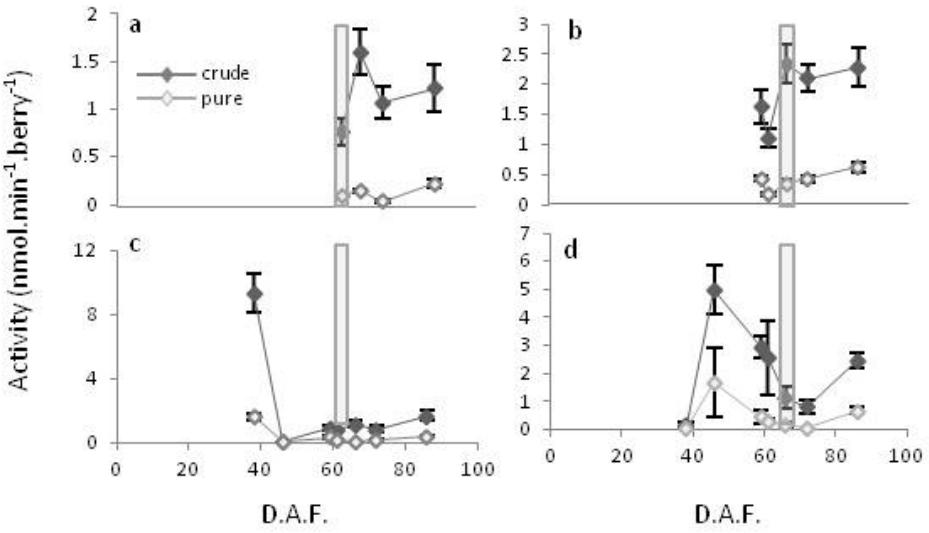

Figure 3.3: Activities of contaminating enzymes in crude and purified preparations of grape berry mitochondria, for a) PEPC [cytosolic], b) NADP-MDH [chloroplastic], c) NADP-ME [cytosolic/chloroplastic] and d) catalase [peroxisomal]. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

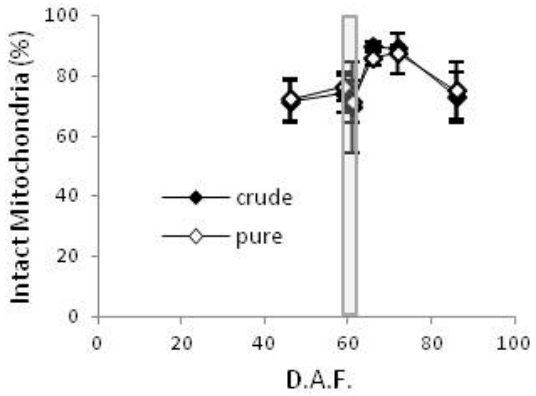

Figure 3.4: Mitochondrial membrane integrity of crude and purified mitochondria. COX activity was measured in mitochondrial preparations before and after osmotic lysis by dilution in water. Integrity was calculated as $[COX_{lysed}–COX_{intact}]/COX_{lysed}$. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

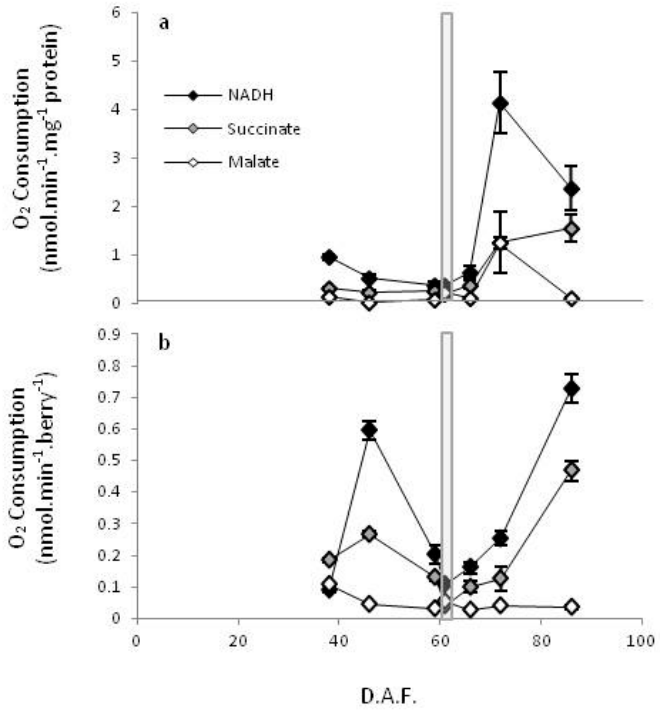

Figure 3.5: State III mitochondrial respiration across berry development in the presence of three combinations of substrates; NADH/succinate/pyruvate/DTT, succinate/ATP and malate/NAD^+, and presented as a) $nmolO_2.min^{-1}.mg^{-1}$ protein and b) $nmolO_2.min^{-1}.berry^{-1}$. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

of respiration than pre-véraison and véraison fruit mitochondria; reaching up to 4.15 nmol.min^{-1}.mg^{-1} protein (0.72 nmol.min^{-1}.berry^{-1}) in the presence of NADH; almost an order of magnitude higher than seen in véraison fruit mitochondria.

3.4.4. Malate-metabolising enzymes in purified mitochondria

Activities of NAD-ME and NAD-MDH from purified mitochondrial preparations were measured spectrophotometrically, with maximum rates at the ripening stage (72 D.A.F.) of approximately 16 and 100 nmol.min^{-1}.mg^{-1} protein, respectively (Figure 3.6). Both enzyme activities showed similar patterns across development, with a large induction post-véraison that was largely lost by the time the fruit were ripe.

3.4.5. COX and AOX activities

Capacities of the COX and AOX enzymes were measured polarographically, although COX was also measured spectrophotometrically. Patterns of COX capacity (Figure 3.7) were seen to increase during ripening, regardless of the substrate used or the expression method (ie. per milligram of protein, or per berry). However, rates were lower with the NADH/succinate substrate combination than with the TMPD/ascorbate substrate, and when expressed per berry, there was an extra peak in COX capacity pre-véraison, with a dip at véraison (Figure 3.7b). Maximum capacities of COX activity were seen to reach 2.51 nmol.min^{-1}.mg^{-1} protein (0.54 nmol.min^{-1}.berry^{-1}) with NADH/succinate substrates. Patterns of AOX capacity were also seen to peak during ripening when expressed as a function of mitochondrial protein, and a dip at véraison when expressed per berry (Figure 3.8). Maximum capacities of AOX activity were seen to reach 2.53 nmol.min^{-1}.mg^{-1} protein (0.34 nmol.min^{-1}.berry^{-1}).When capacities of the COX and AOX enzymes were expressed as proportions of the total respiration rate through NADH/succinate (Figure 3.9), COX capacity was seen to remain steady at approximately 20% pre-véraison, before increasing slightly from véraison throughout ripening to a maximum of 60%. AOX capacity as a proportion of total respiration was high pre-véraison at approximately 70%, before a dip at véraison to a minimum of 20%, before increasing throughout ripening to 60%. When expressed as a proportion of total respiration with malate as the substrate (Figure 3.10), AOX capacity was steady at approximately 60% pre-véraison, before increasing to a peak of 160% post-véraison.

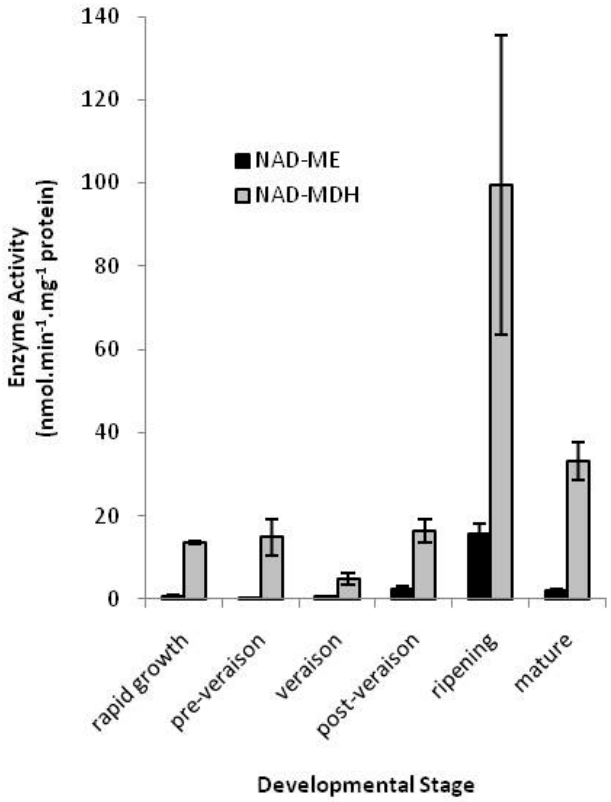

Figure 3.6: Relative rates of mitochondrial malate-catabolic pathways through NAD-dependent malic enzyme (NAD-ME) and NAD-dependent malate dehydrogenase (NAD-MDH) across grape berry development, measured as the spectrophotometric gain or loss of NADH, respectively. Data are presented as means (± SEM) of four vineyard replicates.

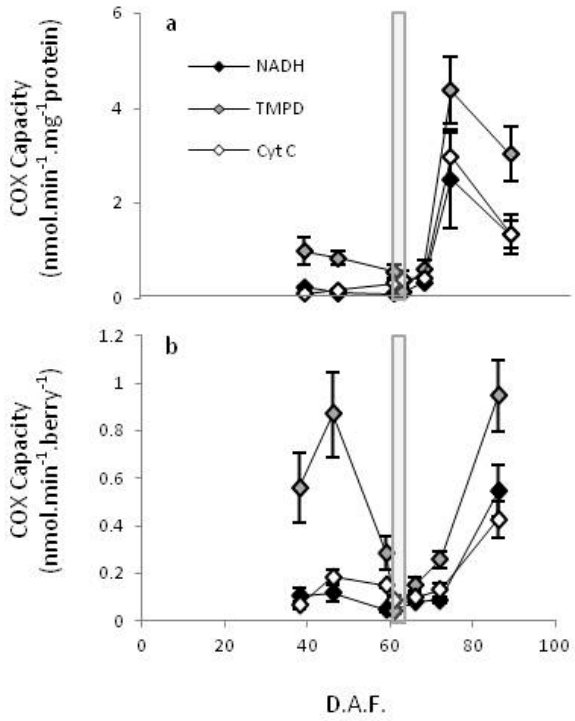

Figure 3.7: Capacity for mitochondrial respiration through COX measured polarographically as the rate of O_2 consumption (in the presence of NADH or TMPD), and spectrophotometrically as the rate of cytochrome c oxidation; presented as a) nmoles.min^{-1}.mg^{-1} mitochondrial protein and b) nmoles.min^{-1}.berry^{-1}. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

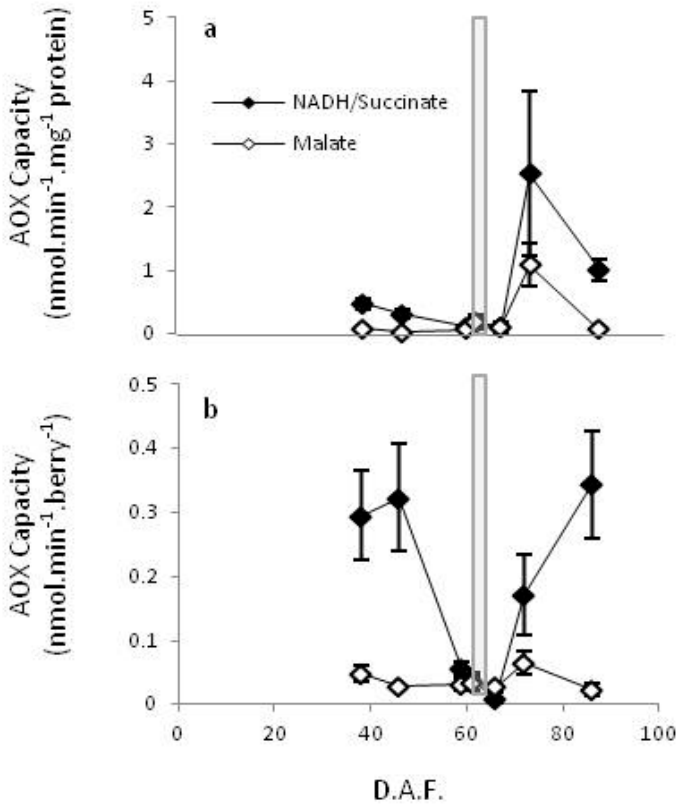

Figure 3.8: Capacity for mitochondrial respiration through AOX in the presence of NADH/succinate/pyruvate/DTT/ADP, and malate/NAD^+/ADP, presented as a) $nmolO_2.min^{-1}.mg^{-1}$ protein and b) $nmolO_2.min^{-1}.berry^{-1}$. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

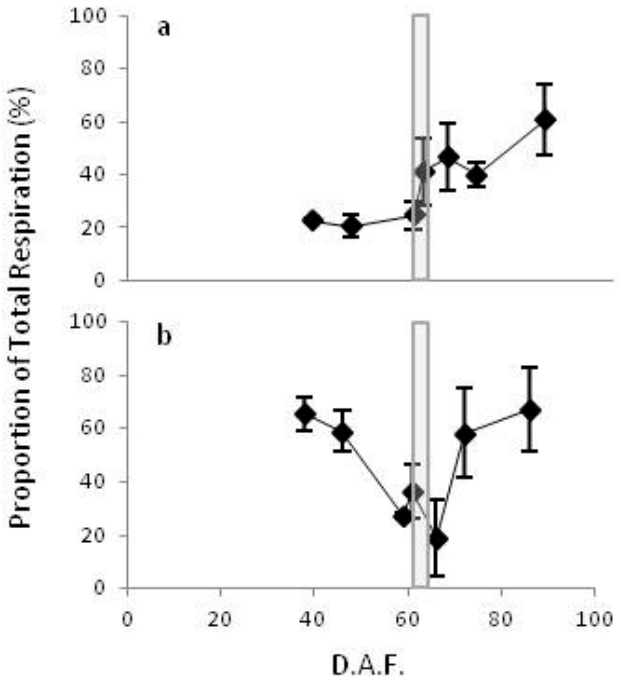

Figure 3.9: Capacity for respiration through a) COX and b) AOX in isolated mitochondria undergoing state III respiration, presented as a proportion of uninhibited respiration. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

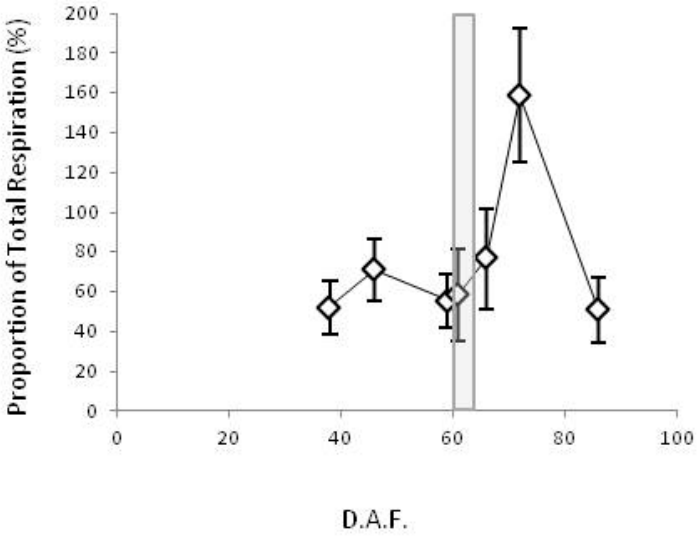

Figure 3.10: Capacity for respiration through AOX in isolated mitochondria respiring malate (in the presence of NAD^+ and ADP), presented as a proportion of uninhibited malate respiration. Data are presented as means (± SEM) of four vineyard replicates. The shaded region indicates veraison.

3.5. Discussion:

3.5.1. Transcripts of the mitochondrial electron transport chain (mETC)

As has been seen before, Arabidopsis genes shared more homology with each other than with orthologous genes from other species (Considine et al., 2002), however there was a large amount of similarity between the translated *VvAox* genes and known AOX protein sequences from *A. thaliana* and *M. indica*. According to the appearance of *V. vinifera* AOX genes in particular EST libraries, it has been previously suggested that *VvAox1a* is limited to fruit and roots, *VvAox1b* to flowers only and that *VvAox2* is present in all tissue types (Costa et al., 2009). However, these new data demonstrated that mRNA copies of all of these genes can exist in the grape berry, with developmental regulation. The relatively steady transcript level seen for VvCox3 across Shiraz berry development suggests that this gene product may be important in cellular respiration regardless of the age of the fruit. However, a small peak pre-véraison may indicate an increased requirement for respiratory flux at this time. Developmental increases in *VvAox1a*, *VvAox1b* and *VvAox2* indicated that these genes might be important for the control of AOX activity levels during more specific stages of berry development. An increase in transcript abundance of *VvAox* genes has been observed in microarray studies with grape berries across broad developmental stages (Pilati et al., 2007; Kobayashi et al., 2009), and is confirmed here with qRT-PCR data.

The sudden induction of *VvAox1a* transcript from veraison suggests that this may play a specific role in ripening fruit. A similar pattern of expression was seen for the *MnAox1a* of mango fruit (Considine et al., 2001). When compared to *A. thaliana* and *M. indica* protein sequences, the translated *VvAox1a* was most similar to *AtAox1a* and *AtAox1b*, although it also aligned very well with *AtAox1c*, consistent with a previous phylogenetic analysis (Costa et al., 2009). In *A. thaliana*, *AtAox1a* is the most highly expressed of all AOX genes. Along with *AtAox1c*, it is particularly high in the flower and leaf, but can be found in virtually every tissue within the plant. In contrast, *AtAox1b* seems to be specific to the floral bud, pollen and stamen (Clifton et al., 2006). Transcript levels of the *VvAox1b* were low relative to the other two *VvAox* genes. The highest level of *VvAox1b* transcript was seen very early in fruit development (10 D.A.F.), at a time when *VvAox1a* and *VvAox2* transcript levels were minimal. As this gene has only previously been seen in ESTs from floral tissues (Costa et al., 2009), the relatively high level of *VvAox1b* transcript early in development may be due to the remaining presence of some floral

tissues on the very young grapes. Therefore this gene product may function in the final stages of flowering, or initial stages of fruit development. The subsequent increase in *VvAox1b* transcript level during berry ripening also implies a role for this gene in the fruit. The most similar *A. thaliana* ortholog *AtAox1d*, has been implicated in *Arabidopsis* leaf senescence. While fruit ripening can be arguably defined as a form of senescence (Coombe, 1976; Brady, 1987), further investigation is required to determine whether there such a role for *VvAox1b*.

VvAox2 transcript was the most abundant of the three isogenes in grape berry EST databases (Costa et al., 2009), and in young Shiraz grape berries (Figure 3.2d). However, after a dramatic developmental induction, the *VvAox1a* transcript took predominance in ripening fruit. Based on homology, *VvAox2* is orthologous to *MiAox2* and *AtAox2*, as well as *Aox2* genes from numerous other plant species (Costa et al., 2009). Unlike *AtAox2*, which is tentatively suggested to play a role in *Arabidopsis* seed maturation and germination (Clifton et al., 2006), *VvAox2* transcripts have been detected in grapevine roots, leaves, flowers and fruit in addition to seeds (Figure 3.2d and Costa et al. 2009), and *MiAox2* transcript has also been detected in mango fruit and vegetative tissues (Considine et al., 2001). This suggests that activities of the*MiAox2* and *VvAox2* gene products may not be limited to seed metabolism.

As three *VvAox* genes were co-ordinately upregulated during ripening of the grape berry, with véraison a potential switch for this upregulation, a role for AOX activity in grape berry respiration during ripening should be further explored, and were thus investigated in purified grape berry mitochondria.

3.5.2. Mitochondrial purity

Mitochondria were isolated from berries of rapid-growth, pre-véraison, véraison, post-véraison, ripening and mature stages of development. Isolation of high-quality mitochondria was difficult from fruit younger than 40 D.A.F., and generally resulted in preparations that had low membrane integrity. At each stage, crude and purified extracts were assayed for contaminating enzyme activities representative of different cellular compartments, including PEPC (cytosolic; Figure 3.3a), NADP-MDH (chloroplastic; Figure 3.3b), NADP-ME (cytosolic/chloroplastic; Figure 3.3c) and catalase (peroxisomal; Figure 3.3d). Mitochondrial preparations had some contamination from cytosolic, chloroplastic and peroxisomal enzymes, although these levels were minimal in

comparison to whole cellular extracts, and were mostly removed upon purification. According to latency assays with COX activity, approximately 70-90% of mitochondria were intact in preparations from the various developmental stages, and the purification process did not reduce membrane integrity. A small increase in membrane integrity of mitochondria in ripening berries may be due to more favourable cellular conditions (i.e. reduced acid and higher sugar levels) that made the extraction process less detrimental to the mitochondria at this time. The decrease in mitochondrial membrane integrity seen in the final time-point (ripe berries) may be due to natural loss of membrane integrity that could occur through cell death in the fruit at this time (Tilbrook and Tyerman, 2008), although this has not been determined experimentally.

3.5.3. Mitochondrial respiration

Rates of state III respiration (ie. respiration that is not limited by ADP levels) were measured from purified mitochondria with three combinations of substrates: malate, NAD^+ and $MnCl_2$ (to activate malic enzyme); succinate and ATP (to activate succinate dehydrogenase), and NADH, succinate, pyruvate and DTT (for maximum rate of oxygen consumption through the mETC). In each case, the rate of oxygen consumption by young fruit mitochondria was low, with an increase in post-véraison fruit that was most pronounced with the addition of NADH. Rates were similar to those described in two previous reports for mitochondrial respiration in grape berry tissue (Romieu and Flanzy, 1988; Romieu et al., 1989), and suggest a ripening-related increase in the capacity for mitochondrial oxygen consumption. The same pattern was observed with malate (and succinate) as the sole electron source. When expressed in fold-change units (data not shown), changes in succinate- and NADH-supported respiration were of a similar relative magnitude. However, summation of the malate- and succinate-driven mitochondrial oxygen consumption rates could not account for the maximum rates seen with NADH. This may be explained by the ability of NADH to donate electrons directly to the mETC, whereas malate and succinate must first donate electrons to matrix NAD^+ and FAD^+, through the activities of metabolic enzymes in the mitochondria. In plants, generation of NADH from mitochondrial malate can occur through oxidation by NAD-dependent malate dehydrogenase (NAD-MDH) activity as part of the TCA cycle, or by decarboxylation to pyruvate through NAD-dependent malic enzyme (NAD-ME) activity. Mitochondrial succinate must be oxidised through succinate dehydrogenase activity, which occurs as part of the TCA cycle and as Complex II of the mETC. Evidently the mETC

at full capacity can accommodate a higher rate of oxygen consumption (and potentially a higher rate of ATP synthesis), than it could with only malate as a source of electrons. Romieu & Flanzy (1988) demonstrated that ripe grape berry mitochondria in the presence of 10 mM malate, 10 mM glutamate, 0.5 mM coenzyme A and excess ADP were capable of a higher rate of oxygen consumption than mitochondria presented directly with 1 mM NADH. In this situation, endogenous glutamic-oxaloacetic transaminase activity removes oxaloacetate from the mitochondrial matrix, pulling the MDH reaction in the direction of malate consumption and therefore generating NADH that can then be oxidised by the mETC. That this process resulted in a more rapid rate of mitochondrial oxygen consumption than direct addition of NADH (Romieu and Flanzy, 1988), suggests that malate, or a by-product of malate metabolism may have had an activation effect on the grape berry mETC relative to NADH in the absence of exogenous pyruvate, succinate and DTT. It is now well known that pyruvate, among other organic acids, can have a stimulatory effect on components of the mETC (Millar et al., 1993).

When expressed per-berry, mitochondrial O_2 consumption was seen to peak at pre-véraison and post-véraison stages, revealing a dip at véraison. This is consistent with observations from daytime gas exchange measurements in whole berries (Niimi and Torikata, 1979; Ollat and Gaudillere, 2000), where respiration rate (expressed per berry) dropped suddenly and dramatically at véraison, then increased towards maturity. This dramatic dip may be due to the slowing of glycolysis around this time (Ruffner and Hawker, 1977), or could be linked to changes in relative growth rate of grape berries, which also shows a similar pattern (Palliotti and Cartechini, 2001). This may also be linked with the véraison switch to malate as primary carbon source (Sweetman et al., 2009), which could necessitate a re-structuring of the respiratory machinery.

3.5.3.1. Respiration of exogenous malate

Evidence for the respiration of malate by grape berries has previously been presented in two forms; recovery of CO_2 from grapes subject to exogenous and radiolabelled malate (Hardy, 1968; Kriedemann, 1968), and; oxygen consumption by purified mitochondria in the presence of malate (Figure 3.5 and Romieu & Flanzy, 1988). As mentioned previously, the respiration of mitochondrial malate requires the activity of either malic enzyme or malate dehydrogenase. Both of these enzymes are present in numerous isoforms that are dependent on either NAD+ or NADP+ for activity. Many isoforms of these enzymes have been clearly identified and characterised in grapes, and their roles in fruit ripening

have been discussed (Hawker, 1969; Ruffner et al., 1984; Franke and Adams, 1992; Gutierrezgranda and Morrison, 1992; Taureilles-Saurel et al., 1995a; b). While the presence of a mitochondrial NAD-dependent malic enzyme (NAD-ME) has been questioned due to the lack of detectable activity in isolated grape mitochondria (Romieu et al., 1992), a grape berry protein that binds an antibody to the *Eleusine coracana* leaf NAD-ME has been detected in grape berry tissue (Famiani et al., 2000), and was seen to increase throughout early fruit growth. Here we report for the first time, evidence of mitochondrial malic enzyme activity in grape berries.

NAD-ME activity can be estimated by rates of mitochondrial oxygen consumption in the presence of malate and NAD^+. An increase in the capacity for mitochondria to respire malate through this mechanism was observed in ripening fruit. This suggests that grape berries can catabolise malate at a higher rate after véraison, at a time when patterns of malate level show a net degradation of the acid. While the kinetics of grape berry NAD-ME activity are yet to be determined, the enzyme generally favours malate decarboxylation in plants (Wedding, 1989). While the cellular environment of grapes around véraison (i.e. low pH and high malate levels) may be expected to favour activity in the direction of malate decarboxylation, the reverse reaction cannot be ruled out for this enzyme. Spectrophotometric measurements of NAD-ME (Figure 3.6) resulted in a similar pattern to that of the malate-driven O_2 consumption described above (Figure 3.5), although at a rate approximately 20 times higher. As the oxidation of two moles of NADH is necessary for the reduction of one mole of O_2 in the mitochondrial electron transport chain, this leaves a 10-fold difference in the apparent rates of NADH production through NAD-ME, and the potential for O_2 consumption from NADH produced by NAD-ME. While the polarographic assay relies on activities of the respiratory machinery, this is certainly not expected to be rate-limiting in this instance, as rates of oxygen consumption through the mitochondrial electron transport chain reached higher rates with other substrates. Therefore this 10-fold difference could be due to characteristics of the enzyme assays, such as the use of lysed mitochondria for spectrophotometric measurements, and intact mitochondrial preparations for polarographic measurements, which may limit the amount of malate and other substrates that are accessible to the enzyme. Or there may be another activity that removes the malate substrate (eg. NAD-MDH), thus reducing the activity of the mETC.

Mitochondrial NAD-MDH activity reached a peak of 100 $nmol.min^{-1}.mg^{-1}$ protein (equivalent to 20 $nmol.min^{-2}.berry^{-1}$), approximately 10% of the rate seen in whole berry extracts (Section 2.4.2). While a direct comparison cannot be made due to potential loss of mitochondrial protein in the extraction process, it can be seen that mitochondrial NAD-MDH activity may account for a relatively large proportion of cellular NAD-MDH activity. As such, it is likely to play a key role in the metabolism of malate in the fruit. In addition, mitochondrial NAD-MDH activity was at least 5-20 times higher than mitochondrial NAD-ME activity at all stages of fruit development. Therefore a large proportion of malate flux through respiration could be directed to oxaloacetate through NAD-MDH as part of the TCA cycle. The simultaneous operation of NAD-ME and NAD-MDH in the mitochondria may be necessary to metabolise malate as it is released the cytosol from veraison, support increased respiration during ripening and provide necessary intermediates such as oxaloacetate and pyruvate to the metabolic pool.

3.5.3.2. Grape berry COX

The rate of COX activity in purified mitochondria was determined using three methods: direct oxidation of exogenous cytochrome c (measured spectrophotometrically), cyanide-sensitive O_2 consumption in the presence of exogenous NADH and octyl-gallate, and cyanide-sensitive O_2 consumption of exogenous ascorbate-TMPD. For every two molecules of cytochrome c oxidised, one half of an O_2 molecule can be reduced to water. Polarographic measurements (ie. the latter two methods employed here) may overestimate the rate of COX activity that occurs *in vivo*, as the use of AOX inhibitors such as octyl gallate can cause an increase in the rate of COX activity. Therefore this method provides information on the capacity of COX and cannot be interpreted as the level of COX "engagement" as occurs in the fruit.

Patterns of COX capacity, measured through assays of O_2 consumption in the presence of NADH/succinate and ascorbate/TMPD and respiratory inhibitors, were very similar, although the rates were lower through NADH/succinate. This may be due to the reliance of NADH/succinate on the flux of electrons along the entire mETC, whereas TMPD, when reduced by ascorbate, donates electrons directly to cytochrome c. In addition, cytochrome c oxidation rates were higher than the rates of COX-supported O_2 consumption, as expected stoichiometrically.

When expressed as a function of mitochondrial protein, COX capacity was seen to peak during ripening. When expressed on a per-berry basis there was an additionally high level of capacity pre-véraison, with a dip at véraison. These patterns are consistent with developmental patterns of respiration as measured in isolated berry mitochondria (Figure 3.5) and in whole berries (Niimi and Torikata, 1979; Ollat and Gaudillere, 2000), and correlate with the pattern of *VvCox3* transcript level across development, which showed a lull before véraison that was bordered by peaks at the pre- and post-véraison stages. Such changes in COX capacity may underline the changes in respiratory pathways that occur during the developmental and ripening phases of berry development.

3.5.3.3. Grape berry AOX

AOX capacity was observed for the first time in grapevine. Rates were determined in the presence of NADH, succinate, pyruvate, DTT and excess ADP (state III respiration), or with malate, NAD^+ and excess ADP (respiration limited by activity of NAD-ME). Developmental changes in AOX capacity within state III respiring mitochondria followed a similar pattern to those seen for total respiration rates and COX capacity. That is, a peak in capacity during ripening when expressed as a function of mitochondrial protein, and peaks pre-véraison and during ripening when expressed per berry. At no stage could AOX capacity alone account for 100% of uninhibited state III respiration. At véraison and early ripening stages both terminal oxidases may be required to maintain sufficient electron flow through the mETC. According to these data, it is tentatively suggested that AOX activity is required in developing grape berries, to support high respiration rates particularly during early stages of fruit ripening; a time when malate may be a key player in berry respiration pathways. Respiration through AOX has also been seen to increase during post-climacteric ripening of tomato (Almeida et al., 2002) and mango (Considine et al., 2001), and during the climacteric phase of apples (Duque and Arrabaca, 1999). While grapes are considered to be non-climacteric, Pilati et al. (2007) uncovered an up-regulation of ROS-scavenging genes and a small accumulation of hydrogen peroxide from véraison, and these new data provide evidence for ripening-related increases in gene transcripts and capacities of respiratory pathway enzymes in grape berries.

While increased capacity for AOX during ripening could be associated with higher transcript levels of *V. vinifera* AOX genes, particularly *VvAox1a* and *VvAox2*, the peak in AOX capacity from pre-véraison berry mitochondria could not. As the level of AOX capacity in younger fruit could not be determined due to the difficulty of isolating intact

mitochondria much before the pre-véraison stages, it may be that the high level of AOX capacity pre-véraison arose from the upregulation of *VvAox1b* transcript observed in very young fruit. Alternatively and perhaps more likely, the plant AOX was simply more active in the young fruit. AOX activity can be regulated post-translationally by oligomerisation (Umbach and Siedow, 1993; Umbach et al., 1994), and activation by carboxylic acids such as pyruvate (Millar et al., 1993), succinate and malate (Liden and Akerlund, 1993). It is possible that the level of AOX protein was in excess during the entirety of grape development, and changes in activity were due mainly to activation state of the enzyme. The dramatic induction of *VvAox* transcripts seen during ripening may ensure even more protein is available, when needed. AOX has recently been hypothesised to function in the minimisation of metabolic fluctuations in plant cells (Rasmusson et al., 2009). In such a scenario, excessive expression of the AOX protein may occur in response to broad developmental or environmental cues, thus providing a means for rapid activation in response to short-term metabolic changes. This would be useful for prevention of oxidative damage that can occur as a result of rapid metabolic changes; a role that is often stipulated for enzymes of the non-phosphorylating pathway of respiration in plants (Umbach et al., 2005; Smith et al., 2009; Vijayraghavan and Soole, 2010). The AOX capacities measured in this study are therefore likely to overestimate the AOX engagement and cannot be used to predict changes in the *in vivo* activities during grape berry development.

The high levels of AOX capacity observed during early development may assist in the actively dividing cells, where a high rate of respiration (mostly through imported sugars) is required to support growth and differentiation (Palliotti and Cartechini, 2001). The cellular environment at this time is also likely to be highly reduced, and carboxylic acids such as malate and pyruvate can be readily synthesised and transported to the mitochondria. With véraison, berry metabolic pathways change to favour solute accumulation and development of complex compounds (Conde et al., 2007). The energy requirement during ripening is thought to be less than that of bud break and early development (Ollat and Gaudillere, 2000), and therefore lower rates of fruit respiration are expected. However, the release of malate from the vacuole at véraison and the shift from sugar-driven to predominantly malate-driven respiration may involve some re-shuffling of the mitochondrial respiratory pathways. This may include some uncoupling of oxygen consumption from ATP synthesis in the mitochondria, which can occur in part by increasing the proportion of electron flux through AOX. It may also necessitate a

further increase in AOX capacity, in order to minimise the oxidative impacts of these sudden metabolic changes. In line with this, the proportion of total respiration that could be accounted for by AOX was seen to increase in mitochondria of ripening fruit. Mitochondria that were limited by NADH supply from NAD-ME activity, showed an even clearer increase in the proportion of AOX capacity, which could account for 60% of the respiration of malate in pre-véraison mitochondria, and 160% of malate respiration after véraison, at a time when malate is being actively metabolised in the berry. That is, the addition of cyanide (an inhibitor of the phosphorylating pathway) stimulated respiration of malate in post-véraison berries. Rotenone, an inhibitor of the NADH dehydrogenase from the phosphorylating pathway, has been seen to inhibit pyruvate formation in cauliflower mitochondria in the presence of exogenous malate and NAD^+ (Rustin et al. 1980), suggesting a specific link between NAD-ME and the non-phosphorylating pathway of respiration, as supported from this current study. A link between malate and the alternative respiratory pathway has long ago been recognised, as summarised by Laties (1982). Increased activity of the NAD-ME in ripening grape berries may facilitate the non-phosphorylating pathway of respiration through the supply of NADH from malate decarboxylation.

The use of alternative oxidase inhibitors in the preservation of grape berries under long-term storage has received a small amount of attention in the past, with variable results (Ramprasad et al., 2004), and may warrant further attention as a means for altering organic acid levels in post-harvest fruit. In addition, to further evaluate the uncoupling of ATP synthesis from oxygen consumption in grape berries, activities of the non-phosphorylating NAD(P)H dehydrogenases should also be evaluated from mitochondria of various fruiting stages.

3.6. Concluding remarks

Changes in the activities and transcripts of mETC components from purified grape berry mitochondria have demonstrated a developmental coordination of phosphorylating and non-phosphorylating pathways of respiration during berry ripening. COX capacity from isolated mitochondria correlated well with gene transcript levels of the COX subunit 3, which were present at all stages throughout development, although with specific increases in pre-véraison berries and during late ripening. Changes in the capacity of

AOX in isolated mitochondria were similar to changes in three grapevine AOX transcripts, suggesting that the activity is regulated, at least in part, by transcriptional activation. However, pre-véraison changes in AOX capacity suggest post-translational activation also play a role. Total respiration could not, at any time, be ascribed to COX or AOX capacities individually, instead requiring input from both pathways.

The importance of malate in grapevine mitochondrial metabolism has also been explored. Grape berry mitochondria have the ability to convert malate directly to oxaloacetate through mitochondrial NAD-MDH, and to pyruvate through NAD-ME. As such, malate may provide an important source of carbon for respiration during ripening of the fruit, when sugar metabolism is reduced. Under such circumstances the presence of malate and the flux of electrons through the mETC may directly induce activity of the AOX, to uncouple oxygen consumption from ATP synthesis and thus enable more rapid flux through mitochondrial pathways and cellular processes overall. Simultaneously, the conversion of malate to oxaloacetate and pyruvate, and further downstream products, may facilitate the biosynthesis of other important compounds involved in grape berry ripening processes.

3.7. References

Almeida, A. M., R. Navet, W. Jarmuszkiewicz, A. E. Vercesi, C. M. Sluse-Goffart and F. E. Sluse (2002). The energy-conserving and energy-dissipating processes in mitochondria isolated from wild type and nonripening tomato fruits during development on the plant. Journal of Bioenergetics and Biomembranes **34**(6): 487-498.

Beers, R. F. and I. W. Sizer (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase Journal of Biological Chemistry **195**(1): 133-140.

Blanke, M. M. and A. Leyhe (1987). Stomatal Activity of the Grape Berry Cv Riesling, Muller-Thurgau and Ehrenfelser. Journal of Plant Physiology **127**(5): 451-460.

Brady, C. J. (1987). Fruit ripening. Annual Review of Plant Physiology and Plant Molecular Biology **38**: 155-178.

Clifton, R., A. H. Millar and J. Whelan (2006). Alternative oxidases in *Arabidopsis*: A comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. Biochimica Et Biophysica Acta-Bioenergetics **1757**(7): 730-741.

Conde, C., P. Silva, N. Fontes, A. C. P. Dias, R. M. Tavares, M. J. Sousa, A. Agasse, S. Delrot and H. Geros (2007). Biochemical changes throughout grape berry development and fruit and wine quality. Food **1**(1): 1-22.

Considine, M. J., D. O. Daley and J. Whelan (2001). The expression of alternative oxidase and uncoupling protein during fruit ripening in mango. Plant Physiology **126**(4): 1619-1629.

Considine, M. J., R. C. Holtzapffel, D. A. Day, J. Whelan and A. H. Millar (2002). Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiology **129**(3): 949-953.

Coombe, B. G. (1976). The development of fleshy fruits. Annual Review of Plant Physiology **27**: 207-228.

Coombe, B. G. (1992). Research on development and ripening of the grape berry. American Journal of Enology and Viticulture **43**(1): 101-110.

Costa, J. H., D. F. de Melo, Z. Gouveia, H. G. Cardoso, A. Peixe and B. Arnholdt-Schmitt (2009). The alternative oxidase family of *Vitis vinifera* reveals an attractive model to study the importance of genomic design. Physiologia Plantarum **137**(4): 553-565.

Davies, C. and S. P. Robinson (1996). Sugar accumulation in grape berries - Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. Plant Physiology **111**(1): 275-283.

Day, D. A., J. Whelan, A. H. Millar, J. N. Siedow and J. T. Wiskich (1995). Regulation of the alternative oxidase in plants and fungi. Australian Journal of Plant Physiology **22**(3): 497-509.

Duque, P. and J. D. Arrabaca (1999). Respiratory metabolism during cold storage of apple fruit. II. Alternative oxidase is induced at the climacteric. Physiologia Plantarum **107**(1): 24-31.

Famiani, F., R. P. Walker, L. Tecsi, Z. H. Chen, P. Proietti and R. C. Leegood (2000). An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries. Journal of Experimental Botany **51**(345): 675-683.

Finnegan, P. M., K. L. Soole and A. L. Umbach (2004). Alternative mitochondrial electron transport proteins in higher plants. Plant Mitochondria: From Genome to Function **17**: 163-230.

Franke, K. E. and D. O. Adams (1992). Inhibition of malic enzyme from grape berries by sulfhydryl-reagents and oxalic-acid. American Journal of Enology and Viticulture **43**(2): 153-158.

Gutierrezgranda, M. J. and J. C. Morrison (1992). Solute distribution and malic enzyme activity in developing grape berries. American Journal of Enology and Viticulture **43**(4): 323-328.

Hale, C. R. (1962). Synthesis of organic acids in the fruit of the grape. Nature **195**(4844): 917-918.

Hardy, P. J. (1968). Metabolism of sugars and organic acids in immature grape berries. Plant Physiology **43**: 224-228.

Harris, J. M., P. E. Kriedemann and J. V. Possingham (1968). Anatomical aspects of grape berry enlargement. Vitis **7**: 106-119.

Harris, J. M., P. E. Kriedemann and J. V. Possingham (1971). Grape berry respiration: Effects of metabolic inhibitors. Vitis **9**: 291-298.

Hatch, M. D., M. Tsuzuki and G. E. Edwards (1982). Determination of NAD malic enzyme in leaves of C_4 plants. Plant Physiology **69**: 483-491.

Hawker, J. S. (1969). Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry **8**: 19-23.

Jacquot, J. P. P., B. B. Buchanan, F. Martin and J. Vidal (1981). Enzyme regulation in C4 photosynthesis. Purification and properties of thioredoxin-linked NADP-malate dehydrogenase from corn leaves. Plant Physiology **68**(2): 300-304.

Kobayashi, H., K. Fujita, S. Suzuki and T. Takayanagi (2009). Molecular characterization of Japanese indigenous grape cultivar 'Koshu' (Vitis vinifera) leaf and berry skin during grape development. Plant Biotechnology Reports **3**(3): 225-241.

Kriedemann, P. E. (1968). Observations on gas exchange in the developing sultana berry. Australian Journal of Biological Sciences **21**: 907-916.

Kumar, A. M. and D. Soll (1992). *Arabidopsis* alternative oxidase sustains *Escherichia coli* respiration. Proceedings of the National Academy of Sciences of the United States of America **89**(22): 10842-10846.

Laties, G. G. (1998). The discovery of the cyanide-resistant alternative path: and its aftermath. Discoveries in Plant Biology. S. D. Kung and S. F. Yang. Singapore, World Scientific Publishing Co. **1**.

Liden, A. C. and H. E. Akerlund (1993). Induction and Activation of the Alternative Oxidase of Potato-Tuber Mitochondria. Physiologia Plantarum **87**(2): 134-141.

Melino, V. J., K. L. Soole and C. M. Ford (2009a). A method for determination of fruit-derived ascorbic, tartaric, oxalic and malic acids, and its application to the study of ascorbic acid catabolism in grapevines. Australian Journal of Grape and Wine Research **15**(3): 293-302.

Melino, V. J., K. L. Soole and C. M. Ford (2009b). Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. Bmc Plant Biology **9**.

Millar, A. H., J. T. Wiskich and J. Whelan (1993). Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Letters **329**(3): 259-262.

Mollering, H. (1974). Determination with malate dehydrogenase and glutamate-oxaloacetate transaminase. Methods of Enzymatic Analysis. H. U. Bergmeyer. New York, Verlag Chemie, Weinheim / Academic Press, Inc. **3:** 1589-1593.

Moore, A. L. and J. N. Siedow (1991). The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. Biochimica et Biophysica Acta **1059**(2): 121-140.

Niimi, Y. and H. Torikata (1979). Changes in photosynthesis and respiration during berry development in relation to the ripening of Delawere grapes. Japanese Society of Horticultural Science **47**(4): 448-453.

Ollat, N. and J. P. Gaudillere (2000). Carbon balance in developing grapevine berries. Proceedings of the V International Symposium on Grapevine Physiology.

Palliotti, A. and A. Cartechini (2001). Developmental changes in gas exchange activity in flowers, berries, and tendrils of field-grown Cabernet Sauvignon. American Journal of Enology and Viticulture **52**(4): 317-323.

Pilati, S., M. Perazzolli, A. Malossini, A. Cestaro, L. Dematte, P. Fontana, A. Dal Ri, R. Viola, R. Velasco and C. Moser (2007). Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at veraison. BMC Genomics **8**.

Ramprasad, V., Y. N. Reddy and M. Reddy (2004). Studies on extension of shelf-life of grape through antioxidants and alternative inhibitors. Proceedings of the Viith International Symposium on Temperate Zone Fruits in the Tropics and Subtropics. K. K. Jindal, R. C. Sharma and A. S. Rehalia**:** 397-402.

Rasmusson, A. G., A. R. Fernie and J. T. van Dongen (2009). Alternative oxidase: a defence against metabolic fluctuations? Physiologia Plantarum **137**(4): 371-382.

Romieu, C. and C. Flanzy (1988). Extraction des mitochondries de baies de raisin (*Vitis vinifera*). Plant Physiology and Biochemistry **26**(5): 589-596.

Romieu, C., C. Tesniere, L. Thanham, C. Flanzy and J. P. Robin (1992). An examination of the importance of anaerobiosis and ethanol in causing injury to grape mitochondria. American Journal of Enology and Viticulture **43**(2): 129-133.

Romieu, C. G., F. X. Sauvage, J. P. Robin and C. Flanzy (1989). Evolution de diverses activities enzymatiques au cours du matabolisme anaerobie de la baie de raisin. Connaissance de la Vigne et du Vin **23**: 165-173.

Ruffner, H. P. (1982). Metabolism of tartaric and malic acids in *Vitis*: A review - Part B. Vitis **21**(4): 346-358.

Ruffner, H. P. and W. M. Kliewer (1975). Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiology **56**: 67-71.

Ruffner, H. P. and J. S. Hawker (1977). Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochemistry **16**: 1171-1175.

Ruffner, H. P., J. S. Hawker and C. R. Hale (1976). Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15**: 1877-1880.

Ruffner, H. P., D. Possner, S. Brem and D. M. Rast (1984). The physiological role of malic enzyme in grape ripening. Planta **160**: 444-448.

Saisho, D., E. Nambara, S. Naito, N. Tsutsumi, A. Hirai and M. Nakazono (1997). Characterization of the gene family for alternative oxidase from Arabidopsis thaliana. Plant Molecular Biology **35**(5): 585-596.

Saitou, N. and M. Nei (1987). The Neighbor-joining methid - a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4**(4): 406-425.

Siedow, J. N. and A. L. Umbach (1995). Plant mitochondrial electron transfer and molecular biology. Plant Cell **7**(7): 821-831.

Smith, C. A., V. J. Melino, C. Sweetman and K. L. Soole (2009). Manipulation of alternative oxidase can influence salt tolerance in *Arabidopsis thaliana*. Physiologia Plantarum **137**(4): 459-472.

Soole, K. L. and R. I. Menz (1995). Functional molecular aspects of the NADH dehydrogenases of plant mitochondria. Journal of Bioenergetics and Biomembranes **27**(4): 397-406.

Sweetman, C., L. G. Deluc, G. R. Cramer, C. M. Ford and K. L. Soole (2009). Regulation of malate metabolism in grape berry and other developing fruits. Phytochemistry **70**(11-12): 1329-1344.

Taureilles-Saurel, C., C. G. Romieu, J. P. Robin and C. Flanzy (1995a). Grape (*Vitis vinifera* L.) malate dehydrogenase .II. Characterization of the major mitochondrial and cytosolic isoforms and their role in ripening. American Journal of Enology and Viticulture **46**(1): 29-36.

Taureilles-Saurel, C., C. G. Romieu, J. P. Robin and C. Flanzy (1995b). Grape (*Vitis vinifera* L.) malate dehydrogenase .I. Intracellular compartmentation of the isoforms. American Journal of Enology and Viticulture **46**(1): 22-28.

Tilbrook, J. and S. D. Tyerman (2008). Cell death in grape berries: varietal differences linked to xylem pressure and berry weight loss. Functional Plant Biology **35**(3): 173-184.

Umbach, A. L. and J. N. Siedow (1993). Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiology **103**(3): 845-854.

Umbach, A. L., J. T. Wiskich and J. N. Siedow (1994). Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Letters **348**(2): 181-184.

Umbach, A. L., F. Fiorani and J. N. Siedow (2005). Characterization of transformed Arabidopsis with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiology **139**(4): 1806-1820.

Vijayraghavan, V. and K. Soole (2010). Effect of short- and long-term phosphate stress on the non-phosphorylating pathway of mitochondrial electron transport in *Arabidopsis thaliana*. Functional Plant Biology **37**(5): 455-466.

Wedding, R. T. (1989). Malic Enzymes of Higher-Plants - Characteristics, Regulation, and Physiological-Function. Plant Physiology **90**(2): 367-371.

Wharton, D. C. and A. Tzagoloff (1967). Cytochrome c oxidase. Methods in Enzymology **10**: 245-250.

Zhang, Q., C. S. Moore, K. L. Soole and J. T. Wiskich (2003). Over-reduction of cultured tobacco cells mediates changes in respiratory activities. Physiologia Plantarum **119**(2): 183-191.

Chapter 4

Effects of elevated temperature on malate and other aspects of fruit primary metabolism in *Vitis vinifera*

Effects of elevated temperature on malate and other aspects of fruit primary metabolism in *Vitis vinifera*

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4.1. Abstract

The composition of fruits is critically important for the production of high-quality, pleasant-tasting crops, and reflects their metabolic capacity to manufacture and store high levels of useful biochemical compounds. The occurrence and proportion of such compounds are dependent on species or cultivar, developmental stage and exposure of the plant to various environmental situations such as changes in climate. To account for this, species selection and even fruit development can be controlled to a degree, through common horticultural practices. However the spontaneity of environmental events is still a large source of variability in the determination of crop yield and quality. It is well known that fruit species that are suited to some regions may not be as well suited to others. Within the common grape species (*Vitis vinifera*), even specific cultivars can be more adapted to specific regions, particularly in the case of grapes used in winemaking. In this notion, the effects of location and climate can actually bring exciting variation to a fruit or wine product, and can enable marketing of particular characteristics for an element of exclusivity or prestige. However, it also demonstrates the sensitivity of fruit biochemistry to changes in environmental circumstances. Therefore changes in climate will affect the compatibility of existing fruit cultivars with their current regions. Temperature can have a dramatic impact on numerous aspects of fruit quality; particularly acidity. Typically, elevated seasonal temperatures can result in low-acid fruit. For winegrapes, regions that are subject to hot summers result in acid loss from the fruit through malate degradation, which can be detrimental to fruit flavour and is costly for winemakers. Despite the importance of acidity in aspects of fruit quality, little is known about the regulation of fruit acid metabolism in response to elevated temperature. In an idyllic vision, the development of new cultivars or exploitation of existing cultivars that are more resilient to environmental change would reduce the impact on fruit industries.

In an attempt to pinpoint the effects of elevated temperature on fruit malate metabolism, this study employed three systems to artificially raise Shiraz grapevine temperatures in the field. Vines were subject to treatments at various developmental stages, with simulations of moderate-length heat-waves as well as longer-term temperature shifts. The effects of separately controlled day and night heating were also investigated. Berries were analysed for malate content and the activities and transcript levels of enzymes potentially involved in

malate metabolism. An analysis of the data demonstrate that the effects of temperature on berry fresh weight and malate content are dependent on developmental and diurnal phases, and are likely a result of changes in photosynthetic and respiratory pathways. Data from heat treatments with young fruit suggest diurnal differences in grape berry malate metabolism that may parallel a CAM-type photosynthesis cycle, where heating at night led to increased malate and heating during the day led to decreased malate in the fruit. Veraison berries exposed to elevated day temperature had lower malate levels, while elevation of both day and night temperatures had no effect on malate level, but did cause accelerated berry development. Treatments with late-ripening fruit did not affect any changes in malate level. Some of the effects of veraison heating may be explained by changes in the malate-synthetic activity of phosphoenolpyruvate carboxylase and the malate-degradative activity of NAD-dependent malic enzyme. Further alterations in pyruvic acid metabolism through changes in activities of the pyruvate kinase, as well as the transcript of pyruvate,orthophosphate dikinase, may also play a role in temperature-sensitive changes in malate metabolism during fruit development and ripening.

4.2. Introduction

Fruits are specialised sink tissues that can accumulate numerous compounds such as vitamins and antioxidants (Melino et al., 2009), pigments (Jackman et al., 1987), and unique aroma and flavour compounds (Song and Bangerth, 1996; Dunlevy et al., 2010). The level of accumulation and degradation of such compounds within the fruit tissue can be dependent on a very large number of biological variables, from the choice of plant cultivar, location and cultivation methods including fertilisation, irrigation, pruning and time of harvest, to environmental conditions such as seasonal temperature, rainfall, humidity, sun exposure and air movement (Winkler *et al.*, 1974). For fruits such as grape, temperature is a powerful environmental determinant of quality. Studies based in Europe have linked increases in temperature over multiple decades to earlier phenological events in grape berry development, and reduced wine production (Duchene and Schneider, 2005; Ramos et al., 2008). In Australia, maturity of grapevines has advanced in parallel with increasing temperature, causing earlier flowering, fruiting and harvest dates (Webb et al., 2007; Petrie

and Sadras, 2008; Sadras and Petrie, 2011b; a; Webb et al., 2011). Such trends have the potential to greatly affect fruit characteristics. One of the clearest relationships between temperature and fruit quality occurs with grape berry acidity (Winkler *et al.*, 1974), whereby a vineyard exposed to higher temperatures (heat summation days) will turn out grapes with reduced acid. As fruit organic acids strongly shape flavour, changes in seasonal temperature will leave lasting effects on the final product; dull, flat-tasting fruit and wines. However, a direct link between elevated vineyard temperature and changes in grape berry organic acids is yet to be comprehensively described at biochemical and molecular levels.

Developing grapes display distinct patterns of organic acid accumulation and degradation. Malate and tartrate are predominant at all stages of development, and at harvest the levels of these acids, as well as cationic groups such as potassium, dictate the acidity and pH of the must to be used for winemaking (Morris *et al.*, 1983). Tartrate is relatively inert and sequestered as calcium tartrate or potassium bitartrate in the plant cell vacuole. It is generally the most abundant organic acid of a grape juice, as after tartrate accumulates, it is usually retained (Amerine, 1956). Due to its chemical stability, additions of tartrate are common practice in wineries for the adjustment of must pH. Malate can also be sequestered in the vacuoles of the fruit cells, but upon release, is susceptible to chemical conversion through a suite of enzyme activities. It accumulates in young berries and is largely lost through metabolism during ripening, thus reducing titratable acidity (Kliewer, 1965) and enhancing the perceptibility of sweetness in the fruit. It is also a loss of malate that causes reduced acidity in grapes grown at warmer temperatures. The strongest relationship between grape berry malate and developmental changes in metabolism occurs with a “switch” from the use of sugars as a source of carbon for respiration in pre-veraison fruit, to an inhibition of glycolysis that lends malate as the primary source of carbon for respiration in post-veraison fruit (Ruffner, 1982). This could affect regulation of the TCA cycle enzymes, as malate can feed directly into this mitochondrial pathway (Fernie et al., 2004). As such, this could also have downstream effects on the mitochondrial electron transport chain, as well as amino acid biosynthesis and other pathways that utilise products from the TCA cycle. There are also dramatic changes in photosynthetic capability and sink strength of aging grape berries (Ollat and Gaudillere, 2000). Developmental differences in enzyme activities from numerous pathways involving malate (Hawker, 1969; Sweetman et

al., 2009) may contribute to some of the different responses of malate metabolism seen with temperature treatments conducted at different developmental stages and at different times of the day. It has been suggested that a loss of malate from grape berries caused by elevated temperature could be attributed to an increase in the rate of degradation during ripening rather than a decrease in the rate of synthesis during early development (Kliewer, 1964; 1973). Synthesis of malate is thought to largely occur from sugars in the berry (Hale, 1962) through the activities of PEP carboxylase (PEPC) and malate dehydrogenase (MDH), which may divert phosphoenolpyruvate (PEP) from glycolysis and hence bypass the formation of pyruvate through pyruvate kinase (PK) (See Sweetman *et al*., 2009 for a review of this topic). Malate catabolism could occur through a large number of pathways. Enzymes directly involved in malate metabolism include the MDH, which catalyses the conversion between malate and oxaloacetate (OAA), and the malic enzyme (ME), which catalyses the conversion between malate and pyruvate; both of which exist as several isoforms in the grape (Taureilles-Saurel *et al.*, 1995; Famiani *et al.*, 2000). However, other activities can indirectly affect malate metabolism. For example, enzymes that use or produce OAA, such as PEP carboxylase (PEPC) and PEP carboxykinase (PEPCK) could alter the reaction equilibria of the MDHs. Similarly, enzymes that use or produce pyruvate, such as pyruvate kinase (PK), pyruvate, orthophosphate dikinase (PPDK) and alcohol dehydrogenase (ADH; after decarboxylation by pyruvate decarboxylase) could alter the reaction equilibria of the MEs. Some of these enzymes have received attention in grapes and other fruits (Hawker, 1969; Tesniere and Verries, 2000; Famiani et al., 2005; Lara et al., 2009), but their roles in temperature-sensitive malate regulation in field-grown grapes are still unclear. The aim of this study was to investigate the effect of elevated temperature on malate content in berries of Shiraz vines, and to pin-point some potential pathways through which the acid could be regulated under such circumstances, through the evaluation of the activities and expression of enzymes mentioned above. Field-grown vines were exposed to temperature elevation strategies at various developmental stages. Fruit were analysed for changes in growth parameters and malate content, as well as activities and transcripts of the enzymes mentioned above.

4.3. Materials and methods

4.3.1. Experimental Design

Trials were conducted using North-South facing, own-rooted Shiraz vines (established in 2004, with 3m row spacing and 2.25m vine spacing), at the Nuriootpa Research Station in the Barossa Valley, South Australia (274m elevation, -34.468˚ latitude, 138.998˚ longitude). Vines were spur-pruned to 40-50 nodes per vine, and drip irrigated weekly from mid-December.

Three experimental setups were used for the elevation of ambient temperature in the vineyard. These were (i) open-top chambers (Sadras and Soar, 2009), (ii) closed chambers (Soar et al., 2009) and (iii) blowers (an adaptation of Tarara et al. 2000). Treatments were applied to vines at three stages of berry development (T1:post-set, T2:veraison, T3:pre-harvest) during 08-09 season (Figure 1). Open-top chambers (OTC) were used to elevate daytime bunch temperature to 2.3-3.2˚C above ambient for 3 weeks across 7 vines, thus emulating a small increase in temperature over an extended period of time. Treatments involving simultaneous use of closed chambers and blowers (CH/BL) were conducted over 10 days, with a temperature increase of approximately 6˚C between heated and control treatments during the day and night. This set-up allowed for a two-by-two factorial design, with day temperature (regulated at the whole-vine level by closed chambers) and night temperature (regulated at individual bunch level by blowers). At each time-point two chambers (Soar *et al.*, 2009) were assembled. One was designated as a control, and the other was heated during the day. Within each of the two chambers, four replicate sets of control and heated blowers were aimed at specific bunches that had been tagged at 0 D.A.F (0 days after flowering), and were operated at night. Control samples were collected from within the controlled temperature chamber/blower setups, as well as “external controls” from nearby, un-treated vines. Samples of 10 berries were taken from each bunch due to the limited amount of available material.

CH/BL treatments were assembled early in the morning and left for 10 days, before deconstruction on the morning of the 11th day. Day and night bunch heating was controlled separately by the chambers and blowers respectively, enabling a test of the effects of day heating only, night heating only, and a combination of day and night heating. Berry samples

were collected immediately after disassembly of the chambers, as well as at veraison (66 D.A.F.) and harvest ripeness (110 D.A.F.). The post-set (T1: PS) stage was conducted early in berry development (13 – 23 D.A.F.), at a stage when the fruit were rapidly accumulating organic acids. The veraison (T2: V) treatment was set up prior to the initiation of veraison (55 D.A.F.), at a time when grape berries contained peak quantities of malate and the highest titratable acidity, shortly preceding the dramatic loss of malate seen throughout ripening. Upon removal of the chambers, the majority of berries had undergone colour change. The pre-harvest (T3: PH) treatment was initiated approximately two weeks after the veraison treatment ended (83-93 D.A.F.), once a significant portion of malate had been lost from the fruit. OTC treatments were also established at these developmental stages, but remained in place for 3 weeks. Each treatment was applied to previously untreated Shiraz vines within the vineyard, such that the effect of each treatment could be determined immediately after treatment and at harvest. This also meant that later treatments were not confounded by effects of the previous treatments.

Fruit (80-100 berries) were collected on a weekly basis from random bunches within the treatments and from nearby, untreated vines. Bunches were tagged at 50% cap-fall for all CH/BL trials. Samples were taken weekly between 6.30-9:30am throughout the season. Berries were removed by cutting through the petiole at the junction between stem and berry, using sewing scissors, then taken to the Nuriootpa Research Centre for freezing in liquid nitrogen. Time between harvest and freezing was approximately 10 mins, and fruit were stored at -80˚C.

4.3.2. Malate Quantification

Organic acids were extracted from 500 mg frozen berry powder, using the method of Melino *et al*. (2009a) in 5 ml of 3% (w/v) metaphosphoric acid (MPA; with 1 mM EDTA) with shaking for 60 mins at 4˚C. The homogenate was centrifuged at 2,500 x *g* for 5 mins and the supernatant was passed through a Millipex 0.45 µm hydrophilic PVDF syringe-driven filter (Millipore, NSW, Australia). The filtrate was diluted in 0.1M MOPS (pH 8.0) and used for enzyme-linked spectrophotometric assays, as modified from (Mollering, 1974). This method measured NADH formation (340 nm) in 200 µl of 0.1 M MOPS (pH 8.0) containing 10 mM NAD^+, 50 mM glutamate, alanine aminotransferase (3 U) and malate dehydrogenase (2 U),

before and after a 30 minute incubation with extract (20 µl). Malate was quantified according to standards of known concentrations dissolved in MPA and diluted 1/10 with 0.1 M MOPS (pH 8.0).

4.3.3. Enzyme Extraction

Active enzymes were extracted using a method adapted from Ruffner & Kliewer (1975). Twelve volumes of extraction buffer (0.5 M Tris-Cl, pH 8.5 with 200 M KCl, 20 mM $MgCl_2$, 10 mM EDTA, 8% (w/v) PEG-4000, 8 mM cysteine-HCl, 7 mM diethyldithiocarbamate, 5 mM DTT, 2% (w/v) PVPP, 0.25% (w/v) BSA, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine) were added to 1 g of frozen grape berry powder and mixed gently at 4˚C for 15 mins. After centrifugation at 2,750 x *g* for 5 mins to remove cell debris, PEG-4000 was added to a final concentration of 65% (w/v) in the supernatant, then mixed by gentle shaking at room temperature until dissolved. After centrifugation at 30,000 x *g*, the precipitated protein was carefully resuspended in 2mL final volume (5 mM Tris-Cl, pH 7.0, with 20 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, 3% (v/v) Triton X-100, 0.5 mM PMSF and 0.5 mM p-aminobenzamidine), re-centrifuged at 3,000 *x g* and the supernatant used in enzyme activity assays as described below.

4.3.4. Enzyme Activity Assays

All assays were carried out at 25˚C using a FLUOstar UV/vis plate reader (BMG Labtech, Victoria, Australia), in a final volume of 200 µl. The rate of change in absorbance at 340nm was used to measure the reduction of 2 mM $NAD(P)^+$ or oxidation of 0.5 mM NAD(P)H, as outlined below. In each case the assay was initiated by the addition of the reagent listed last.

NAD-malate dehydrogenase activity was quantified as the rate of NADH oxidation at pH 6.0 (50 mM MES), in the presence of 5 mM oxaloacetate, as described previously (Ruffner *et al.*, 1976).

NADP-malate dehydrogenase activity was quantified as the rate of NADPH oxidation at pH 8.0 (50 mM TES), in the presence of 5 mM DTT and 5mM oxaloacetate, a modification from Jacquot *et al*. (1981).

NAD-malic enzyme activity was quantified as the rate of NAD reduction at pH 7.4 (50 mM TES) in the presence of 5 mM $MnCl_2$, 5 mM DTT, 2.5 mM potassium cyanide, 0.3 µM octyl gallate (OG), 5 mM malate and 75 µM Coenzyme A (CoA), a modification from Hatch *et al*. (1982). A temporary rate in the absence of CoA was ascribed to NAD-MDH activity.

NADP-malic enzyme activity was quantified as the rate of NADP reduction at pH 6.0 (50 mM MES) in the presence of 8 mM $MnCl_2$, 2.5 mM potassium cyanide, 0.3 µM octyl gallate and 5 mM malate, a modification from Ruffner *et al*. (1976).

PEP carboxylase activity was quantified as the rate of NADH oxidation at pH 8.0 (50 mM TES), in the presence of 10 mM $MgCl_2$, 5 mM DTT, 5 mM $KHCO_3$, 6 U malate dehydrogenase (Sigma) and 2.5 mM PEP, a modification from Ruffner et al. (1976).

PEP carboxykinase activity was quantified as the rate of NADH oxidation at pH 6.7 (50 mM MES), in the presence of 0.1 M KCl, 6 mM $MnCl_2$, 25 mM DTT, 90 mM $KHCO_3$, 6 U malate dehydrogenase (Sigma), 6 mM PEP and 1 mM ADP, as described previously (Walker *et al.*, 1999).

Alcohol dehydrogenase activity was quantified as the rate of NADH oxidation at pH 6.0 (50 mM MES) in the presence of 50 µM acetaldehyde, as described previously (Molina *et al.*, 1987).

Pyruvate kinase activity was quantified as the rate of NADH oxidation at pH 7.2 (50 mM TES) in the presence of 70 mM KCl, 30 mM $MgCl_2$, 6 U lactate dehydrogenase, 5 mM PEP and 8 mM ADP, as described previously (Turner and Plaxton, 2000).

4.3.5. Quantitative Real-time Reverse Transcriptional PCR

Grape berry RNA was extracted using a method modified from Davies and Robinson (1996). The resultant RNA was purified using an RNeasy Mini Kit (Qiagen, Australia), as described by Melino *et al*. (2009b). Genomic DNA contamination was removed by an on-column DNase step, using RNase-free DNase I (Qiagen, Australia). Grape RNA quality was assessed with agarose gel electrophoresis, and quantified with a Nanodrop spectrometer (Thermo Scientific, Biolab, Australia). First-strand cDNA synthesis from grape berry RNA was achieved with oligo$(dT)_{20}$ primer and Superscript III reverse transcriptase (Invitrogen), according to

the manufacturer's instructions. The resultant cDNA was diluted in DNase free water and 50 ng used for each qRT-PCR assay.

Reactions were set up in Faststart Universal Probe Master (Rox) master mix (Roche, Australia) with gene-specific primers and Universal ProbeLibrary probes (Roche, Australia) in a final volume of 16 µl (For details of gene accessions and probe sets, see Table 4.1). Thermal cycling conditions for all qRT-PCR involved an initial 95˚C melt step (10 mins), followed by 45 cycles of; 95˚C (15 secs), 57˚C (1 min). Assays were conducted with a C1000 Thermal Cycler fitted with a CFX96 Real-time PCR detection system (BioRad), and analysed using the CFX Manager software (BioRad). All transcripts were normalized to a reference number derived from transcript levels of the two housekeeping genes, ubiquitin and ankyrin, for each cDNA sample.

4.3.6. Statistical analyses

Data were analysed using GraphPad Prism version 5.04 for Windows (California, USA), through 2-way ANOVA for CH/BL treatments and 1-way ANOVA and two-tailed t-tests for the OTC treatments. Findings were considered significant when $p \leq 0.05$.

4.4. Results

4.4.1. Berry weight and malate content

Fruit exposed to warmer nights during early development was heavier than fruit grown at control night temperatures (Figure 4.1). At veraison the CH/BLs had a negative effect on berry weight, relative to the external control. Heating during the day amplified this effect unless the fruit were also heated at night. Treatment later during ripening had no effect on berry fresh weight.

Malate content (mg/berry) of Shiraz berries from the Nuriootpa Research Vineyard displayed the usual pattern of pre-veraison accumulation and post-veraison degradation. However, the fruit subjected to the CH/BL control treatment had altered berry malate regardless of treatment temperature. Pre-veraison vines exposed to elevated day

Table 4.1: List of accession numbers, primer sets and probes used for qRT-PCR. Probe numbers are from the Human Universal Probe Library (Roche, Australia).

Gene	Accession #	Protein	Primer Sequences (5'-3')	Probe
ADH1	AF194173 (CDS)	AAG01381	F: tttagtgaatacaccgtcgttca R: ggttttgccacattgagagtg	76
ADH2	AF194174 (CDS)	AAG01382	F: tgtgttcttagctgtggaatctct R: cgattgatgaacccttcgat	145
ADH3	AF194175 (CDS)	AAG01383	F: aaccccaaagatcatgacaaa R: gcaacgcttccagtacactct	25
ADH4	XM_002263489.1	XP_002263525.1	F: tctggaagaacgttgagagga R: tgaattcgtcaatctggatttc	88
PEPC1	AF236126 (mRNA)	AAL83719	F: cgccagttattatcaagtggaa R: caagcacagagtgatctatagcaga	34
PEPC2	XM_002280806	CAN62388	F: atgcccgacttgctgttagt R: aatagaggaaccacccgtagc	113
PEPC3	XM_002285406	CBI20195	F: aatagaggaaccacccgtagc R: caagaaggtccttgtgtccag	25
PEPCkin1	XM_002285126.1	CAN72597.1	F: aacggcagcaaaggatctc R: ttgtctcaccaacgcttgtc	88
PEPCkin2	XM_002265916.1	CAN74792.1	F: ggatgtttcaaggcgattgt R: ttaaccgtttgagtttgagttcac	91
PEPCK	XM_002277794.1	CBI35761	F: cattgatctctcaagggagaaag R: tcgaacaccacattttccaac	147
NAD-ME	XM_002266661	XP_002266697	F: gactgactttgtcaaccaaaagatag R: gccatattaagcacaccaatcc	4
PPDK	CA808125 (EST)	CBI26150.3	F: tgggaaccatgattgagattc R: gaagaattctgcctccttcg	164
AOX1a	EU165202	ACI28876.1	F: aatggaaatggagctgtttca R: aagctactttctcagtccataccc	60
AOX1b	EU165203	ACI28877.1	F: tcttcaacgcatacttcctaacat R: tccttgaggaattcggtgtgag	25
AOX2	EU523224	ACB45425.1	F: ggaattcagcggcctaaga R: ttgaagtgtctgcatgat	126
COX3	XR_078009	YP_002608380.1	F: agggggtgcaacacttctc R: gccaccatacgaacatggta	105
Ankyrin	XM_002283462	XP_002283498	F: ggttatggcaggaaggagtg R: ggtgtcttgccatccatgtt	120
Ubiquitin	BN000705 (mRNA)	CAI56329	F: gtggccacagcaaccagt R: gcaacctccaatccagtcat	143

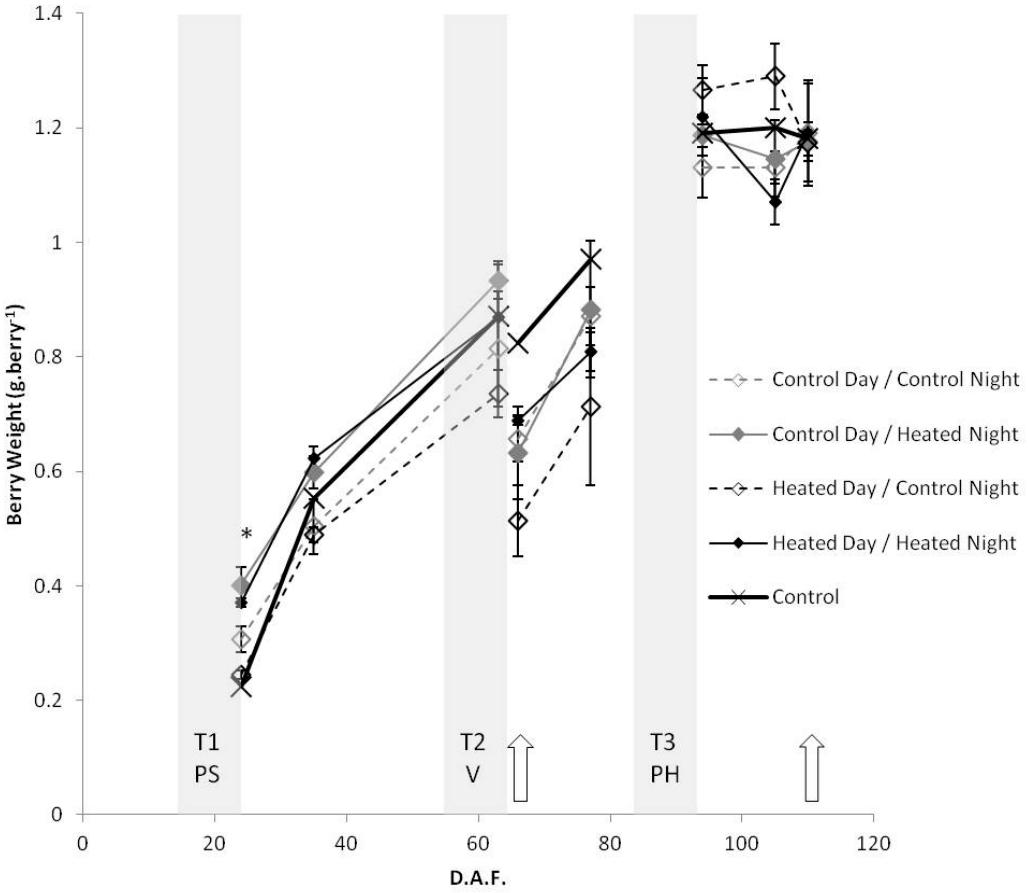


Figure 4.1: Developmental changes in berry weight in response to Chamber/Blower treatments. Shaded boxes indicate timing of treatment events. White arrows indicate véraison and harvest dates. Treatment 1: Post-set, Treatment 2: Véraison, Treatment 3: Pre-harvest. Data are presented as means (± SEM) of four bunch replicates (i.e. four separate bunches from four separate blowers, within one chamber). Data were subject to two-way ANOVA at each developmental stage, for comparison of day treatments and night treatments simultaneous with a comparison of control and heated temperatures, and for interaction between these two factors. * significant differences ($p \leq 0.05$).

temperature had fruit with less malate, and vines exposed to elevated night temperature had fruit with more malate, immediately after the treatment (Figure 4.2a). At veraison, increased day temperature also resulted in lower malate content, although increased night temperature appeared to remove this effect (Figure 4.2b). However, these differences were not statistically significant. Late in ripening the chamber/blower treatment had no discernible effect on grape berry malate content (Figure 4.2c), whereas some of the post-set and veraison CH/BL effects were still evident at harvest (Figure 4.2d-f). In particular, fruit exposed to elevated day temperature contained approximately half of the malate seen in control fruit.

Berries from OTC heat treatments showed somewhat similar results, which were still evident at harvest (Figure 4.2g). While the malate content of berries from the pre-veraison treatment (2.2 mg/berry) was similar to the control (2.5 mg/berry), treatments conducted at veraison and pre-harvest resulted in significantly lower levels of malate at harvest (1.8 and 1.7 mg/berry, respectively).

4.4.2. Enzyme activities and gene transcripts

Activities of enzymes potentially involved in the regulation of malate metabolism in response to elevated temperature were measured in samples exposed to veraison and post-veraison OTC treatments, and veraison CH/BL treatments. Furthermore, putative gene transcripts of some of these enzymes were assessed in post-set and veraison CH/BL samples.

4.4.2.1. NAD(P)-dependent malate dehydrogenases and malic enzymes

Figure 4.3(a-h) represents activities of the malate dehydrogenases and malic enzymes. These activities are dependent on NAD and/or NADP (or their reduced derivatives). In all cases, NAD-MDH activity was considerably higher than other activities measured in this study. Veraison NAD(P)-MDH and NADP-ME activities showed little response to heat treatments by OTCs and CH/BLs, although there was a slight decrease in post-veraison NAD-MDH in fruit exposed to OTC heating. NAD-ME activity increased in response to OTC and CH/BL heating, although for the latter this effect was only seen when both day and night temperatures were raised.

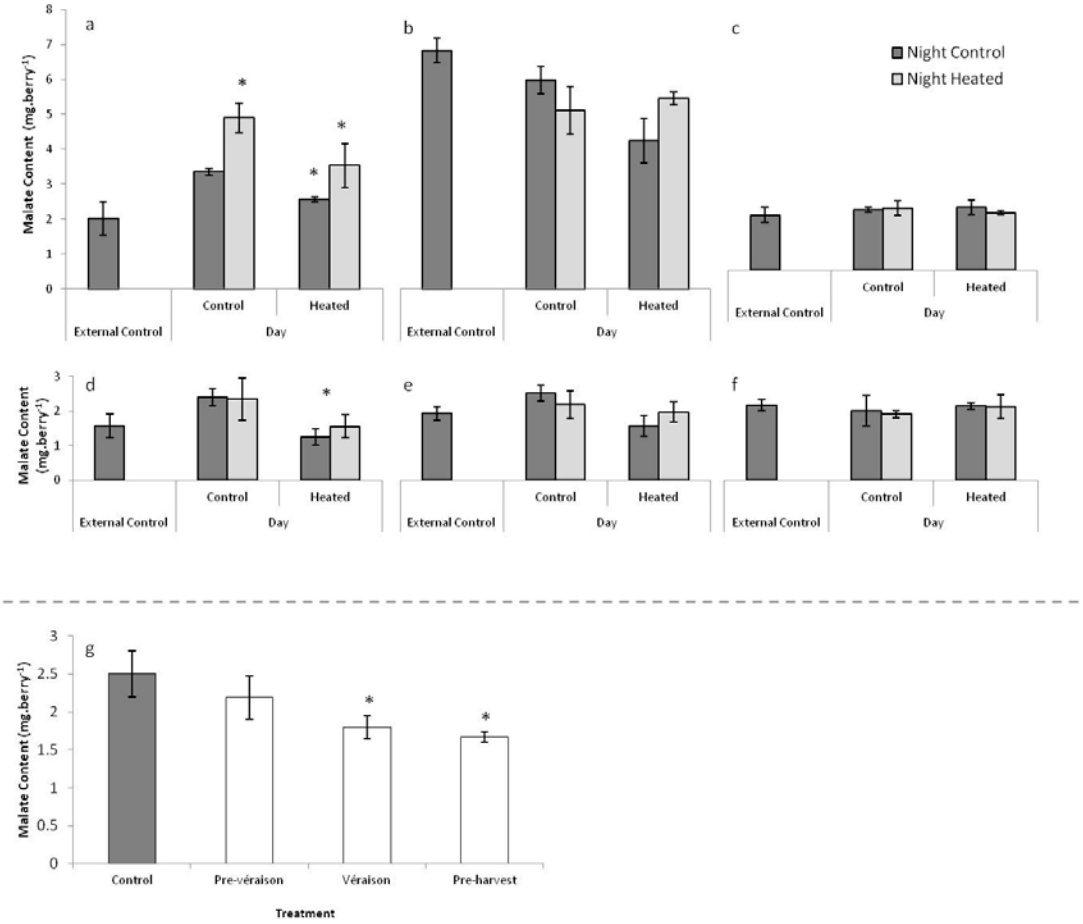


Figure 4.2: Effect of elevated temperature treatments on amount of malate per berry.

Upper panel: Malate content of fruit from CH/BL treatments conducted at post-set (a and d), véraison (b and e) and pre-harvest (c and f) developmental stages, for berries sampled immediately after treatment (top) and at harvest (bottom). Lower panel: Effect of OTC treatments on malate at harvest. CH-BLs were used to alter day and night temperature. OTCs altered day temperature only. Data from the CH-BL experiment are presented as means (± SEM) of four bunch replicates (i.e. four separate bunches from separate blowers, within one chamber), and subjected to two-way ANOVA for comparison of day treatments and night treatments simultaneous with a comparison of control and heated temperatures. Data from the OTC experiment are presented as means (± SEM) of three vine replicates, and treatments were compared using non-parametric t-tests. * significant differences ($p \leq 0.05$).

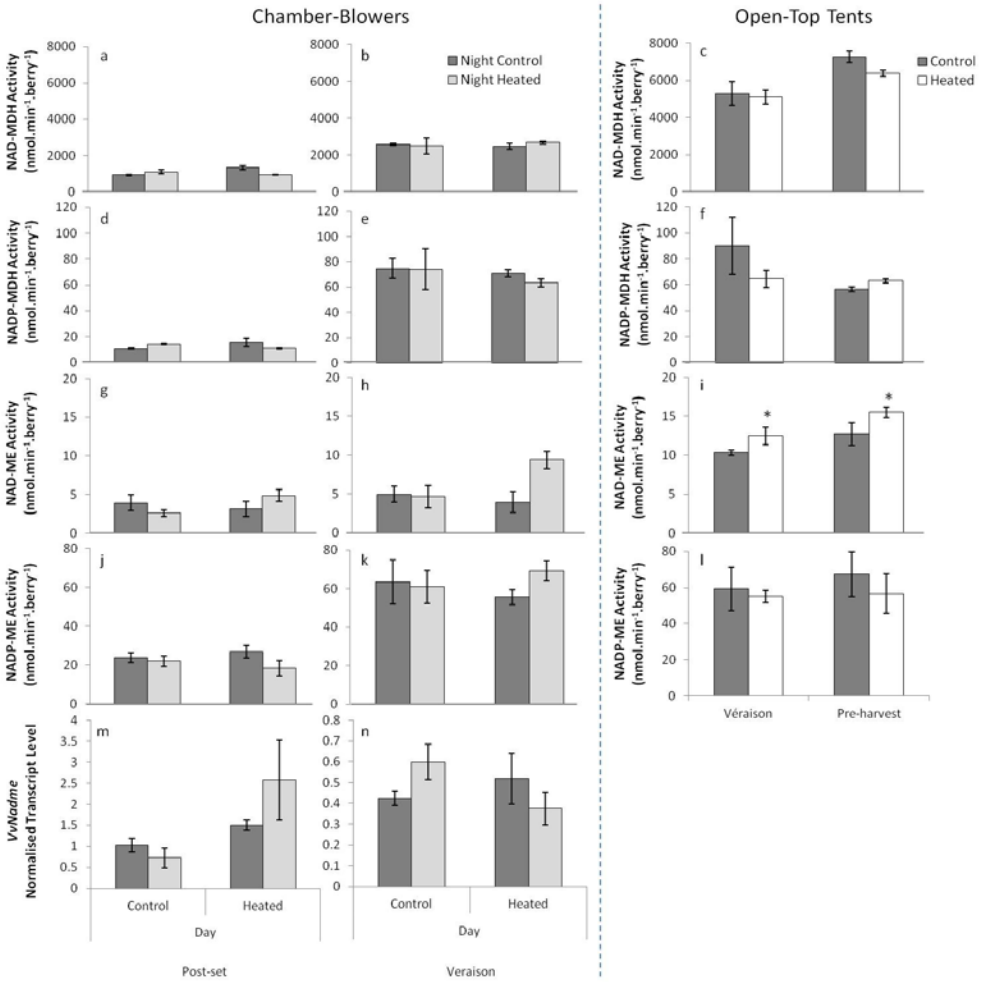


Figure 4.3: Effect of elevated temperature treatments on activities of enzymes directly involved in malate metabolism. Activities of NAD-MDH (a, b, c), NADP-MDH (d, e, f), NAD-ME (g, h, i), NADP-ME (j, k, l), and transcript level of *VvNadme* (m, n) at different developmental stages, including post-set CH-BLs (a, d, g, j, m), véraison CH-BLs (b, e, h, k, n), veraison OTCs (c, f, j, l), and pre-harvest OTCs (c, f, j, l). Data are presented as means (± SEM) of four bunch replicates for CH-BL treatments, and three vine replicates for OTCs. CH-BL data were analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with a comparison of day and night treatments, while OTC data were analysed with two-way ANOVA for comparison of control and heated temperatures at two developmental stages. * significant differences ($p \leq 0.05$).

VvNad-me transcript levels were slightly lower at veraison than post-set (Figure 4.3i-j). At the post-set stage, CH/BL berries heated during the day had more *VvNad-me* transcript, particularly if also heated at night. At veraison, separate day and night heating led to elevated *VvNad-me*, but continuous heating had no effect. This was not consistent with CH/BL activity data, whereby continuous heating during both day and night caused an increase in active NAD-ME.

4.4.2.2. Phosphoenolpyruvate carboxylase

PEPC activity decreased significantly in response to increased daytime temperature in CH/BL and OTC treatments conducted over veraison and ripening (Figure 4.4), although this effect was not evident when night temperature was also increased in the CH/BL experiments (Figure 4.4l). This pattern reflected malate content in the fruit (Figure 4.2), and linear regression showed a positive correlation between berry malate content and PEPC activity (R^2 = 0.78, Figure A.4.6). Therefore a decrease in PEPC activity may be responsible, at least in part, for the decrease in malate seen with increased day temperature and the recovery of malate in response to increased night temperature. Of the three putative Vitis PEPC gene transcripts measured here, *VvPepc3* showed the most similar pattern to that of PEPC activity and berry malate content from the veraison CH-BL experiment, although the changes in this transcript were not significant. At the post-set treatment this transcript showed no relationship to the berry malate content, thereby suggesting a level of developmental control that may override the temperature response.

The heat-response of *VvPepc1* and *VvPepc2* transcripts in CH/BL experiments was also dependent on developmental stage. In post-set fruit, both of these transcripts tended to decrease when berries were heated at night (Figure 4.4a,c), whereas at veraison elevated night temperature had more of a stimulatory effect (Figure 4.4b,d). *VvPepc2* and *VvPepc3* transcripts were approximately 5-10 times higher than *VvPepc1* at both stages of development.

PEPC kinase is a regulatory enzyme that activates PEPC through phosphorylation, thereby potentially increasing the rate of conversion from PEP to OAA. Two putative transcripts for PEPC kinase were quantified in this study. Relative to post-set levels, *VvPepckin1* transcript decreased slightly at veraison (Figure 4.4g,h), consistent with previously explored

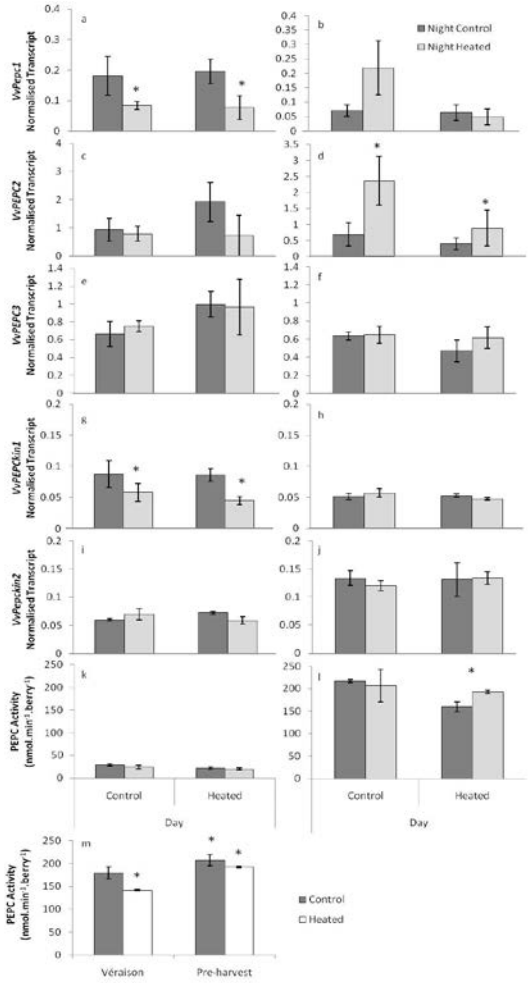


Figure 4.4: Effect of elevated temperature treatments on PEPC transcripts and activity. Transcript abundance of *VvPepc1* (a, b), *VvPepc2* (c, d), *VvPepc3* (e, f), VvPepckin1 (g, h) and VvPepckin2 (i, j), and enzyme activity (k, l) of PEPC in response to CH/BL heat treatments in fruit at post-set (a, c, e, g, i, k) and véraison (b, d, f, h, j, l) developmental stages, as well as OTC treatments at veraison and pre-harvest (m). Data are presented as means (± SEM) of four bunch replicates for CH-BL treatments (a-l), and three vine replicates for OTCs (m). CH/BL data were analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with a comparison of day and night treatments, while OTC data were analysed with two-way ANOVA for comparison of control and heated temperatures at the two developmental stages. * significant differences ($p \leq 0.05$).

microarray data (Sweetman *et al.*, 2009), while *VvPepckin2* transcript was higher at veraison (Figure 4.4i,j). In post-set fruit, increased night temperature led to decreased *VvPepckin1* (Figure 4.4g); similar to *VvPepc1* (Figure 4.4a).

4.4.2.3. Phosphoenolpyruvate carboxykinase

Berries from vines exposed to increased day temperature at the post-set developmental stage showed an increase in *VvPepck* transcript abundance, particularly when night temperature was also elevated (Figure 4.5a). These fruit also displayed higher malate content when heated at night (Figure 4.2a), although PEPCK activity (Figure 4.5c) did not reflect the changes in transcript. At veraison, elevated day temperature significantly reduced PEPCK transcript and activity in CH/BL treatments (Figure4. 5b,d). Elevated night temperature at veraison resulted in a significant increase in *VvPepck* transcript, although this had no effect on the activity in these fruit. OTC treatments did not alter PEPCK activity (data not shown).

4.4.2.4. Pyruvate kinase

While temperature had no consistent effect on PK activity in post-set fruit (Figure 4.6a), at veraison elevated day temperature led to a significant decrease in the activity of PK (Figure 4.6b) suggesting a decrease in the conversion of PEP to pyruvate.

4.4.2.5. Pyruvate, orthophosphate dikinase

In post-set fruit, heated days led to increased *VvPpdk* transcript (Figure 4.6c), while warmer nights had the opposite effect. Alternatively at veraison, levels of *VvPpdk* were unaffected by elevated day temperature and warmer nights showed consistent and significant induction of this transcript (Figure 4.6d).

4.4.2.6. Alcohol dehydrogenase

VvAdh1 was expressed at a lower level than all other ADH transcripts, though stimulated slightly by elevated day temperature (Figure 4.7a,b). Warmer days led to significantly lower *VvAdh2* transcript levels, while elevated night temperature resulted in increased *VvAdh2* (Figure 4.7c,d), particularly at veraison; similar to the pattern of ADH activity (Figure 7j).

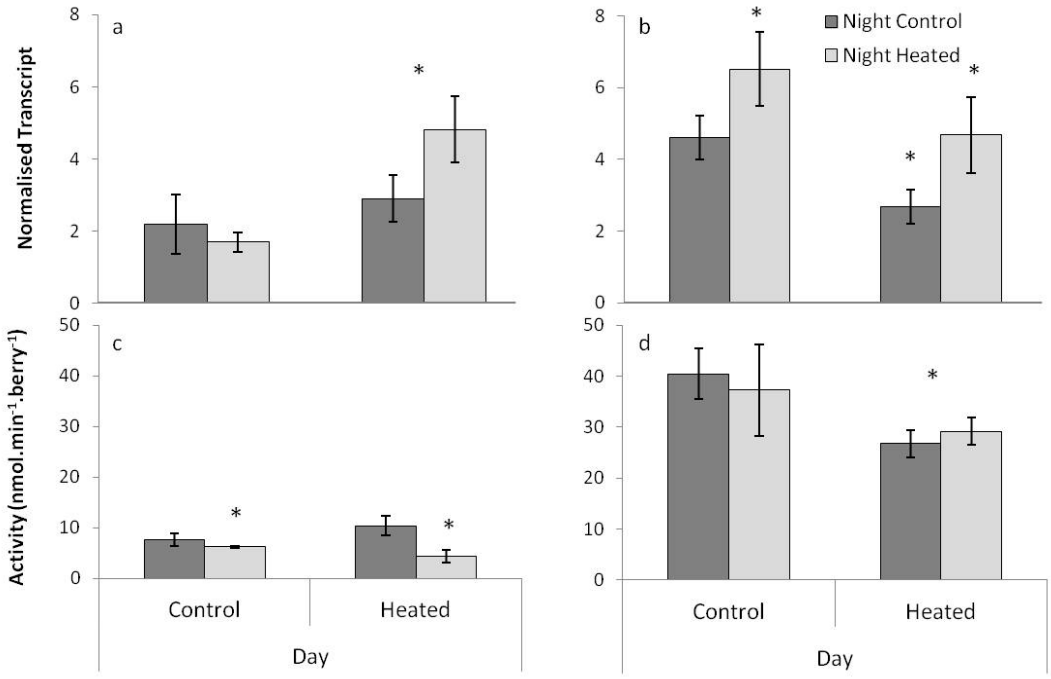


Figure 4.5: Effect of elevated temperature treatments on PEPCK transcript and activity. *VvPepck* transcript abundance (a, b) and PEPCK enzyme activity (c, d) for CH/BL treated fruit at post-set (a, c) and véraison (b, d). Data are presented as means (± SEM) of four bunch replicates and analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with a comparison of day and night treatments. * significant differences ($p \leq 0.05$).

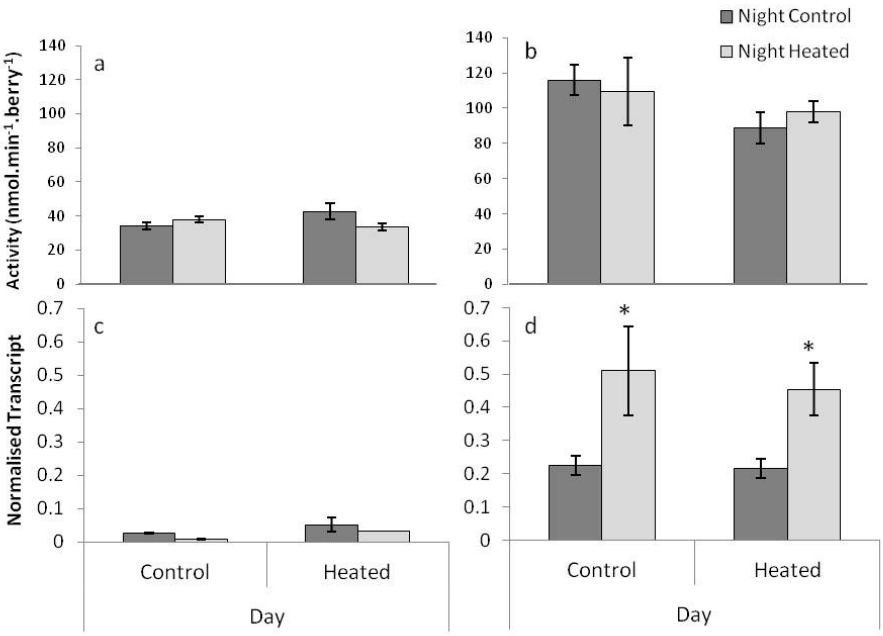


Figure 4.6: Effect of elevated temperature treatments on aspects of pyruvate metabolism. Fruit from CH/BL treatments measured for PK enzyme activity (a, b) and *VvPpdk* transcript abundance (c, d) at post-set (a, c) and véraison (b, d) developmental stages. Data are presented as means (± SEM) of four bunch replicates and analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with a comparison of day and night treatments. * significant differences ($p \leq 0.05$).

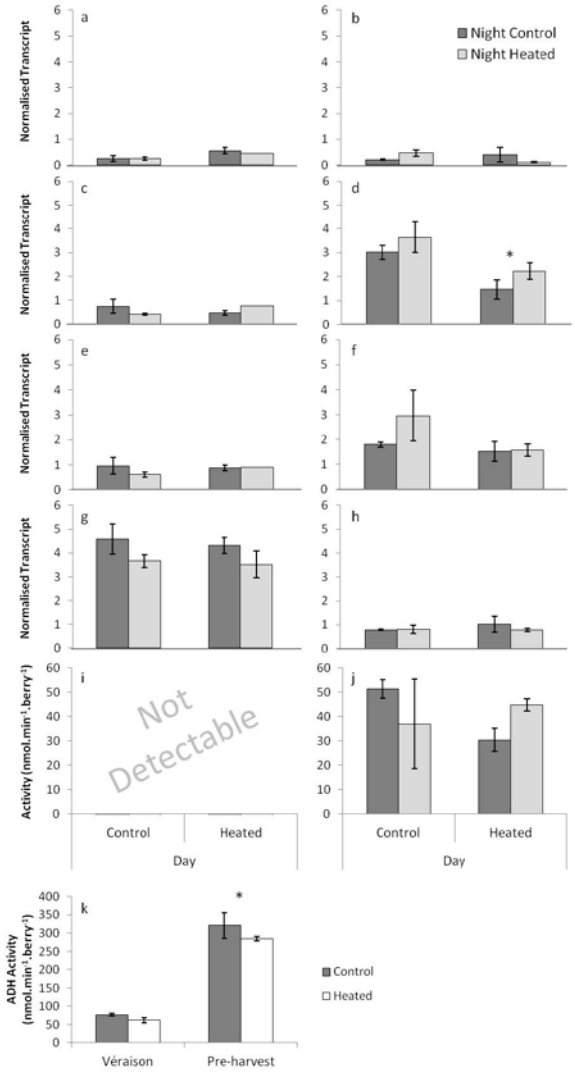


Figure 4.7: Effect of elevated temperature treatments on ADH transcripts and activity. Transcript abundance of *VvAdh1* (a, b), *VvAdh2* (c, d), *VvAdh3* (e, f) and *VvAdh4* (g, h), and enzyme activity of ADH (i, j) at different developmental stages; post-set CH-BLs (a, c, e, g, i), véraison CH-BLs (b, d, f, h, j) and veraison OTCs (k), and pre-harvest OTCs (k). Data are presented as means (± SEM) of four bunch replicates for CH-BL treatments (a-j), and three vine replicates for OTCs (k). CH-BL data were analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with day and night treatments, while OTC data were analysed with two-way ANOVA for comparison of control and heated temperatures at the two developmental stages. * significant differences ($p \leq 0.05$).

VvAdh2 was also higher at veraison than post-set, therefore displaying the expected developmental stimulation (Tesniere and Verries, 2000). *VvAdh3* was developmentally induced but not significantly affected by temperature treatments (Figure 4.7e,f), while *VvAdh4* was negatively regulated with development and also when night temperatures were raised in post-set fruit (Figure 4.7g,h). ADH activity decreased in response to increased daytime temperature in both CH/BL and OTC treatments, unless night temperature was also increased (Figure 4.7j,k).

4.4.2.7. Terminal oxidases of mitochondrial electron transport chain

VvAox1a, *VvAox1b* and *VvAox2* transcripts were all induced by high day temperature in post-set fruit (Figure 4.8a,c,e). The veraison pattern of *VvAox2*, which decreased with elevated day temperature unless night temperature was also increased, was similar to that of malate content in the fruit (Figure 4.2b). Transcript levels of the COX subunit 3 (Figure 4.8g,h) appeared to be negatively affected by heated nights in post-set fruit and heated days in veraison fruit, although this effect was not significant with two-way ANOVA.

4.5. Discussion

Until now, the direct effect of elevating ambient temperature with field grapevines at different physiologically important stages of grape berry development has gone without investigation. In addition, the effect of diurnal temperature range on aspects of grape berry acid metabolism has had little attention. To determine the effect of elevated temperature on metabolic changes in malate, three vineyard-based systems were employed at three developmental stages. With the use of OTCs, day temperature was elevated by 2-4˚C (Sadras and Soar, 2009), while closed chambers (Soar et al., 2009) were used for controlling day temperature in concert with individual bunch “blowers” that regulated night temperature. The effects of these treatments at post-set, veraison and pre-harvest stages were measured in terms of berry growth and malate level (mg/berry). Fruit displayed the typical double sigmoid pattern of fresh weight accumulation during development, as reviewed by Coombe (1992), although various temperature treatments led to some slight variations (Figure 4.1). Representative activities and putative transcripts of several enzymes

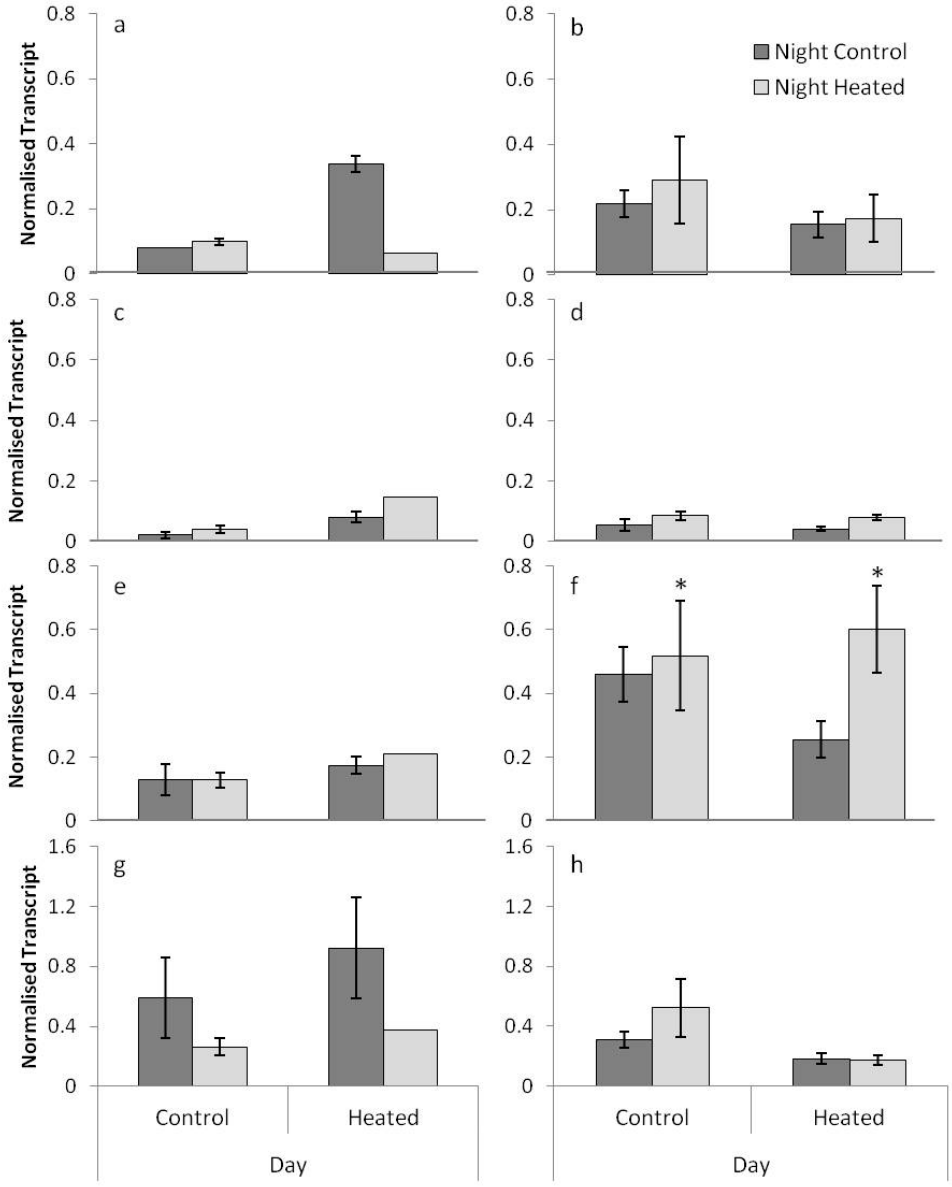


Figure 4.8: Effect of elevated temperature treatments on transcripts of terminal oxidases of the mitochondrial electron transport chain. Transcript abundance in fruit treated at T1:post-set (a, c, e, g) and T2:véraison (b, d, f, h) developmental stages, for *VvAox1a* (a, b), *VvAox1b* (c, d), *VvAox2* (e, f) and *VvCox3* (g, h). Data are presented as means (± SEM) of four bunch replicates and analysed with two-way ANOVA for comparison of control and heat temperatures simultaneous with a comparison of day and night treatments. * significant differences ($p \leq 0.05$).

were analysed in an attempt to explain the mechanisms behind changes in malate content in response to the field temperature trials.

4.5.1. Effect of elevated temperature on post-set berries

When young (post-set) Shiraz vines were heated during the day, berries had lower malate levels than those from control temperature vines. A radiotracer study involving potted canes suggested that heating led to a higher proportion of $^{14}CO_2$ incorporation into malate in young grapes (Kliewer, 1964). However, continued incubation of the potted vines for more than 24 hours between 20 and 37˚C decreased the proportion of label recovered as malate (Kliewer, 1964). In the present study, the use of field-grown vines, for which ambient temperatures were already frequently above 20˚C, and the use of treatments that carried over several days or weeks, resulted in net losses of malate. Therefore, while a higher proportion of CO_2 may be assimilated in grapes exposed to temperatures up to 20˚C, the resulting malate may also be more rapidly processed under continued exposure to high temperatures. In general, the effects of elevated day temperature on post-set berry malate were still evident at harvest. Therefore heat events during early grape berry development are likely to influence characteristics of the ripe berries. This is despite the dramatic metabolic changes occurring after the treatments at developmental stages such as the veraison switch from net malate accumulation to degradation, the loss of fruit photosynthetic capacity and the import of sugars for provision of carbon to the ripening berry (Ollat and Gaudillere, 2000).

Young Shiraz berries grown under a heated night regime were larger and contained more malate than fruit from the control temperature regime (Figures 4.1 and 4.2). As elevated night and day temperatures caused a respective increase and decrease in malate content of young grape berries, it follows that these differences in malate levels were due to differential regulation of metabolism rather than (or in addition to) advancement of berry development. This also suggests that a proportion of grape berry carbon assimilation may occur at night, may involve malate, and may be heat-responsive. As such, we hypothesise that the young Shiraz fruit undergo a CAM-like photosynthesis cycle, whereby CO_2 is granted passage into the berry at night and captured as malate, then released to the chloroplasts during the day for light-driven synthesis of sugars. Such a role for fruit malate is not unheard

of. In young cherry tomatoes, CO_2 from radiolabelled malate administered to the fruit in the dark, was utilised in the pentose phosphate pathway for photosynthetic production of sugars and starch (Farineau and Laval-Martin, 1977). The possibility for a CAM-like photosynthetic pathway has been suggested previously for grape (Ruffner, 1982), and young grapes have demonstrated a similar photosynthetic capacity to young leaves and are capable of re-assimilating respired CO_2 (Ollat and Gaudillere, 2000; Palliotti et al., 2010). In CAM plants, the demand for PEP during net dark CO_2 assimilation is met by a breakdown of stored starch. In grapevine, sugars and starch can be mobilized from storage pools present in the leaf, stem or wood tissues (Downton and Hawker, 1973; Bates et al., 2002), and the rate of transport in the phloem has been seen to increase during the night (Gholami et al., 2004). Whole vines exposed to chamber heat-treatments displayed increased stomatal conductance and photosynthesis, particularly early in the morning (Soar *et al.*, 2009). As yet, little research has been applied to diurnal changes in grape berry metabolism at different developmental stages.

C4 and CAM photosynthesis both employ a CO_2 concentrating mechanism, such that CO_2 can be released to coincide spatially (C4; bundle sheath cells) or temporally (CAM; light cycle) with the RUBISCO and Calvin cycle for carbon assimilation and carbohydrate synthesis. Carbon dioxide taken up through the stomata is used in the β-carboxylation of PEP to OAA (via PEPC), which can then be converted to malate (via MDH) and transported or stored until required. Malate is then decarboxylated to pyruvate (via ME), thus releasing CO_2 to RUBISCO for assimilation into sugars. To support a role of grape berry malate in a CAM-type photosynthesis, activities of PEPC and MDH may be expected to increase with elevated night temperature (consistent with CO_2 assimilation), while ME and PPDK activities may be expected to increase with elevated day temperature (consistent with CO_2 release). While the β-carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) serves as a major step in the synthesis of malate in young grape berries (Hawker, 1969), there were no significant changes to MDH activity, PEPC activity nor PEPC transcript levels in response to post-set heat treatments, although a slight decrease in PEPC activity was seen in response to elevated day temperature. While the transcript of *VvPepckin1* showed similar patterns to the *VvPepc1* transcript, neither of these reflected changes in PEPC activity. As such, post-transcriptional activation of PEPC through phosphorylation by PEPC kinase does not appear

to play a regulatory role in this circumstance. ME activities also did not show any significant changes in response to post-set temperature elevation. However, despite the relatively low levels of *VvPpdk* transcript in young fruit, there was a pattern of increased transcript with warmer days and decreased transcript with warmer nights. This was essentially opposite to the pattern of malate level seen at this time. If these changes in *VvPpdk* were translated to changes in PPDK activity in the fruit, the increased conversion of pyruvate to PEP with warmer days could be linked to an increased rate of malate degradation or decreased rate of malate synthesis. In general, plant PPDK activity is activated by light, and the chloroplastic isoform is involved in photosynthetic carbon assimilation in C4 and CAM plants (Holtum and Osmond, 1981; Burnell and Hatch, 1985; Jenkins and Hatch, 1985). A temperature-sensitive increase in PPDK transcript simultaneous to a decline in berry malate levels provides some support for the involvement of malate in a CAM-type photosynthesis in young grape berries.

In addition to the proposed role of malate in grape berry photosynthesis, malate is also a common plant respiratory intermediate (Sweetman et al., 2009). As respiration rates are generally higher in young fruit (Harris *et al.*, 1971), changes in respiratory enzymes at this time could have a large impact on grape berry malate metabolism. In this study, transcripts of four putative genes have been measured. One of these genes encodes a subunit of the cytochrome oxidase (COX), which acts as the terminal oxidase of the phosphorylating pathway. The other three are isogenes of the alternative oxidase (AOX), the terminal oxidase of the non-phosphorylating pathway. The presence of alternative oxidase genes in grape has only recently been determined (Pilati *et al.*, 2007; Costa *et al.*, 2009), and their involvement in the regulation of plant respiration may also affect intermediates of the TCA cycle, including malate. A potential increase in COX and AOX transcripts was observed in young fruit exposed to elevated day temperature (Figure 4.8a,c,e,g), suggesting an increase in the capacity for respiration through both phosphorylating and non-phosphorylating respiratory pathways. This may be required to support the accelerated changes in fruit development also seen when temperatures were increased. The same fruit samples also had lower fresh weight and less malate per berry, which could be a direct effect of respiratory carbon loss. On the other hand, COX transcript was reduced in post-set fruit exposed to elevated night temperature (Figure 4.8g,h). These fruit also had higher fresh weight (Figure 4.1) and malate content (Figure 4.2a), which may be partly attributed to an

increase in photosynthetic carbon assimilation but could also be an effect of decreased respiration caused by a decrease in the presence of COX.

4.5.2. Effect of elevated temperature on veraison berries

Shiraz berries exposed to a heated day regime around veraison were smaller than controls and contained less malate, consistent with veraison OTC treatments. In general, the veraison heating effects on berry weight and malate were still evident at harvest (Figure 4.2e). Despite their smaller size, berries exposed to elevated temperatures showed accelerated development. Data from some modeling work have led to predictions of earlier grape berry harvests in Australia under realistic climate change scenarios (Webb et al., 2007), which may be largely due to an earlier onset of veraison (Sadras and Petrie, 2011a). The small fruit of the elevated temperature treatments may be a result of carbon redirection from cell growth and division to the production of ripening-related compounds, although this possibility is yet to be explored.

The loss of malate caused by OTC heating in post-veraison and ripening berries (Figure 4.2g) was consistent with loss of malate from CH/BL veraison day-heating (Figure 4.2b), as well as previous reports with potted vines grown in temperature-regulated growth chambers (Kliewer, 1964; 1973). The diurnal temperature range (i.e. the difference between the maximum day temperature and the minimum night temperature) may have had a significant effect on berry malate level. Increasing the diurnal temperature range by elevation of day temperature only, led to decreased berry malate. Maintaining the diurnal temperature range by elevation of both day and night temperatures, led to no change in berry malate level. Very similar patterns were observed for berry fresh weight (data not shown). These new data suggest that malate lost during warmer days may be recovered if berries are also exposed to warmer nights.

As malate undergoes a ripening-related degradation that begins just prior to berry softening (Section 2.4.2), the loss of malate observed with elevated veraison temperatures may be due to the acceleration of this degradation. MDH and ME activities catalyse the direct, reversible conversion of malate to oxaloacetate (OAA) and pyruvate, respectively. With isozymes represented in most, if not all cellular compartments, these activities could regulate malate levels through altered synthesis or degradation of the acid (Sweetman et

al., 2009). Plant NAD-ME activity is confined to the mitochondria (Artus and Edwards, 1985), while NADP-ME activity can be found in the cytosol and chloroplasts (Edwards and Andreo, 1992). Increased NAD-ME activity, as seen in response to elevated temperature in the longer-tern OTC treatments, may explain the loss of malate from these fruit through accelerated conversion of malate to pyruvate and reduction of NAD to NADH in the mitochondria. The shorter-term CH-BL treatments, where day and night temperature were controlled separately, demonstrated that elevated day or night temperatures alone were insufficient to affect NAD-ME activity, but when the fruit were continuously heated during the day and night, NAD-ME activity doubled. It may be that heat-sensitive NAD-ME activity is a slow, adaptive response that can be accelerated if both day and night temperatures are elevated. NAD-ME activity is also very susceptible to post-transcriptional regulation, such as translational control (Long and Berry, 1996), subunit association and allosteric regulation by metabolic intermediates including succinate, fumarate and PEP (Tronconi *et al.*, 2010). Such regulators could alter activity in response to short-term changes in temperature *in planta*.

NADP-ME and PK activities decreased slightly in response to increased daytime temperature (Figures 4.3k and 4.6b), similar to results from heat-treated peach (Lara *et al.*, 2009). Changes in malate levels followed a similar pattern, suggesting that temperature may cause an alteration to flux between malate, pyruvate and PEP. There was no correlation between PPDK transcript and malate content, although the *VvPpdk* showed a striking response to elevated night temperature. While PPDK activity was not measured, increased transcript level gives this activity the potential to increase when environmental temperatures are raised. In peach, PPDK protein level was seen to increase with heat treatment of the fruit (Lara *et al.*, 2009). PPDK-driven metabolism of pyruvate could place an increased demand on the malic enzymes to supply pyruvate during elevated day temperature, thus utilising malate more rapidly.

In a pattern similar to *VvPpdk*, *VvPepck* transcript was significantly higher in veraison fruit that were heated at night, although this was not reflected in the activity data for this enzyme. PEPCK can provide PEP to the gluconeogenic pathway. According to radiolabelling studies, at least a small amount of malate can be utilized for sugar synthesis in grapes (Beriashvili and Beriashvili, 1996). However, it seems unlikely that PEPCK plays a role in the exaggerated degradation of malate in response to increased day temperature, as PEPCK

activity and transcript were actually less represented under such conditions. These data do not preclude post-translational regulatory mechanisms that are well known to occur with PEPCK in other plants (Walker and Leegood, 1995; Bailey *et al.*, 2007). Such mechanisms are evidenced in a light-response, although yet to be tested under an altered temperature regime. Alternatively, PEPCK, which is reversible, may function in malate recovery along with PEPC (favouring OAA synthesis instead of PEP). In *Brassica napus* seedlings, a PEPCK homolog was seen to be cold-induced (Saezvasquez *et al.*, 1995), and colder temperatures caused the PEPCK of *Cucurbita pepo* to become less sensitive to chemical inhibition (Trevanion *et al.*, 1995). The down-regulation of *V. vinifera* PEPCK transcript and activity in response to elevated temperature (Figure 4.5) support these findings.

The decrease in PEPCK and PK activities with day heating and potential increase in PPDK activity (through increased transcript) with night heating could alter flux between OAA, PEP and pyruvate, and may increase PEP:pyruvate (through decreased PK and increased PPDK) and ADP:ATP (through decreased PK and PEPCK). As PEP can be a branching point for the biosynthesis of secondary metabolites within the cell, such a situation may enable the fruit to support elevated synthesis of complex, ripening-related intermediates, but will also likely draw on malate. An increased ADP:ATP ratio may facilitate an increase in other respiratory pathways, such as oxidative phosphorylation through the mitochondrial electron transport chain.

Data presented by Koch and Alleweldt (1978) demonstrated that elevated growth temperature led to increased RQ values (CO_2/O_2) in numerous *V. vinifera* varieties, particularly during ripening, although this has also seen for developing grapes (Harris *et al.*, 1971). An increase in RQ values suggests that more molecules of CO_2 are released per molecule of O_2 consumed, likely due to an increase in the respiration of malate relative to sugars (as malate feeds directly into the TCA cycle), or an increase in the rate of fermentation (Romieu *et al.*, 1992). Grapevine clusters showed slight increases in respiration with increased temperature (from 10-35˚C) at veraison and ripening, while clusters at anthesis (flowering) showed drastic increases in respiration rate with increased temperature (Palliotti *et al.*, 2005). Such increases in respiration rate may require upregulation of both phosphorylating and non-phosphorylating pathways. Transcripts for *VvAox1a*, *VvAox1b*, *VvAox2* and *VvCox3* from the CH-BL treatments showed some

developmental differences, consistent with previous reports (Pilati *et al.*, 2007). Veraison fruit demonstrated a decreasing trend of COX and AOX transcripts in response to elevated day temperature. This may slow flux through the phosphorylating pathway, and potentially increase NADH:NAD and ADP:ATP. On the other hand, high night temperature generally increased AOX transcripts (particularly *VvAox1b* and *VaAox2*), potentially increasing the capacity of the non-phosphorylating pathway. This may lead to an increase in NADH oxidation without affecting ADP:ATP. Intuitively, an increase in temperature would be expected to increase the oxidation of carbon compounds. It may be, under these circumstances, that lower COX and AOX activities are still sufficient to maintain the same (or elevated) rates of respiration. However, the increase in AOX transcript with night heating suggests that the capacity to favour non-phosphorylating respiration may be relevant to the temperature effect. By removing machinery responsible for the phosphorylating pathway (i.e. COX) and producing more machinery responsible for the non-phosphorylating pathway (i.e. AOX), NADH can continue to be oxidised without the metabolic constraints of high ATP:ADP. Therefore the cell can continue to metabolise fuels and release biosynthetic precursors in response to increased temperature, without altering the energetic status of the cell.

Grapes accumulate a small level of ethanol later in development (Romieu *et al.*, 1992), likely due to post-veraison increases of ADH activity (Section 2.4.2). In the present study, elevated day temperature at veraison with the use of OTCs and CH-BLs led to a decline in ADH activity, although elevated night temperature did show some stimulation of *VvAdh2* transcript and ADH activity. Such patterns correlated positively with malate levels, and are consistent with ADH activity changes in response to heat-treated peach (Lara *et al.*, 2009). They also may be expected due to the potential changes in PEP:pyruvate discussed above. Nevertheless, these data do not support our original hypothesis that elevated temperature would increase the utilization of malate through the provision of pyruvate to alcohol fermentation. It may be that grapes exposed to heat stress accumulate acetaldehyde rather than ethanol, as seen in peach (Lara *et al.*, 2009), through elevated pyruvate decarboxylase (PDC) activity (not measured here) and decreased ADH activity. Transcripts and activity of the PDC, which converts pyruvate to acetaldehyde, are present throughout grape berry

development (Hawker, 1969; Or et al., 2000), although the occurrence and regulation of acetaldehyde in grapes awaits evaluation.

In addition to changes in enzymes potentially involved in malate degradation, the effects of elevated temperature regimes on activity and transcript levels of the malate-synthetic enzyme PEPC, were also measured. The veraison decrease in malate content observed with moderate-term CH/BL (Figure 4.2b) and long-term OTC (Figure 4.2g) heat treatments correlate with decreases in PEPC activity (Figure 4.4l,m), as well as lower levels of *VvPepc3* transcript (Figure 4.4e). As the synthesis of malate can occur directly from OAA (through MDH), a decrease in PEPC activity could reduce the rate of malate synthesis *in vivo*, by limiting the substrate OAA. A slight decline in NAD-MDH activity was also seen with OTC heating, consistent with activity data of ripening, heat-treated peach (Lara *et al.*, 2009), and may indicate a decrease in either cytosolic or mitochondrial NAD-MDH activity. However, the rate of NAD-MDH activity far exceeded that of any other enzyme and may be limited only by availability of substrates or cofactors. The loss of malate from berries heated during ripening has in the past been attributed to accelerated degradation (Kliewer, 1964; 1973). These new data bring back into question whether the noticeable decrease in malate content in response to increased temperature is, at least in part, due to decreased synthesis. As ripening grapes will inevitably continue the standard developmental loss of malate from the vacuolar storage pool, berries that are exposed to elevated temperatures any time from veraison may actually have a decreased capacity to re-synthesise malate from OAA in the metabolic pool.

4.5.3. Grape berry metabolism in the face of climatic change

Overall, the main effects of elevated day temperature included an increase in NAD-linked malic enzyme activity and a decrease in PEP carboxylase activity, PEP carboxykinase activity and ADH activity. These results suggest decreased cycling between OAA and PEP, and could indicate a general flux of malate to pyruvate through respiratory pathways. Overall, the main effects of elevated night temperature were observed as an increase in NAD-linked malic enzyme, PEP carboxylase and PEP carboxykinase activities and pyruvate, orthophosphate dikinase transcript, as well as a decrease in pyruvate kinase and potential uncoupling of respiratory pathways from ATP feedback control. These results suggest

increased cycling between OAA and PEP, and general flux of malate to pyruvate (NAD-ME) rather than PEP to pyruvate (PK). Pyruvate formed from malate may be converted to PEP by pyruvate, orthophosphate dikinase (perhaps destined for biosynthesis of complex secondary compounds), or ethanol via alcohol dehydrogenase (for recycling of NADH). Due to decreased activities of PK and ADH with elevated day temperature and an increase in PPDK transcript level with elevated night temperature, pyruvate catabolism may decline when vines are heated, however under particular conditions (ie. during the night), pyruvate may be shunted towards PEP.

In order to reduce the heat-responsive effects of malate metabolism in fruit, it is necessary to further investigate the influence of individual genes. With the discovery of the dwarfed L1 mutant Pinot Meunier variety, which can be successfully transformed and is capable of rapid development, such investigations are becoming more feasible in grapevine (Chaib *et al.*, 2010). Potential targets should involve enzymes that are subject to transcriptional regulation, with the ability to alter malate metabolism without adversely affecting other pathways of metabolism. Likely candidates established from this study in response to elevated temperature may include; the NAD-linked malic enzyme, which could negatively affect malate content through degradation in response to elevated temperature, particularly over longer time periods, or; PEP carboxylase and PEP carboxykinase, which could be involved in malate recovery due to the positive, linear relationship of PEPC activity and malate content of berries used in these experiments. However, it may also be beneficial to investigate genes that are known to play a role in regulating temperature responses at a transcriptional level (e.g. transcription factors). One such example is the ARP gene which, when targeted in *Arabidopsis* knockout lines, produced plants with properties synonymous with heat stress, through the modification of histone binding (Kumar and Wigge, 2010). Upregulation of the expression of the ARP gene could thereby potentially reduce effects of heat stress. However, this is likely to result in broad changes in many aspects of plant biochemistry and physiology, rather than a targeted approach to modifying acid metabolism of the fruit.

4.6. Concluding remarks

Increased diurnal temperature range (warmer days and cooler nights) has been correlated with increased wine quality in Northeast Spain (Ramos *et al.*, 2008). However, the artificial increase in diurnal temperature range imposed by this study (i.e. elevated day temperature and control level night temperature) led to decreased malate levels; a characteristic that is not favourable in South Australian wine regions. Furthermore, the fruit were smaller and veraison occurred earlier in these treatments, supporting accelerated development in the face of climatic warming. However, when these fruit were also heated at night, berries contained malate at levels comparable to controls, implying some balancing effect that may be due to the maintenance of the diurnal temperature range in this treatment. Although the average South Australian summer diurnal temperature range has decreased (mean night temperatures have increased more rapidly than mean day temperatures), most Australian wine regions have typically shown increasing diurnal temperature ranges from 1910 to 2010 (ABM, 2011). It can be expected, should these trends remain unchanged, that grapevines in these areas will continually produce fruits with lower malate content at harvest, thereby increasing the need for expensive acid additions during winemaking, and increasing interest in the mechanisms of temperature-driven fruit acid loss.

4.7. References

ABM. (2011). Climate Change. Australian Bureau of Meteorology Retrieved 10th April 2011, from http://www.bom.gov.au/climate/.

Amerine, M. A. (1956). The maturation of wine grapes. Wine and Vines **37**: 27-36, 53-55.

Artus, N. N. and G. E. Edwards (1985). Properties of leaf NAD-malic enzyme from the inducible crassulacean acid metabolism species *Mesembryanthemum crystallinum*. Plant and Cell Physiology **26**(2): 341-350.

Bailey, K. J., J. E. Gray, R. P. Walker and R. C. Leegood (2007). Coordinate regulation of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase by light and CO2 during C-4 photosynthesis. Plant Physiology **144**(1): 479-486.

Bates, T. R., R. M. Dunst and P. Joy (2002). Seasonal dry matter, starch, and nutrient distribution in 'Concord' grapevine roots. Hortscience **37**(2): 313-316.

Beriashvili, T. V. and L. T. Beriashvili (1996). Metabolism of malic and tartaric acids in grape berries. Biochemistry (Moscow) **61**(10): 1316-1321.

Burnell, J. N. and M. D. Hatch (1985). Light-dark modulation of leaf pyruvate, P_i dikinase. Trends in Biochemical Sciences **10**(7): 288-291.

Chaib, J., L. Torregrosa, D. Mackenzie, P. Corena, A. Bouquet and M. R. Thomas (2010). The grape microvine - a model system for rapid forward and reverse genetics of grapevines. Plant Journal **62**(6): 1083-1092.

Coombe, B. G. (1992). Research on development and ripening of the grape berry. American Journal of Enology and Viticulture **43**(1): 101-110.

Costa, J. H., D. F. de Melo, Z. Gouveia, H. G. Cardoso, A. Peixe and B. Arnholdt-Schmitt (2009). The alternative oxidase family of *Vitis vinifera* reveals an attractive model to study the importance of genomic design. Physiologia Plantarum **137**(4): 553-565.

Davies, C. and S. P. Robinson (1996). Sugar accumulation in grape berries - Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. Plant Physiology **111**(1): 275-283.

Downton, W. J. S. and J. S. Hawker (1973). Enzymes of starch metabolism in leaves and berries of *Vitis vinifera*. Phytochemistry **12**(7): 1557-1563.

Duchene, E. and C. Schneider (2005). Grapevine and climatic changes: a glance at the situation in Alsace. Agronomy for Sustainable Development **25**(1): 93-99.

Dunlevy, J. D., K. L. Soole, M. V. Perkins, E. G. Dennis, R. A. Keyzers, C. M. Kalua and P. K. Boss (2010). Two O-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavour. Plant Molecular Biology **74**(1-2): 77-89.

Edwards, G. E. and C. S. Andreo (1992). NADP-malic enzyme from plants. Phytochemistry **31**(6): 1845-1857.

Famiani, F., R. P. Walker, L. Tecsi, Z. H. Chen, P. Proietti and R. C. Leegood (2000). An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries. Journal of Experimental Botany **51**(345): 675-683.

Famiani, F., N. G. M. Cultrera, A. Battistelli, V. Casulli, P. Proietti, A. Standardi, Z. H. Chen, R. C. Leegood and R. P. Walker (2005). Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. Journal of Experimental Botany **56**(421): 2959-2969.

Farineau, J. and D. Laval-Martin (1977). Light *versus* dark carbon metabolism in cherry tomato fruits. II. Relationship between malate metabolism and photsynthetic activity. Plant Physiology **60**(6): 877-880.

Fernie, A. R., F. Carrari and L. J. Sweetlove (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Current Opinions in Plant Biology **7**(3): 254-261.

Gholami, M., B. G. Coombe and S. R. Robinson (2004). Grapevine phloem sap analysis: 1-sucrose, amino acids, potassium concentrations, seasonal and diurnal patterns. Viticulture - Living with Limitations. A. G. Reynolds and P. Bowen**:** 143-153.

Hale, C. R. (1962). Synthesis of organic acids in the fruit of the grape. Nature **195**(4844): 917-918.

Harris, J. M., P. E. Kriedemann and J. V. Possingham (1968). Anatomical aspects of grape berry enlargement. Vitis **7**: 106-119.

Harris, J. M., P. E. Kriedemann and J. V. Possingham (1971). Grape berry respiration: Effects of metabolic inhibitors. Vitis **9**: 291-298.

Hatch, M. D., M. Tsuzuki and G. E. Edwards (1982). Determination of NAD malic enzyme in leaves of C_4 plants. Plant Physiology **69**: 483-491.

Hawker, J. S. (1969). Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry **8**: 19-23.

Holtum, J. A. M. and C. B. Osmond (1981). The gluconeogenic metabolism of pyruvate during deacidification in plants with Crassulacean acid metabolism Australian Journal of Plant Physiology **8**(1): 31-44.

Jackman, R. L., R. Y. Yada, M. A. Tung and R. A. Speers (1987). Anthocyanins as food colorants - a review. Journal of Food Biochemistry **11**(3): 201-247.

Jacquot, J. P. P., B. B. Buchanan, F. Martin and J. Vidal (1981). Enzyme regulation in C4 photosynthesis. Purification and properties of thioredoxin-linked NADP-malate dehydrogenase from corn leaves. Plant Physiology **68**(2): 300-304.

Jenkins, C. L. D. and M. D. Hatch (1985). Properties and reaction mechanism of C4 leaf pyruvate, P_i dikinase. Archives of Biochemistry and Biophysics **239**(1): 53-62.

Kliewer, W. M. (1964). Influence of environment on metabolism of organic acids and carbohydrates in *Vitis vinifera*. I. Temperature. Plant Physiology **39**(6): 869-880.

Kliewer, W. M. (1965). Changes in the concentration of malates, tartrates and total free acids in flowers and berries of *Vitis vinifera*. American Journal of Enology and Viticulture **16**(2): 92-100.

Kliewer, W. M. (1973). Berry composition of *Vitis vinifera* cultivars as influenced by photo- and nycto- temperatures during maturation. Journal of the American Society for Horticultural Science **98**: 153-159.

Koch, R. and G. Alleweldt (1978). Gas exchange of ripening berries. Vitis **17**: 30-44.

Kumar, S. V. and P. A. Wigge (2010). H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. Cell **140**(1): 136-147.

Lara, M. V., J. Borsani, C. O. Budde, M. A. Lauxmann, V. A. Lombardo, R. Murray, C. S. Andreo and M. F. Drincovich (2009). Biochemical and proteomic analysis of 'Dixiland' peach fruit (Prunus persica) upon heat treatment. Journal of Experimental Botany **60**(15): 4315-4333.

Long, J. J. and J. O. Berry (1996). Tissue-specific and light-mediated expression of the C-4 photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. Plant Physiology **112**(2): 473-482.

Melino, V. J., K. L. Soole and C. M. Ford (2009a). A method for determination of fruit-derived ascorbic, tartaric, oxalic and malic acids, and its application to the study of ascorbic acid catabolism in grapevines. Australian Journal of Grape and Wine Research **15**(3): 293-302.

Melino, V. J., K. L. Soole and C. M. Ford (2009b). Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. Bmc Plant Biology **9**.

Molina, I., C. Salles, M. Nicolas and J. Crouzet (1987). Grape alcohol dehydrogenase. II. Kinetic studies: mechanism, substrate, and coenzyme specificity. American Journal of Enology and Viticulture **38**(1): 60-64.

Mollering, H. (1974). Determination with malate dehydrogenase and glutamate-oxaloacetate transaminase. Methods of Enzymatic Analysis. H. U. Bergmeyer. New York, Verlag Chemie, Weinheim / Academic Press, Inc. **3:** 1589-1593.

Morris, J. R., C. A. Sims and D. L. Cawthon (1983). Effects of excessive potassium levels on pH, acidity, and colour of fresh and stored grape juice. American Journal Of Enology And Viticulture **34**: 35-39.

Ollat, N. and J. P. Gaudillere (2000). Carbon balance in developing grapevine berries. Proceedings of the V International Symposium on Grapevine Physiology.

Or, E., J. Baybik, A. Sadka and A. Ogrodovitch (2000). Fermentative metabolism in grape berries: isolation and characterization of pyruvate decarboxylase cDNA and analysis of its expression throughout berry development. Plant Science **156**(2): 151-158.

Palliotti, A., O. Silvestroni and D. Petoumenou (2010). Seasonal Patterns of Growth Rate and Morphophysiological Features in Green Organs of Cabernet Sauvignon Grapevines. American Journal of Enology and Viticulture **61**(1): 74-82.

Palliotti, A., A. Cartechini, O. Silvestroni and S. Mattioli (2005). Respiration activity in different above-ground organs of *Vitis vinifera* L. in response to temperature and developmental stage. Proceedings of the Seventh International Symposium on Grapevine Physiology and Biotechnology. L. E. Williams**:** 159-165.

Petrie, P. R. and V. O. Sadras (2008). Advancement of grapevine maturity in Australia between 1993 and 2006: putative causes, magnitude of trends and viticultural consequences. Australian Journal of Grape and Wine Research **14**(1): 33-45.

Pilati, S., M. Perazzolli, A. Malossini, A. Cestaro, L. Dematte, P. Fontana, A. Dal Ri, R. Viola, R. Velasco and C. Moser (2007). Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at veraison. BMC Genomics **8**.

Ramos, M. C., G. V. Jones and J. A. Martinez-Casasnovas (2008). Structure and trends in climate parameters affecting winegrape production in northeast Spain. Climate Research **38**(1): 1-15.

Romieu, C., C. Tesniere, L. Thanham, C. Flanzy and J. P. Robin (1992). An examination of the importance of anaerobiosis and ethanol in causing injury to grape mitochondria. American Journal of Enology and Viticulture **43**(2): 129-133.

Ruffner, H. P. (1982). Metabolism of tartaric and malic acids in *Vitis*: A review - Part B. Vitis **21**(4): 346-358.

Ruffner, H. P. and W. M. Kliewer (1975). Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiology **56**: 67-71.

Ruffner, H. P., J. S. Hawker and C. R. Hale (1976). Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15**: 1877-1880.

Sadras, V. O. and C. J. Soar (2009). Shiraz vines maintain yield in response to a 2-4 degrees C increase in maximum temperature using an open-top heating system at key phenostages. European Journal of Agronomy **31**(4): 250-258.

Sadras, V. O. and P. R. Petrie (2011a). Climate shifts in south-eastern Australia: early maturity of Chardonnay, Shiraz and Cabernet Sauvignon is associated with early onset rather than faster ripening. Australian Journal of Grape and Wine Research **17**(2): 199-205.

Sadras, V. O. and P. R. Petrie (2011b). Quantifying the onset, rate and duration of sugar accumulation in berries from commercial vineyards in contrasting climates of Australia. Australian Journal of Grape and Wine Research **17**(2): 190-198.

Saezvasquez, J., M. Raynal and M. Delseny (1995). A rapeseed cold-inducible transcript encodes a phosphoenolpyruvate carboxykinase. Plant Physiology **109**(2): 611-618.

Soar, C. J., M. J. Collins and V. O. Sadras (2009). Irrigated Shiraz vines (Vitis vinifera) upregulate gas exchange and maintain berry growth in response to short spells of high maximum temperature in the field. Functional Plant Biology **36**(9): 801-814.

Song, J. and F. Bangerth (1996). The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. Postharvest Biology and Technology **8**(4): 259-269.

Sweetman, C., L. G. Deluc, G. R. Cramer, C. M. Ford and K. L. Soole (2009). Regulation of malate metabolism in grape berry and other developing fruits. Phytochemistry **70**(11-12): 1329-1344.

Tarara, J. M., J. C. Ferguson and S. E. Spayd (2000). A chamber-free method of heating and cooling grape clusters in the vineyard. American Journal of Enology and Viticulture **51**(2): 182-188.

Taureilles-Saurel, C., C. G. Romieu, J. P. Robin and C. Flanzy (1995). Grape (*Vitis vinifera* L.) malate dehydrogenase .I. Intracellular compartmentation of the isoforms. American Journal of Enology and Viticulture **46**(1): 22-28.

Tesniere, C. and C. Verries (2000). Molecular cloning and expression of cDNAs encoding alcohol dehydrogenases from *Vitis vinifera* L. during berry development. Plant Science **157**: 77-88.

Trevanion, S. J., A. L. Brooks and R. C. Leegood (1995). The effect of temperature on the growth and metabolism of germinating cotyledons of marrow (*Cucurbita-pepo* L). Journal of Plant Physiology **145**(5-6): 741-749.

Tronconi, M. A., M. C. G. Wheeler, V. G. Maurino, M. F. Drincovich and C. S. Andreo (2010). NAD-malic enzymes of *Arabidopsis thaliana* display distinct kinetic mechanisms that support differences in physiological control. Biochemical Journal **430**: 295-303.

Turner, W. L. and W. C. Plaxton (2000). Purification and characterization of cytosolic pyruvate kinase from banana fruit. Biochemistry Journal **352**: 875-882.

Walker, R. P. and R. C. Leegood (1995). Purification, and phosphorylation *in-vivo* and *in-vitro*, of phosphoenolpyruvate carboxykinase from cucumber cotyledons. Febs Letters **362**(1): 70-74.

Walker, R. P., Z. H. Chen, L. I. Tecsi, F. Famiani, P. J. Lea and R. C. Leegood (1999). Phosphoenolpyruvate carboxykinase plays a role in interactions of carbon and nitrogen metabolism during grape seed development. Planta **210**(1): 9-18.

Webb, L. B., P. H. Whetton and E. W. R. Barlow (2007). Modelled impact of future climate change on the phenology of winegrapes in Australia. Australian Journal of Grape and Wine Research **13**(3): 165-175.

Webb, L. B., P. H. Whetton and E. W. R. Barlow (2011). Observed trends in winegrape maturity in Australia. Global Change Biology **17**(8): 2707-2719.

Winkler, A. J., J. A. Cook, W. M. Kliewer and L. A. Lider, Eds. (1974). General Viticulture. CA, University of California Press.

Chapter 5

Summary and future directions

5.1. Experimental summary

To address the aims of this thesis (outlined in Section 1.10), three main studies were undertaken. First, grape berries were harvested from replicate plots of developmentally synchronous bunches within the Coombe vineyard (University of Adelaide, Urrbrae, S.A.), from fruit set until harvest maturity (see Appendix 1 for details). These fruit were frozen and used to measure the occurrence and changes in activities and transcripts of enzymes involved in numerous primary metabolic processes (deemed as relevant to malate metabolism through an initial review of previous literature). Changes in activities and transcripts were compared with concurrent changes in the amount of malate present per berry. As a result, conclusions could be drawn surrounding the developmental influence of particular enzymes on grape berry malate metabolism. In addition, metabolic profiling was undertaken with samples from particular developmental stages, to analyse potential flux of malate metabolism. During this first study it became clear that aspects of fruit respiration were likely to be relevant to developmental changes in malate metabolism. Furthermore, some of the enzymes were known to exist as compartmentalised isoforms. Thus, the second study involved purification of mitochondria from a new developmental series of fresh grape berries, to measure the occurrence and changes in mitochondrial isoforms of the malate dehydrogenase and malic enzyme, as well as changes in rates of mitochondrial respiration and activities of enzymes involved in the mitochondrial electron transport chain of respiration. Thirdly, the effect of artificially elevating vine temperature using replicated field-based treatments, on the same sets of enzyme activities and gene transcripts tested in the first study, was evaluated using fruit from a vineyard at the Nuriootpa Research Station (Nuriootpa, S.A.). These data were again compared with changes in fruit malate, to draw conclusions on the temperature-sensitive regulation of malate metabolism. Due to the clear changes seen in the developmental regulation of grape berry malate from the first study, temperature experiments were conducted at three distinct fruit developmental stages. Finally, data from each of these studies were assembled for comparative analysis by linear regression (see Appendix 4). A summary of findings from these studies is outlined below, followed by suggestions for future research in this area.

5.2. Developmental regulation of grape berry malate

Potential changes in biochemical flux at three stages of berry development are shown diagrammatically in Appendix 3 (Figures A3.1, 3.2, 3.3), and discussed below.

5.2.1. Malate accumulation phase

Synthesis of malate is favoured during pre-veraison grape berry development, enabling malate accumulation until the initiation of ripening (Figure 2.1). Enzyme activities and gene transcripts that were present in high levels during this time included PEPC activity (although this activity was even higher during ripening), and the *VvPepc1* and *VvPepc2* transcripts; NAD-ME activity and *VvNadme* transcript; NAD- and NADP-dependent MDH activities, (although these activities were also higher during ripening), and *VvAox1b* transcript. These findings are summarised in Figure A.3.1) generally not surprising, as many of these enzymes have been previously suggested as key players in malate synthesis (Ruffner, 1982). PEPC activity was apparently not limited to transcriptional regulation (Figure 2.2), however the increase in activity through pre-veraison berry development does support its role in malate synthesis, and highlights the importance of post-transcriptional regulation on activity of this enzyme. For this reason, two transcripts encoding potential PEPC kinases, which activate PEPC activity through phosphorylation, were investigated in subsequent experiments (See Section 4.4.2.2). *VvNadme* transcript decreased early during berry development, followed thereafter by a decline in NAD-ME activity (Figure 2.4). The pattern of NAD-ME transcript and activity suggest a role of this enzyme in the synthesis of malate during the very early stages of berry development, and may be reliant on transcriptional regulation at this time. Of the NAD(P)-MDH activities; due to the high rates and little change (~1-fold increase) throughout early development (Figure 2.3a,b), these enzymes are likely to be involved in the synthesis of malate, but perhaps not in the regulation of developmental changes in its metabolism. The *VvAox1b* transcript was rapidly lost in very young fruit (Figure 3.2b), and is therefore likely to be involved in respiration during fruit set and early berry development.

5.2.2. Malate degradation phase

Malate that accumulates during early grape berry development is almost completely lost throughout fruit ripening. While this phase of malate degradation continues throughout the entirety of ripening, much of the loss occurs at, or just after, véraison (Figure 2.1).

Unexpectedly, from purified mitochondria, State III respiration (in the presence of excess ADP), AOX capacity and COX capacity each showed a dip with véraison (Figures 3.5, 3.7 and 3.8). This suggests that the initial loss of malate does not occur through upregulation of respiration. However, the decreases in respiration rate and the capacities of COX and AOX were temporary, and showed ripening-related increases similar to changes in respiration seen with whole grape bunches (Ollat and Gaudillere, 2000). This increase in respiratory capacity may be related to the use of malate throughout berry ripening. It should be noted that the mitochondria were purified from berries collected in a different season to the malate data outlined here, although the timing of developmental stages (eg. véraison) was normalised between the two seasons. The most interesting change that coincided with the véraison switch between malate accumulation and loss was from mitochondrial NAD-ME activity, which showed a very dramatic induction, although this gradually declined during ripening (Figure 2.4). The change in NAD-ME activity was not reflected by a change in *VvNadme* transcript, thus implying a post-translational level of regulation of this enzyme at this time (cf. translational regulation of this activity in pre-véraison fruit; Section 5.2.1). A summary of the potential metabolic flux of malate in Shiraz berries at veraison is given in Figure A.3.2.

The grape berry continues to exhibit a loss of malate throughout ripening, although at a more gradual rate than that of the veraison “switch”. Enzyme extracts of Shiraz berries at this developmental stage displayed dramatically increased rates of PK activity (Figure 2.3d). This does not support the notion of a post-véraison reduction in grape berry glycolysis reported previously (Ruffner and Hawker, 1977), although this could indicate another important role for this activity during ripening, rather than an induction of the entire glycolytic pathway. Increased activities of NADP-MDH and -ME with fruit ripening (Figure 2.6a,c) are inconsistent with a reduction in grape photosynthetic capacity that occurs at this time (Ollat and Gaudillere, 2000). This may highlight roles for these enzymes outside of a photosynthetic function perhaps in the continued synthesis of malate throughout ripening (despite net losses of the acid at this time). High NAD-MDH and NAD-ME activities (including an increase in mitochondrial-specific NAD-MDH activity; Figures 2.6b,e and 2.5), as well as increased state III respiration rates (Figure 3.5), increased capacities for COX and AOX (Figures 3.7 and 3.8) (and increases of some of these transcripts; Figure 3.2), agree with changes in respiration rate (per berry) in ripening bunches of grapes (Ollat and Gaudillere, 2000). The reason for this increase is

unknown, but could contribute to the provision of intermediates (and ATP) to biosynthetic pathways that are essential for fruit ripening. High levels of PEPCK activity were stimulated dramatically after veraison, following a gradual increase in *VvPepck* transcript that began during early fruit development (Figure 2.6). The reason for the dramatic induction of this activity when transcript was present at a much earlier stage may be due to post-translational modification the enzyme (eg. alteration of phosphorylation status), or could be due to changes in substrate availability such as a sudden abundance of OAA from malate dehydrogenase. *VvPpdk* transcript showed a very similar developmental pattern to *VvPepck* transcript (Figure 2.5). Together, these changes suggest increased capacity for gluconeogenesis in the ripening fruit, in accordance with previous research (Ruffner et al. 1975, cited in Ruffner, 1982; Famiani et al. 2005). This may therefore be a viable pathway through which malate is catabolised during grape berry ripening. In addition, elevated ADH activity and transcript levels were observed with grape berry ripening (Figure 2.7), suggesting a stimulation of alcohol fermentation during grape berry maturation, consistent with previous work (Tesniere and Verries, 2000) and in accordance with the hypothesis that malate decarboxylation provides pyruvate for aerobic fermentation during late berry ripening (Figure A.3.3).

Overall, changes in the rates of many of these enzyme activities, (particularly for those without a coinciding change in transcript), may be due to sudden availability of excess malate at veraison, once the acid has been liberated from the vacuole. It is still unclear what proportion of malate is used for each of the above-mentioned pathways. To address this, metabolic profiling of samples from five stages of berry development was undertaken (Figure 2.8), in a search for other compounds that may also be dramatically regulated at veraison. The switch from net malate accumulation to degradation occurred between the second and third boxes of this figure. As discussed previously (Section 2.5.2), the potential for malate flux into gluconeogenesis was apparent, as well as a likely role in providing carbon to the biosynthesis of secondary intermediates such as benzoate, through the shikimate pathway. Such pathways would require the metabolism of malate to PEP through MDH and PEPCK, or through ME and PPDK, and may support the synthesis of compounds important for the development of fruit aroma and flavour.

5.3. Temperature regulation of grape berry malate

Data accumulated throughout the developmental series of grape berry samples were plotted against minimum temperature recorded the morning of each sampling time from a nearby meteorology station (ABM, 2008). When malate content was plotted against temperature, there was no correlation (Figure A.4.1). Clearly, developmental stage had more of an influence on malate content than temperature during berry development, which is evident from the dramatic developmental changes of malate in the fruit (Figure 2.1). Heat treatments with field-grown vines showed that malate content was affected by elevated temperature, but that this was also influenced by the age of the fruit and time of heating. Post-set treatments resulted in lower berry malate with day heating, but higher berry malate with night heating, while véraison and pre-harvest treatments generally resulted in less malate, particularly with longer-term treatments. To determine whether some of the transcripts and activities could be positively or negatively associated with temperature and malate from the developmental and temperature treatment experiments, data were assembled into scatter plots (see Appendix 4). In many cases these plots have low R^2 values, likely due to the strong influence of development on gene expression and enzyme activity. Results are summarised in Table 5.1 and discussed below.

5.3.1. NAD(P)-MDH/ME

NAD-MDH, NADP-MDH and NADP-ME activities showed very little correlation with temperature and malate content. As expected, these reversible activities may have little to do with the regulation of malate, despite their direct involvement in metabolism of the acid, as the activities are unlikely to be rate-limiting. However, activities of these enzymes were developmentally regulated (see Section 2). In addition, the NAD-MDH activity represents multiple cytosolic and mitochondrial isoforms, therefore changes in any of these activities may be masked by the presence of the other isoforms.

VvNadme transcript and NAD-ME activity displayed some interesting patterns in response to temperature treatments. *VvNadme* was positively correlated with malate content throughout post-véraison development (Table 5.1, Figure A.4.4). Therefore NAD-ME may play a role in malate synthesis, even during the malate degradation phase, but particularly during the initial stages of berry development, due to high levels of transcript and activity in early development (see Figure 2.4 and Section 2.5 for further

Table 5.1. Summary of scatter plot comparisons between temperature, malate, gene transcript levels and enzyme activities.

Transcript or Activity	Experiment	Correlation with temperature (R^2 value)	Correlation with malate (R^2 value)
VvNadme	Development	Weakly positive, post-set (0.22) Negative, véraison (0.33)	Positive, véraison through ripening (0.51)
VvNadme	Treatment	Up-regulated, post-set	Negative, post-set (0.44)
NAD-ME Activity	Treatment	Up-regulated, véraison, post-veraison	Weakly negative, véraison (0.13)
NAD-MDH Activity	Treatment	No change	Weakly negative, post-set (0.21)
NADP-MDH Activity	Treatment	No change	Weakly positive, véraison (0.17)
NADP-ME Activity	Treatment	No change	No correlation
VvPepc1	Development	No correlation	Positive, throughout development (0.58)
VvPepc1	Treatment	Down-regulated, post-set (night only)	Negative, post-set (0.32)
VvPepc2	Development	No correlation	Positive, throughout development (0.55)
VvPepc2	Treatment	Up-regulated, véraison (night only)	Negative, post-set (0.39) Weakly negative, véraison (0.14)
VvPepc3	Development	No correlation	Positive, pre-véraison (0.38)
VvPepc3	Treatment	Up-regulated, post-set	Weakly negative, post-set (0.25) Weakly positive, véraison (0.11)
VvPepckinase1	Treatment	Down-regulated, post-set (night only)	Weakly negative, véraison (0.25)
VvPepckinase2	Treatment	No change	No correlation
PEPC Activity	Treatment	Down-regulated, véraison, post-véraison	Positive, véraison (0.78)
VvPepck	Development	Weakly positive, post-set (0.19) Weakly negative, post-véraison (0.25)	Positive, pre-véraison, véraison (0.71)
VvPepck	Treatment	Up-regulated, post-set Down-regulated, veraison	Positive, véraison (0.33)
PEPCK Activity	Treatment	Down-regulated, veraison	Slightly negative, post-set (0.12) Positive, véraison (0.43)

Continued on next page

Table 5.1. Continued

Transcript or Activity	Experiment	Correlation with temperature	Correlation with malate
VvPpdk	Development	Positive, pre-véraison (0.66) Weakly positive, post-véraison (0.27)	Positive, post-set (0.51) Weakly negative, véraison through ripening (0.13)
VvPpdk	Treatment	Up-regulated, post-set Up-regulated, véraison (night only)	Weakly negative, véraison (0.13)
VvAdh1	Development	Positive, véraison through ripening (0.40)	Weakly positive, post-véraison (0.26)
VvAdh1	Treatment	Up-regulated, post-set	No correlation
VvAdh2	Development	Weakly positive, post-set (0.30)	Positive, pre-véraison (0.50) Weakly negative, véraison through ripening (0.17)
VvAdh2	Treatment	Down-regulated, veraison	Positive, véraison (0.41)
VvAdh3	Development	Weakly positive, post-set (0.22) Weakly negative, véraison (0.19)	Weakly negative, véraison through ripening (0.29)
VvAdh3	Treatment	Down-regulated, post-set (night only)	No correlation
VvAdh4	Treatment	Down-regulated, post-set	Weakly negative, post-set (0.25)
ADH Activity	Treatment	Down-regulated, véraison	No correlation
VvAox1a	Development	Weakly negative, post-véraison (0.24)	No correlation
VvAox1a	Treatment	Down-regulated, véraison	Negative, post-set (0.36) Weakly positive, véraison (0.14)
VvAox1b	Development	No correlation	No correlation
VvAox1b	Treatment	Up-regulated, post-set Down-regulated, véraison (night only)	No correlation
VvAox2	Development	Weakly positive, pre-véraison (0.25)	Weakly positive, pre-véraison (0.27)
VvAox2	Treatment	Up-regulated, post-set	Weakly negative, post-set (0.13)
VvCox	Development	No correlation	Weakly positive, véraison through ripening (0.28)
VvCox	Treatment	Up-regulated, post-set Down-regulated, veraison	Weakly negative, post-set (0.30) Weakly positive, véraison (0.16)

discussion). On the other hand, during post-véraison temperature treatments, NAD-ME transcript and activity were positively correlated with temperature and negatively correlated with malate content (Table 5.1, Figure A.4.4). This suggests that an increase in temperature during ripening increases NAD-ME activity through transcriptional activation, thus increasing the flux of malate to pyruvate and potentially resulting in decreased berry malate content. As such, NAD-ME may be a key player in the temperature response of malate metabolism in grape berries.

5.3.2. PEPC / PEPCK

VvPepc1 transcript was positively linked with malate content during the developmental study (Figure A.4.5a), but negatively linked with malate content and increased night temperature during the heat treatment study (Figure A.4.6a,b). Post-veraison *VvPepc2* was positively correlated with temperature as well as malate content (Figure A.4.5.b,c). However, during the heat treatment *VvPepc2* was negatively linked to temperature, and showed a weak negative correlation with malate content (Figure A.4.6.c,d). *VvPepc3* was positively correlated with malate during development (Figure A.4.5.d), and positively correlated with increased temperature in the heat treatments (Figure A.4.6). Malate content was negatively correlated with this gene early in development and positively correlated during véraison. *VvPepckinase1* transcript was negatively associated with temperature and malate content. PEPC activity was correlated negatively with

temperature, and positively with malate content in véraison CH/BL and UVT trials. These data are consistent with a role for PEPC in malate synthesis, which appears to be maintained throughout ripening, not just during the pre-veraison malate accumulation phase. Overall, elevated temperature may cause a decrease in PEPC activity, which may reduce the synthesis and recovery of malate in these fruit. The gene most likely to be associated with the temperature response of the PEPC activity was the *VvPepc1*, according to a slight positive correlation.

VvPepck transcript was negatively correlated with temperature around véraison and post-veraison, and showed a very strong positive correlation with malate content in véraison CH/BL treatments and during the developmental season. PEPCK activity was also negatively correlated with temperature and positively correlated with malate content. This suggests that an increase in temperature will decrease PEPCK transcript and activity, and may result in decreased malate content. Therefore flux through PEPCK

cannot explain loss of malate in response to elevated temperatures, and increased PEPCK may facilitate the maintenance of higher malate levels.

5.3.3. PK/ PPDK

PK activity may be negatively associated with temperature at véraison, but showed no correlation with malate content.

VvPpdk transcript was positively correlated with temperature, particularly in young fruit, although elevated night temperature was also seen to increase *VvPpdk* transcript in veraison fruit. Changes in transcript were positively correlated with malate in young fruit, and negatively correlated with malate around veraison. This suggests that temperature-related elevation in *VvPpdk* may result in increased malate content in young fruit, but decreased malate content in ripening fruit. This could be due to a photosynthetic role of the PPDK enzyme in green fruit, and a gluconeogenic role in ripening fruit. The activity may be sensitive to developmental regulators through post-transcriptional modification (e.g. alternative splicing), post-translational modification (e.g. phosphorylation), or substrate availability (e.g. the amount of PEP formed by PEPCK). The enzyme, if active in the berries, may play different metabolic roles depending on the stage of development.

5.3.4. ADH

VvAdh1 was positively associated with temperature in fruit at all stages, and slightly positively correlated with malate content in post-véraison fruit. *VvAdh2* data were variable. *VvAdh3* and *VvAdh4* were negatively correlated with temperature and malate content. However, ADH activity was slightly reduced in response to elevated temperature and positively correlated with malate content. Therefore ADH activity is unlikely to be linked with the temperature-sensitive reduction in malate content in developing grape berries.

5.3.5. mETC

VvAox1a transcript was negatively correlated with temperature and also somewhat, with malate content. *VvAox1b* was slightly positively correlated with temperature in post-set fruit, but negatively associated with elevated night temperature in véraison fruit. *VvAox2* could be positively correlated with temperature and malate content. VvAox2 was also positively correlated with temperature in the heat treatments, although slightly negatively correlated with malate content. *VvCox* showed a potential

positive association with temperature in post-set fruit, but a negative association at véraison. During véraison and ripening there were positive correlations between *VvCox* and malate content.

5.4. Implications of research

As long as a ripening grape is left on the vine, it may continue to accumulate sugars (although at the very late stages of ripening the increased concentration of sugars is likely due to water loss and shrivel), and other favourable characters, such as complex pigment, aroma and flavour compounds, but it will lose acids. While it is appealing for a grape to taste sweet, it is also desirable to retain some acidity to promote a fresh, tart flavour. As such, one of the challenges of viticulture is deciding when to harvest, to ensure the optimal ratio of sugar and acid in grapes destined for the table or the wine barrel. Depending on the rates of sugar accumulation and acid loss, there may be a very narrow window for harvesting fruit with optimal sugar-acid balance. This may depend on the grapevine cultivar (some varieties ripen more quickly than others), or environmental particulars. Seasonal temperature is well known to influence the rate of fruit development (Webb et al., 2007), and has a strong impact on grape acidity (Ruffner et al., 1976). As such, it is important to understand how the regulation of fruit ripening may be controlled, to maximise the potential for harvesting fruit with a good sugar-acid balance, as well as high quality colour, aroma and flavour characteristics (ie. broadening the “window of opportunity”). The research described in this thesis has dealt with one aspect of ripening; acid metabolism. The main foci have been the metabolism of malate during grape berry development and ripening, and the effect of altered seasonal temperature. Malate was chosen, as it is one of two predominant acids in the grape berry along with tartrate, and unlike tartrate, it is metabolically active. Therefore changes in grape berry acidity during development and in response to changes in environmental conditions are almost entirely due to changes in malate content.

Overall, it can be seen that numerous activities interact to synthesise and catabolise malate, and likely facilitate the development and ripening processes. Key players in this study included the NAD-dependent malic enzyme (mitochondrial respiration), PEP carboxylase (CO_2 assimilation and glycolysis), PEP carboxykinase (gluconeogenesis), and the pyruvate, orthophosphate dikinase (photosynthesis). NAD-ME showed potential as a

heat-responsive factor in malate loss, due to some positive correlation with temperature (ie. increased temperature led to increased transcript and activity of this enzyme), and negative correlation with malate (i.e. higher levels of transcript and activity could generally be associated with lower levels of malate). This activity, once thought to be absent from grape berries (Romieu et al., 1992), but evidenced here for the first time, may be partially responsible for altering the rate of malate degradation in response to changes in fruit temperature during ripening. The involvement of PEPC in grape berry malate synthesis has long been recognised (Ruffner, 1982), thus the positive correlation of PEPC activity with malate throughout this study was not unexpected. The PEPCK transcript and activity showed very strong correlations with malate, in developmental and heat treatment studies. This enzyme, which catalyses a key step in gluconeogenesis (although is reversible), may instead play a role in malate synthesis or recovery during times of net malate accumulation and degradation, and in response to changes in fruit temperature, rather than utilisation of organic acids for sugar synthesis. Activity of the PPDK is yet to be demonstrated in grapevine and the presence of PPDK protein in fleshy fruits has been sought, but not found (Famiani et al., 2005). However, here a putative transcript of the gene was highly developmentally and environmentally regulated. It is unlikely that the transcript of a gene so dramatically regulated is not translated to active protein. It may be rewarding to pursue an investigation of this enzyme in grape, as there is potential for PPDK to have a large influence on malate metabolism during véraison and ripening, potentially in a diurnal fashion.

5.5. Future Directions

To reduce the impact of an uncertain climatic future on grape berry acidity, some mechanisms of temperature-sensitive malate metabolism could be exploited. Enzymes such as the PEPC, PEPCK, PPDK and NAD-ME may be targeted for crop enhancement through traditional plant breeding or genetic modification approaches. It may also be of scientific interest to explore further the involvement of some of these enzymes in a CAM-like photosynthesis system within this C3 plant.

At this stage, further investigation into the proportion of malate that feeds into various metabolic pathways is required. Some targeted metabolite flux analyses would be useful. Radiotracer studies with labelled malate or fumarate are an option, as has been used in

the past to demonstrate the presence of gluconeogenic flux in grape berries (Ruffner et al. 1975, cited in Ruffner, 1982). Such experiments may be misleading due to the high levels of exogenous malate. This can be overcome by applying labelled CO_2 instead, although for adequate assimilation, CO_2 may need be applied to whole plants (rather than directly to the fruit), which then requires translocation of labelled intermediates to the fruit, and will likely increase background activity. Furthermore, some of the important metabolites in fruit malate metabolism, such as OAA and PEP (as implied by regulated activities of the PEPC, PEPCK, PPDK and NAD-ME) are transitory, and rapidly lost to complex biochemical pathways. By searching for label in end-products of pathways involving these metabolites (e.g. ethanol or other alcohols), information may be obtained regarding the proportion of malate that may be fed into particular pathways (e.g. alcohol fermentation). Perhaps a more useful approach to this problem is through metabolic flux analyses with ^{13}C labelled sugars. While this approach has been very successful with the use of sterile, *in vitro* plant cultures (Iyer et al. 2008) the application to fruit again poses a challenge.

It would also be useful to explore the effects of genetically modifying some key genes that may influence malate metabolism in the fruit (e.g. *VvPepc1*, *VvPepck*, *VvPpdk*, *VvNadme*). With the improvement of tools and techniques for grapevine molecular biology (Chaib et al., 2010), this is becoming increasingly possible in *Vitis*. Effects of knocking down or over expressing these genes on the level of malate and other organic acids in the fruit will provide an evaluation of their usefulness as targets for marker-assisted breeding strategies. In addition, the mechanism of gene regulation could be explored. With the increasing availability of high-quality grapevine genomic sequences, the characterisation of gene promoters may prove useful, and could lead to a screening method for determination of genes that may be temperature-responsive, and thereby linked to changes in metabolism that could ultimately affect grape quality. Transcription factors, or other transcriptional controllers (e.g. histone binding proteins; Kumar and Wigge, 2010) responsible for the regulation of these genes may also be of interest.

The characterisation of these genes and their regulation in response to developmental and environmental stimuli in various grapevine cultivars could provide new criteria for the selection of optimal cultivars according to predicted climate changes. Furthermore, it will enhance current knowledge on malate metabolism in a model non-climacteric fruit, which may be extrapolated to studies in other plants.

5.7. References

ABM. (2008). Adelaide, SA - Daily Weather Observations. Australian Bureau of Meteorology, 2007-2008, from http://www.bom.gov.au/climate/dwo/IDCJDW5002.latest.shtml.

Chaib, J., L. Torregrosa, D. Mackenzie, P. Corena, A. Bouquet and M. R. Thomas (2010). The grape microvine - a model system for rapid forward and reverse genetics of grapevines. Plant Journal **62**(6): 1083-1092.

Famiani, F., N. G. M. Cultrera, A. Battistelli, V. Casulli, P. Proietti, A. Standardi, Z. H. Chen, R. C. Leegood and R. P. Walker (2005). Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. Journal of Experimental Botany **56**(421): 2959-2969.

Kumar, S. V. and P. A. Wigge (2010). H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. Cell **140**(1): 136-147.

Ollat, N. and J. P. Gaudillere (2000). Carbon balance in developing grapevine berries. Proceedings of the V International Symposium on Grapevine Physiology.

Romieu, C., C. Tesniere, L. Thanham, C. Flanzy and J. P. Robin (1992). An examination of the importance of anaerobiosis and ethanol in causing injury to grape mitochondria. American Journal of Enology and Viticulture **43**(2): 129-133.

Ruffner, H. P. (1982). Metabolism of tartaric and malic acids in *Vitis*: A review - Part B. Vitis **21**(4): 346-358.

Ruffner, H. P. and J. S. Hawker (1977). Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochemistry **16**: 1171-1175.

Ruffner, H. P., J. S. Hawker and C. R. Hale (1976). Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15**: 1877-1880.

Tesniere, C. and C. Verries (2000). Molecular cloning and expression of cDNAs encoding alcohol dehydrogenases from *Vitis vinifera* L. during berry development. Plant Science **157**: 77-88.

Webb, L. B., P. H. Whetton and E. W. R. Barlow (2007). Modelled impact of future climate change on the phenology of winegrapes in Australia. Australian Journal of Grape and Wine Research **13**(3): 165-175.

Appendices

Appendix 1. Berry Sampling

A1.1 Developmental series sampling regime and experimental design

Data for the developmental series were assembled from four replicate samples of the Coombe Vineyard (University of Adelaide, Waite campus), South Australia, during the 2007-08 season. This experimental design meant that all four replicates at each time-point consisted of berries from 5 designated vines; one in each row of the trial site (Figure A.1.1). Approximately 6 bunches on 20 vines were tagged at 50% cap-fall (Figure A.1.2) Therefore approximately 120 individually tagged bunches were used for sampling at each time point. Each replicate was made up of berries from approximately 30 bunches, which, despite their differing locations within the vineyard, were developmentally synchronous. Berries were taken twice per week between 8am and 10 am, from just after fruit set, until maturity. At every sampling date, at least one, but as many as four berries (depending on the size of the fruit) were harvested from various positions in each bunch. Characteristic patterns of berry development were observed (Figure A.1.3).

A1.2 Heat treatment sampling regime and experimental design

Data for the heat experiments reflect samples exposed to various treatments in a vineyard of the Nuriootpa Research Station (SARDI), South Australia, during the 2008-09 season. Three experimental setups were used. Single closed chambers were used to mimic short-term “heat-wave” events (Figure A.1.4.a), open-top chambers were used for longer-term temperature shifts (Figure A.1.4.b), and two closed chambers and twelve bunch blowers (“chamber/blowers”) were used to investigate day and night heating responses (Figure A.1.4.c,d). Treatments were conducted at three stages of the season. Bunches (four per treatment) were tagged at 50% cap-fall for the chamber-blower treatments only. For chamber and open-top chamber treatments, berries were taken from random bunches within the treated and control vines (conducted in triplicate).

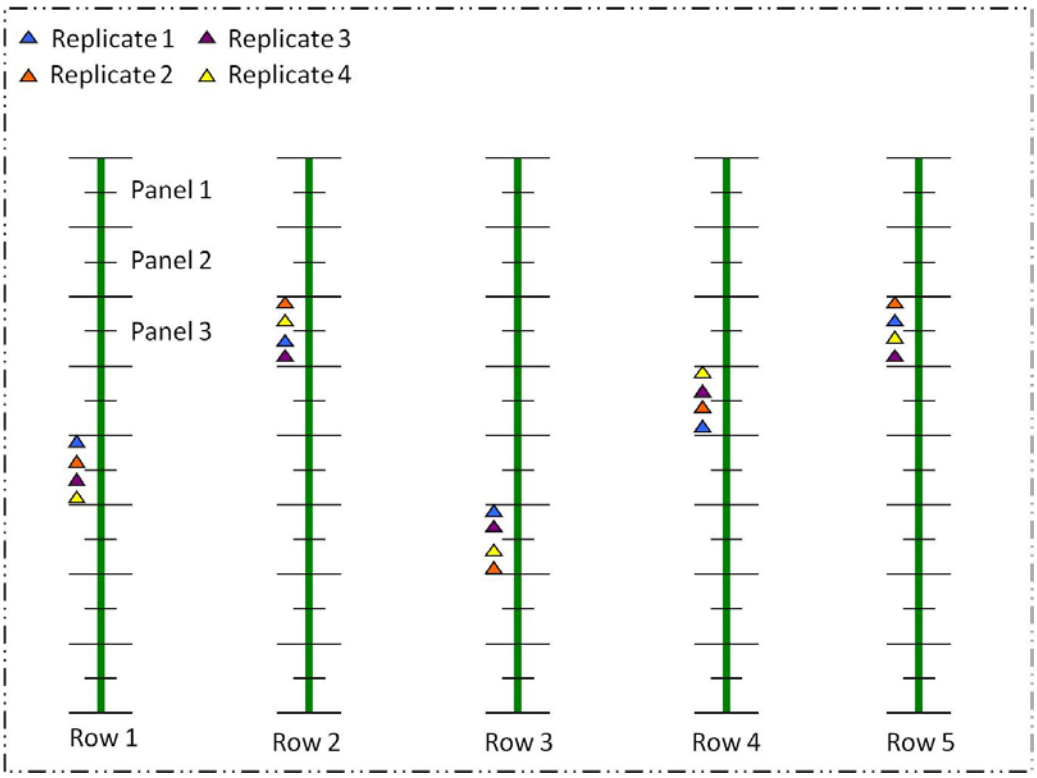


Figure A.1.1. Berry sampling design for Coombe developmental series. Five rows of Shiraz vines were used. Each row was made up of panels of four vines. In each row, one panel was selected, and each of the four vines in that panel was designated as a different replicate (i.e. four replicates).

Figure A.1.2. Tagged bunches for the developmental series. A panel of four Shiraz vines from the Coombe vineyard, with bunches tagged at 50% capfall (anthesis). Different coloured tags were used to identify bunches belonging to the four different replicates.

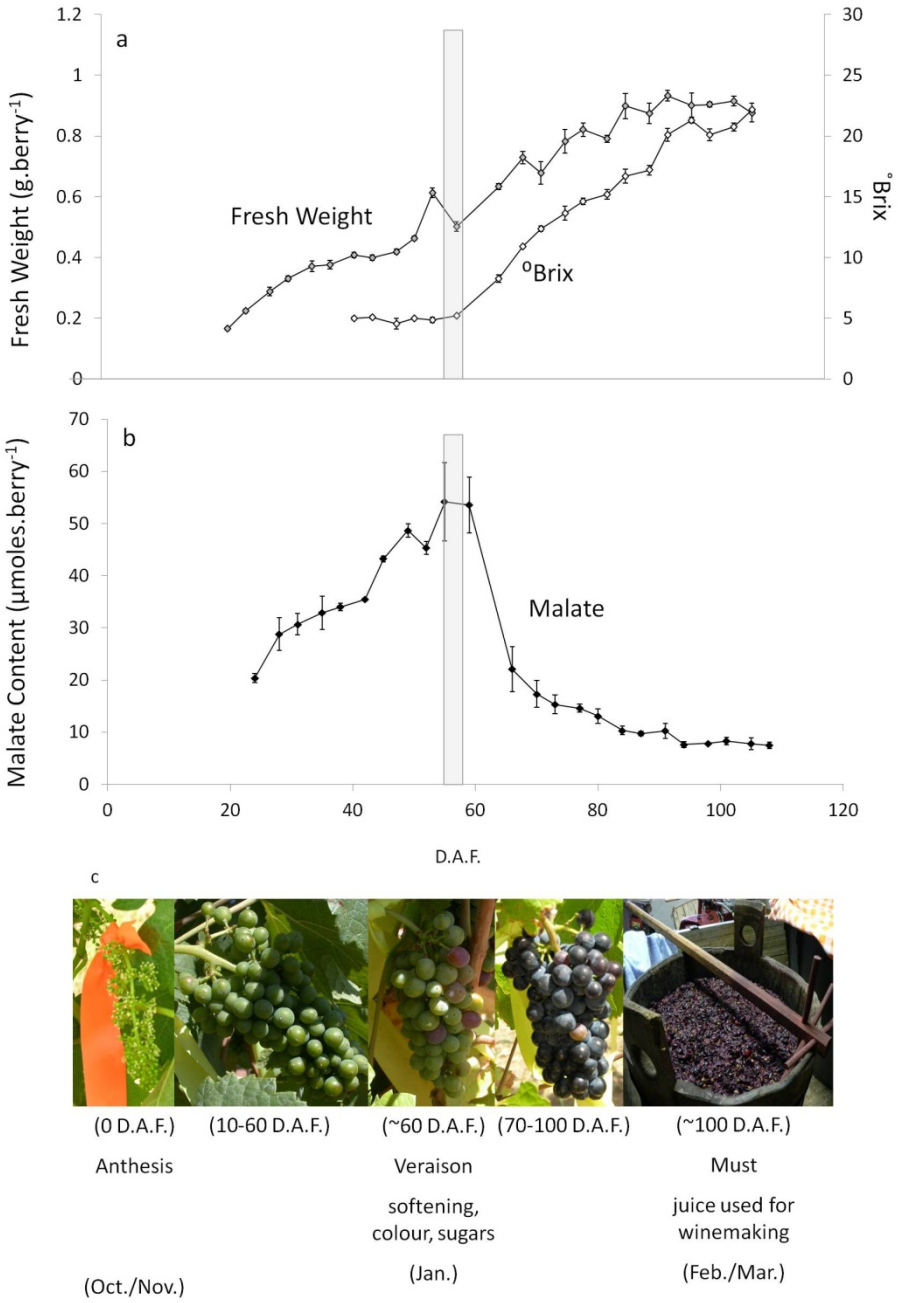


Figure A.1.3. A developmental scale of fruit from Coombe vineyard during 07-08 season. Changes in (a) berry fresh weight and Brix, (b) berry malate content and (c) bunch phenology during the developmental season. D.A.F. Days After Flowering. Véraison indicated by shaded box. Data are presented as means of four biological replicates with error bars representing standard error of the mean.

Figure A.1.4. Equipment used for heat treatment of vines in the field. Single, closed chambers (a) were used for 3 days at a time, open-top chambers (b) were assembled for 3 weeks at a time, while “control” and “heated” closed chambers (c), each containing “control” and “heated” bunch blowers (d) were assembled for 10 days at a time.

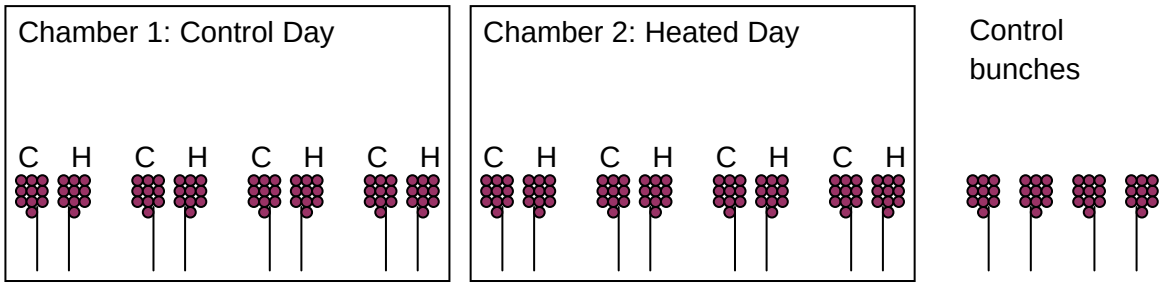


Figure A.1.5. Experimental design of chamber/blower (ie. day/night heating) treatments. Chambers used solar radiation and fans to regulate day temperature (one to maintain ambient temperature and another to elevate vine temperature) and heated fans (blowers) were assembled inside the chambers to regulate temperature of individual bunches overnight (C=control, H=heated). Four replicate bunches were tagged and used for sampling at each time-point.

Table A.1.1: Location and climatic data for Coombe and Nuriootpa Research Station vineyards

	Coombe		**Nuriootpa**
Elevation (m)	123		274
Row spacing, Vine spacing (m)	3, 1.8		3, 2.25
Latitude, Longitude (˚)	-34.964 138.648		-34.468 138.998
	2007-2008	**2008-2009**	**2008-2009**
Mean October Temperature (˚C)	23.2	24.1	23.1
Mean November Temperature (˚C)	28	24.7	23.8
Mean December Temperature (˚C)	28.9	25.5	24.3
Mean January Temperature (˚C)	31	32	32.1
Mean February Temperature (˚C)	27.6	31.4	31.3
Mean Growing Season Temperature (Nov-Feb)	28.9	28.4	27.8
Growing Season Degree Days (days above 18˚C)	118	119	117
Cumulative Growing Degrees (degrees above 18˚C)	1320.9	1242.7	1147.7
Annual Rainfall (Jan-Dec, leading up to the season)	459	395	430.2
Growing Season Rainfall (mm; Nov-Feb)	79.2	56.2	112
Growing Season Rain Days (mm; Nov-Feb)	16	20	25
Growing Season Mean Evaporation (mm)	11.15	10.5	8.6
Mean Relative Humidity (%, 9am for Nov)	45	52	61
Mean Relative Humidity (%, 9am for Dec)	44	55	63
Mean Relative Humidity (%, 9am for Jan)	49	37	38
Mean Relative Humidity (%, 9am for Feb)	48	42	47
Mean Relative Humidity (%, 9am for Nov-Feb)	46.5	46.5	52
Sunshine Days (h/day; Nov-Feb)	9.82	10.2	10.18

Climate data obtained from the Australian Bureau of Meteorology (ABM, 2008).

Appendix 2: Enzyme extraction and assay optimisation

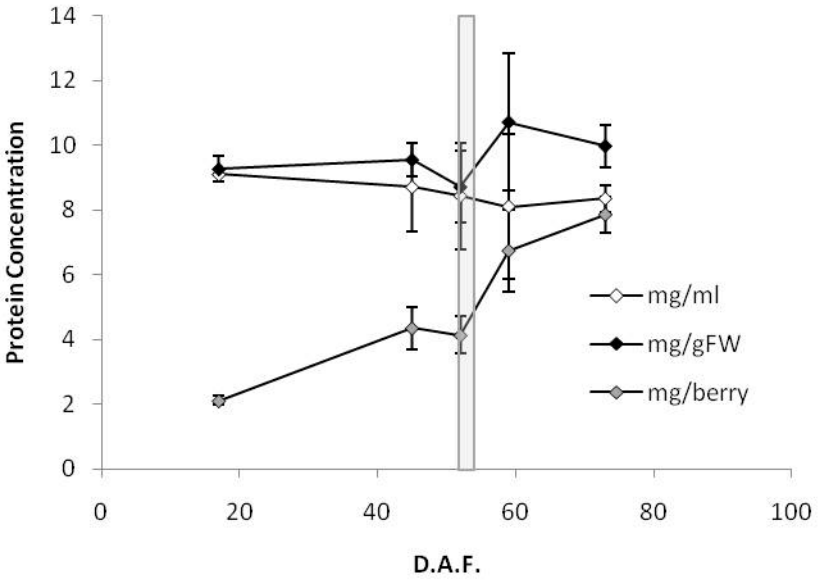


Figure A.2.1: Protein concentration in berry enzyme extracts. Measured as the amount of protein remaining in enzyme extracts after 65% (w/v) PEG precipitation, and presented according to the milligram concentration of protein per ml of extract, per gram fresh weight and per berry. Data are presented as means of four replicates, with error bars representing standard error of the mean.

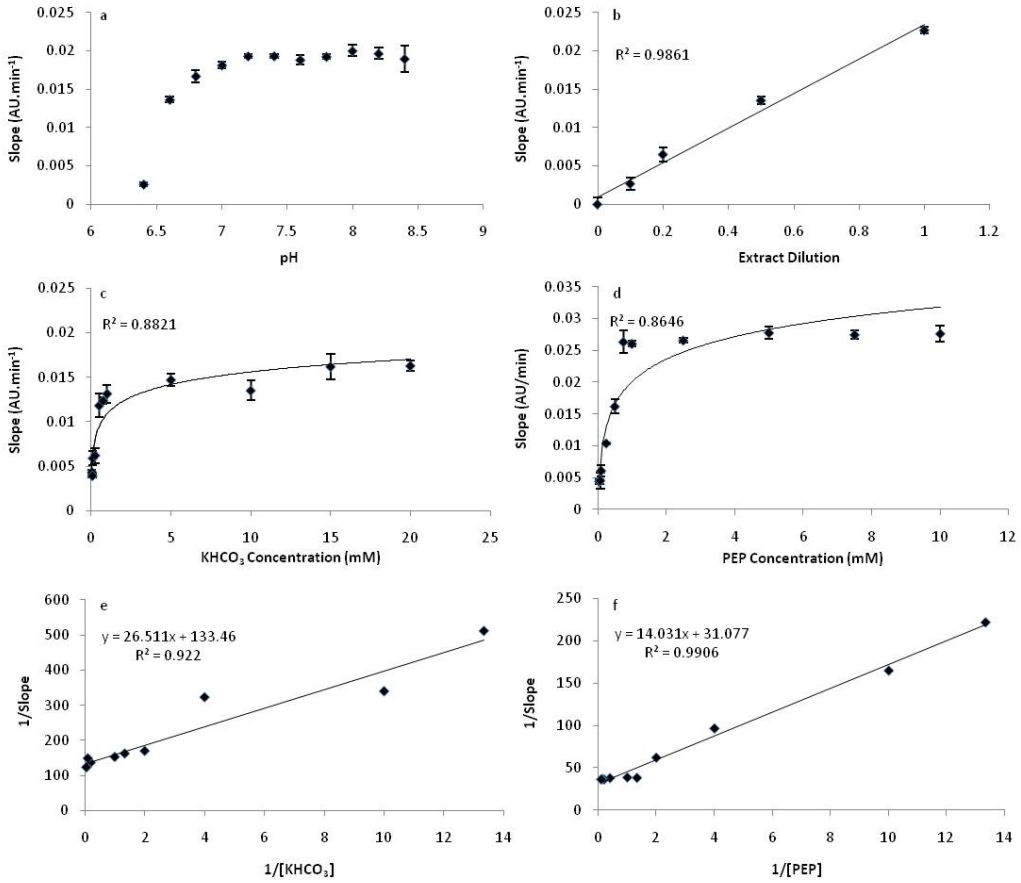
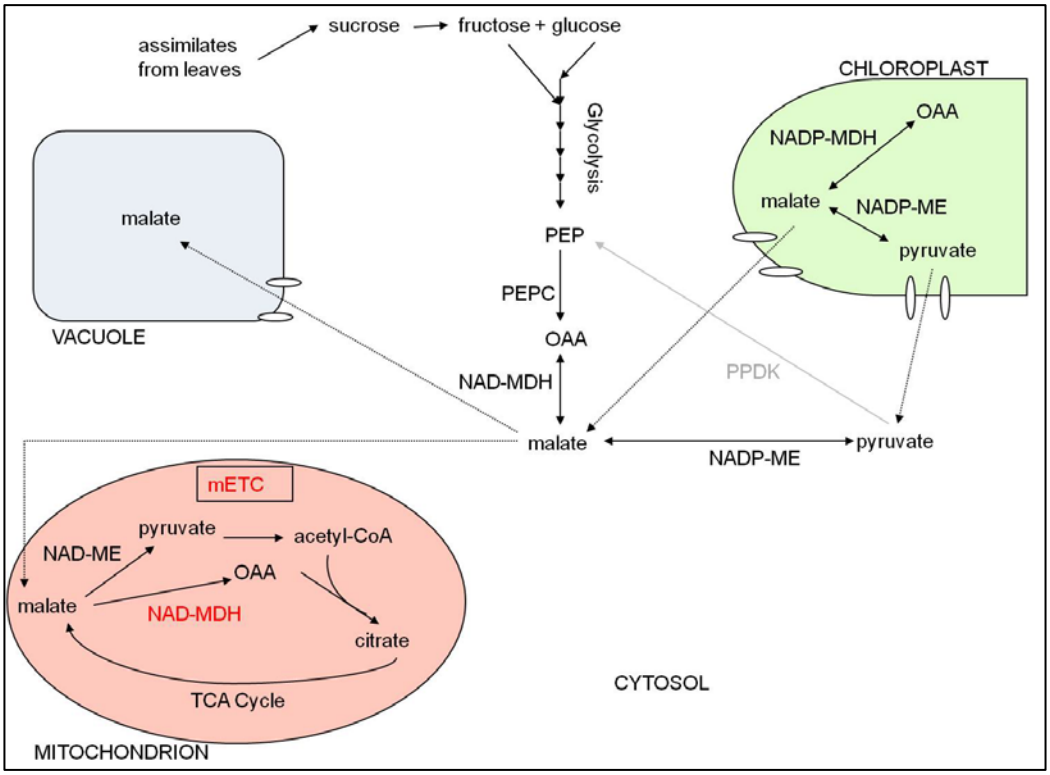


Figure A.2.2. Example of enzyme assay optimisation data. PEPC enzyme assay data for determination of optimal pH (a), enzyme extract dilution (b), $KHCO_3$ concentration (c), and PEP Concentration (d), and Lineweaver-Burk plots for determination of Km values for $KHCO_3$ (e) and PEP (f). Data for optimisation plots are presented as means of three replicates, with error bars representing standard error of the mean.

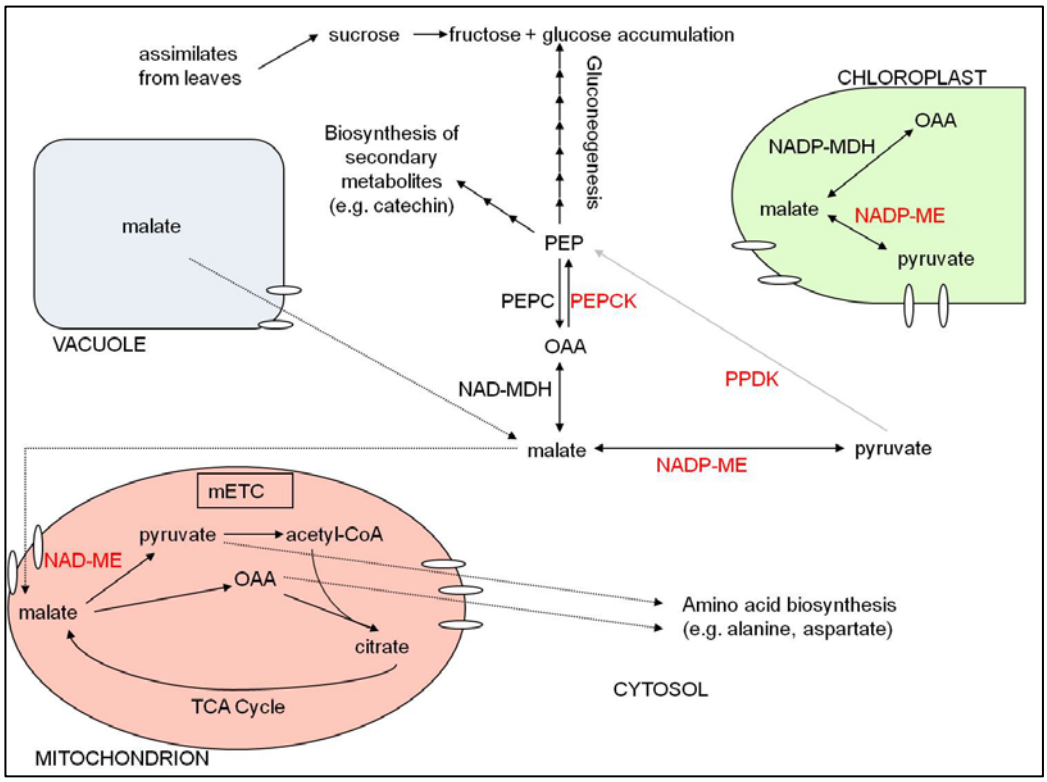
Table A.2.1: Substrate affinities of enzymes from mature Shiraz grape berries

Enzyme	Substrate	Km (mM)
PEPC	$KHCO_3$	0.198
	PEP	0.451
	NADH	2.889
PEPCK	$KHCO_3$	14.307
	PEP	1.69
	ADP	0.100
	NADH	2.889
NAD-MDH	OAA	0.266
	NADH	0.015
NAD-ME	Malate	0.426
	CoA	0.016
	NAD^+	0.116
ADH	Acetaldehyde	0.251
	NADH	0.826

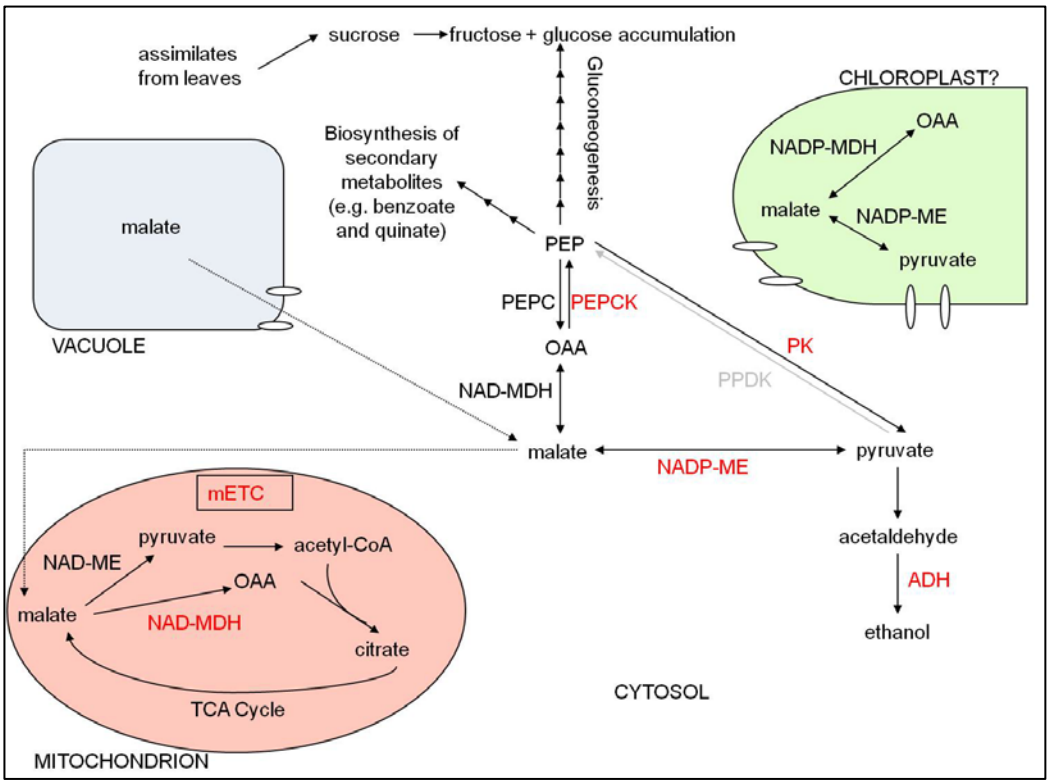
Appendix 3. Networks of potential flux at three stages of berry development



A.3.1. Potential Pre-veraison pathways of malate metabolism. Cytosolic, mitochondrial, chloroplastic and vacuolar malate and potential pathways that are highly represented during pre-veraison Shiraz berry development, according to activity, transcript and metabolite data (Sections 2 and 3). Intermediates are presented in black, lower case text. Activities are presented in black if activity was present, or red if activity was upregulated relative to other stages of development. Where evidence of developmental regulation was only gained through transcriptional data, the enzyme is presented in grey (although red, if upregulated). Transport of intermediates between cellular compartments is indicated with a broken line.



A.3.2. Potential véraison pathways of malate metabolism. Cytosolic, mitochondrial, chloroplastic and vacuolar malate and potential pathways that are highly represented in Shiraz berries at veraison, according to activity, transcript and metabolite data (Sections 2 and 3). Intermediates are presented in black, lower case text. Activities are presented in black if activity was present, or red if activity was upregulated relative to other stages of development. Where evidence of developmental regulation was only gained through transcriptional data, the enzyme is presented in grey (although red, if upregulated). Transport of intermediates between cellular compartments is indicated with a broken line.



A.3.3. Potential post-véraison pathways of malate metabolism. Cytosolic, mitochondrial, chloroplastic and vacuolar malate and potential pathways that are highly represented during post-veraison ripening of Shiraz berries, according to activity, transcript and metabolite data (Sections 2 and 3). Intermediates are presented in black, lower case text. Activities are presented in black if activity was present, or red if activity was upregulated relative to other stages of development. Where evidence of developmental regulation was only gained through transcriptional data, the enzyme is presented in grey (although red, if upregulated). Transport of intermediates between cellular compartments is indicated with a broken line.

Appendix 4. Gene transcript and enzyme activity scatter plots

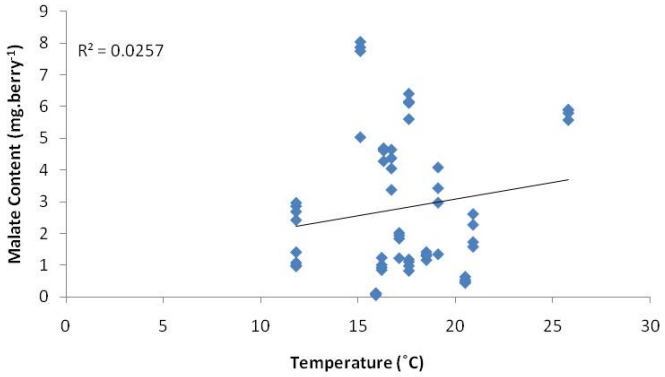


Figure A.4.1. Malate content Vs ambient temperature from developmental series. Scatter plot comparison of individual measurements of malate content in fruit of the developmental data series (see Sections 2 and A1.1), with ambient temperature from the night before each sampling point, collected from a nearby meteorological station (ABM, 2008). A trend line of linear regression was fitted, with R^2 value indicated in the top left corner of the plot.

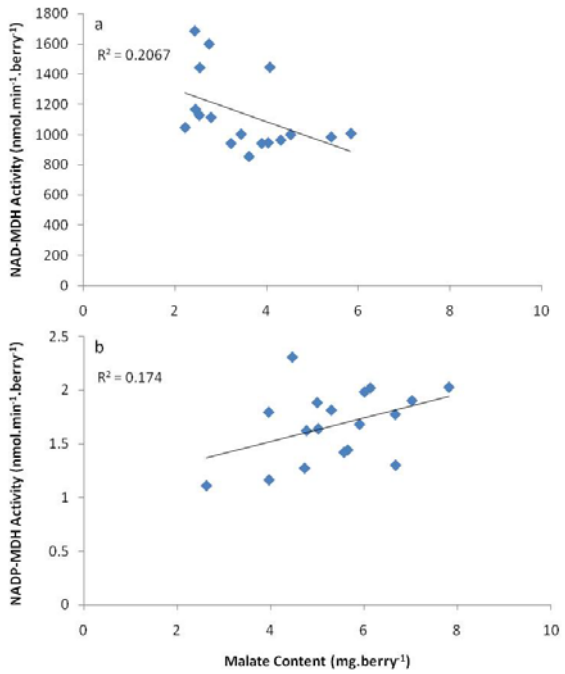


Figure A.4.2. NAD(P)-MDH activities Vs malate content from heat treatments. Scatter plot comparison of individual measurements of NAD-MDH (post-set) and NADP-MDH (véraison) activities, with malate content of the heat treatment data series (see Section 3). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

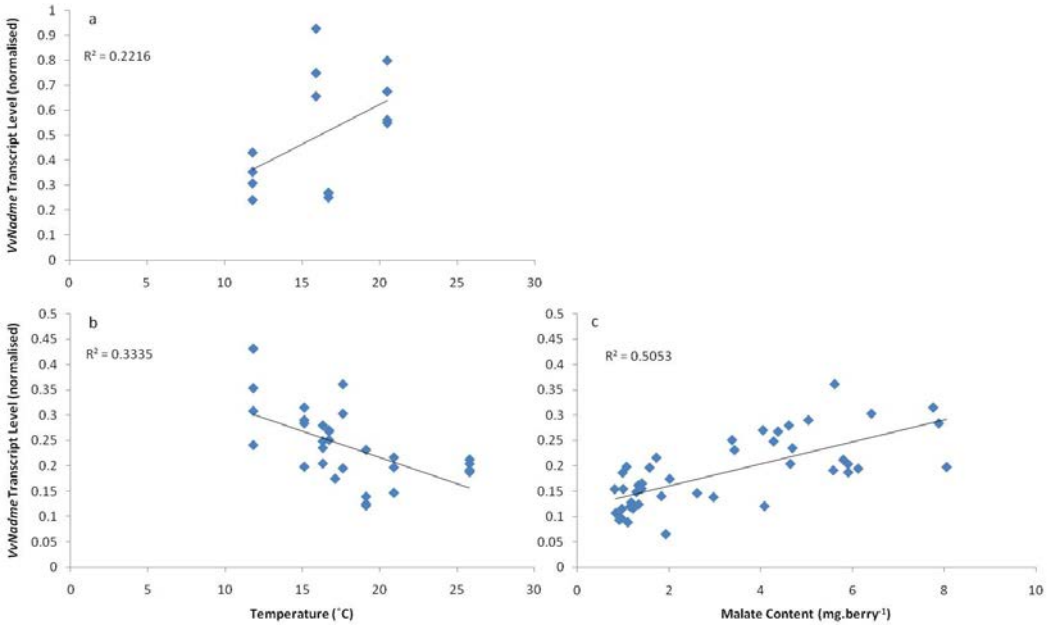


Figure A.4.3. NAD-ME transcript Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of NAD-ME gene transcript with temperature data (left; ABM, 2008) and malate content (right), for samples collected during the pre-véraison (top) and post-véraison (bottom) stages of berry development. of the developmental data series (see Section 2). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

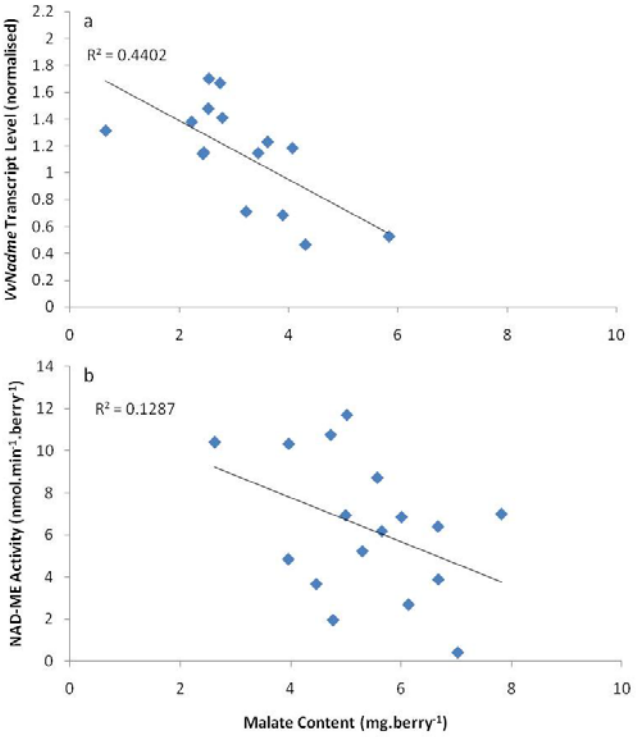


Figure A.4.4. NAD-ME transcript and activity Vs malate content from heat treatments. Scatter plot comparison of individual measurements of NAD-ME gene transcript (post-set) and activity (véraison), with malate content of the heat treatment data series (see Section 3). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

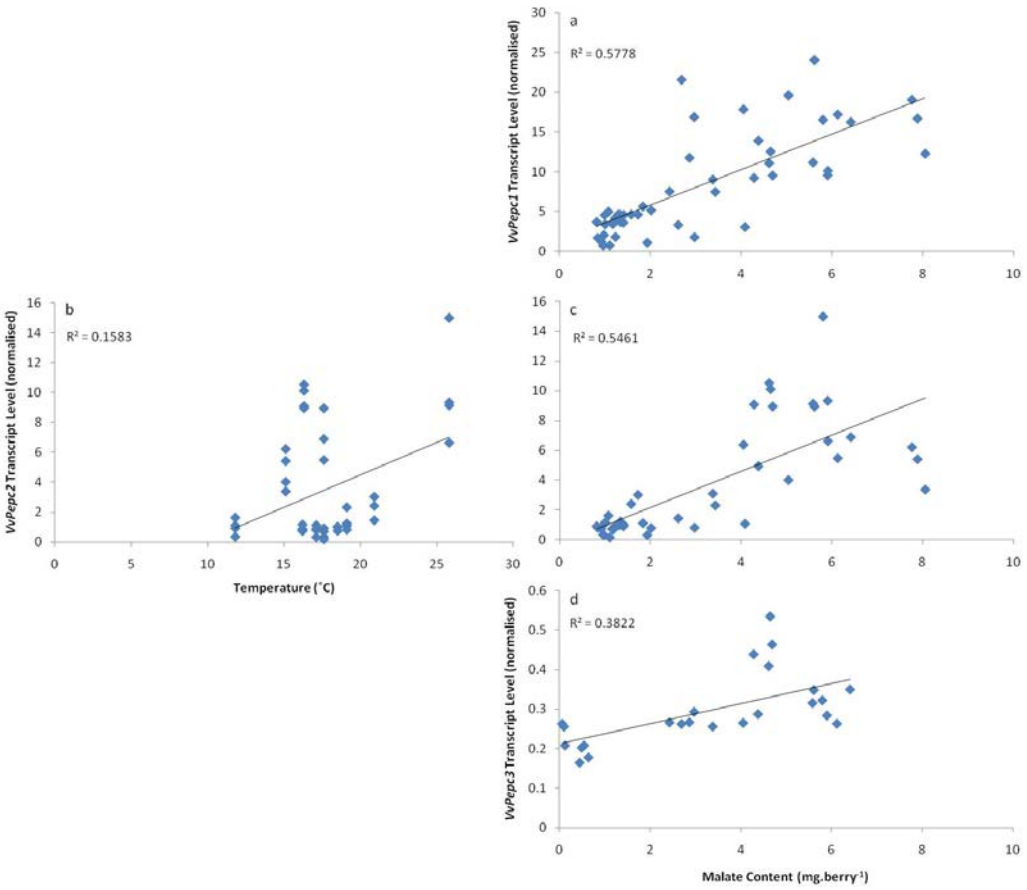


Figure A.4.5. PEPC transcripts Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of PEPC gene transcripts with temperature data (left; ABM, 2008) and malate content (right) of the developmental data series (see Section 2). Data were taken from pre-véraison (d) and post-véraison (a,b,c) stages. A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

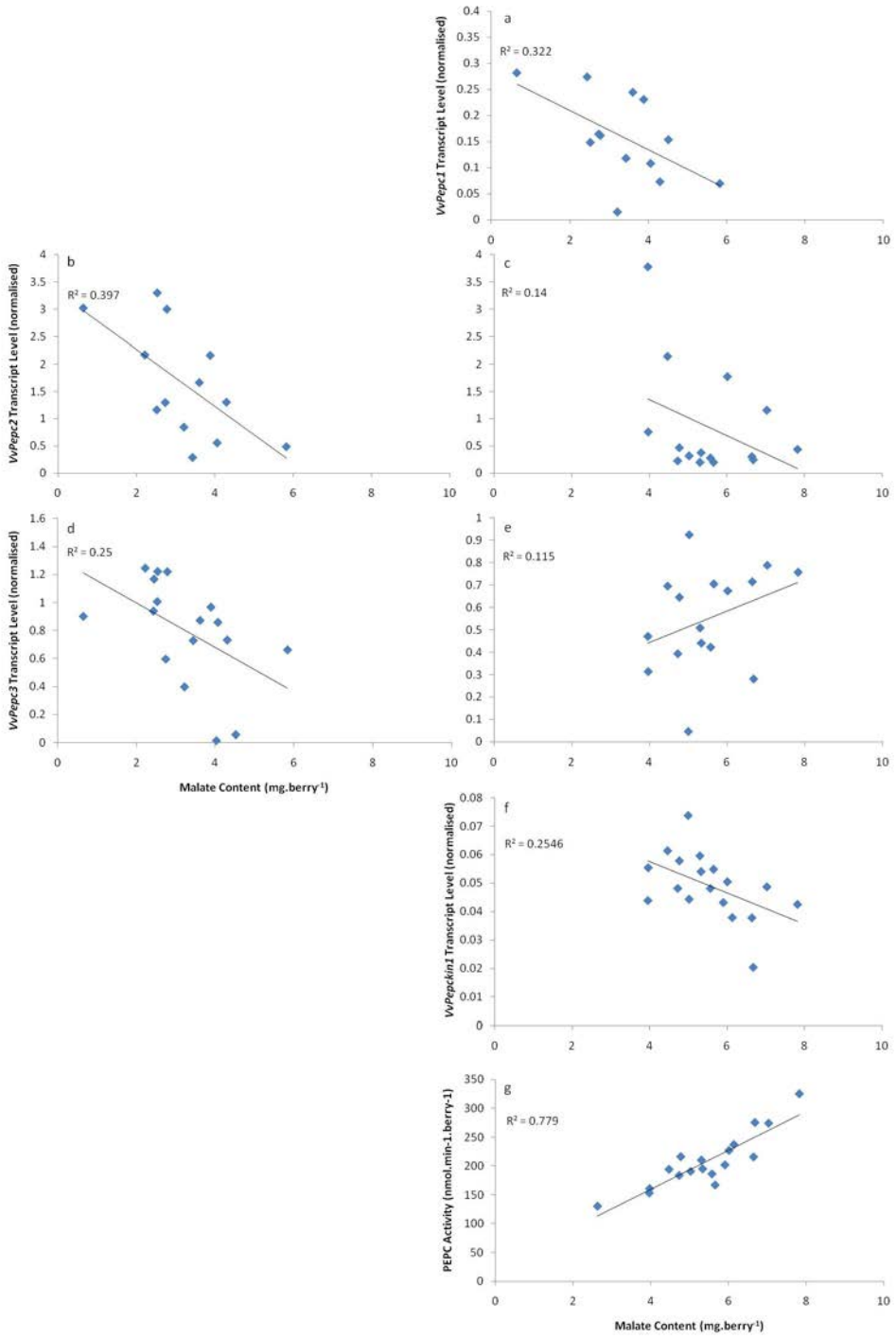


Figure A.4.6. PEPC transcripts and activity Vs malate content from heat treatments. Scatter plot comparison of individual measurements of PEPC gene transcripts and activity, with malate content of the heat treatment data series (see Section 3). Data were used from post-set (left) and véraison (right) treatments separately. A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

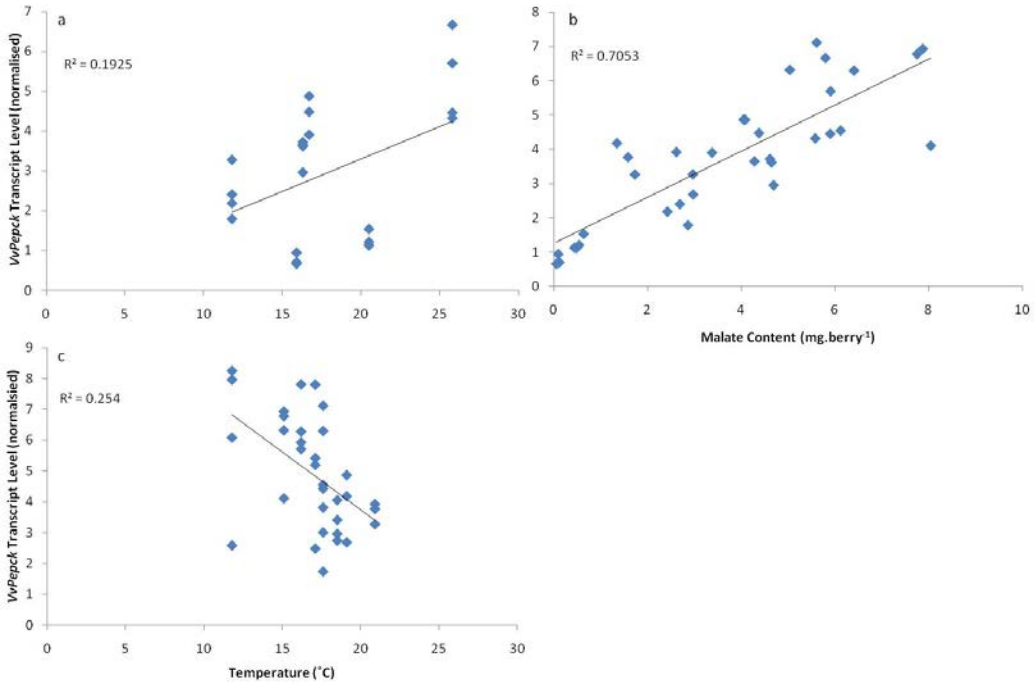


Figure A.4.7. PEPCK transcript Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of PEPCK gene transcript with temperature data (left; ABM, 2008) and malate content (right) of the developmental data series (see Section 2). Data were used from pre-véraison (a), post-véraison (b,c) developmental stages separately. A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

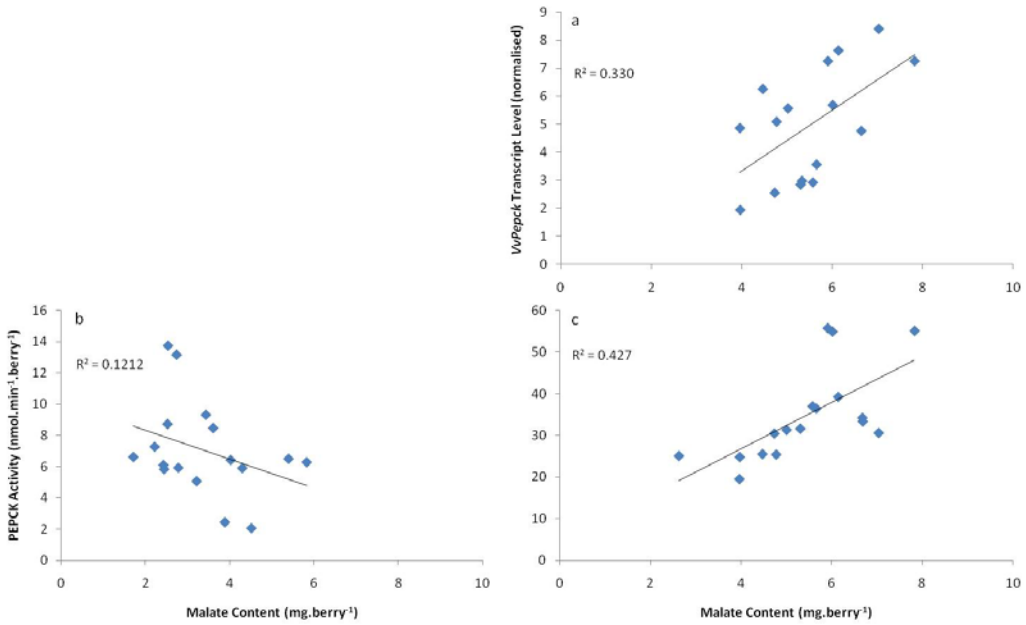


Figure A.4.8. PEPCK transcript and activity Vs malate content from heat treatments. Scatter plot comparison of individual measurements of PEPCK gene transcript (top) and activity (bottom), with malate content of the heat treatment data series (see Section 3) from post-set (left) and véraison (right). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

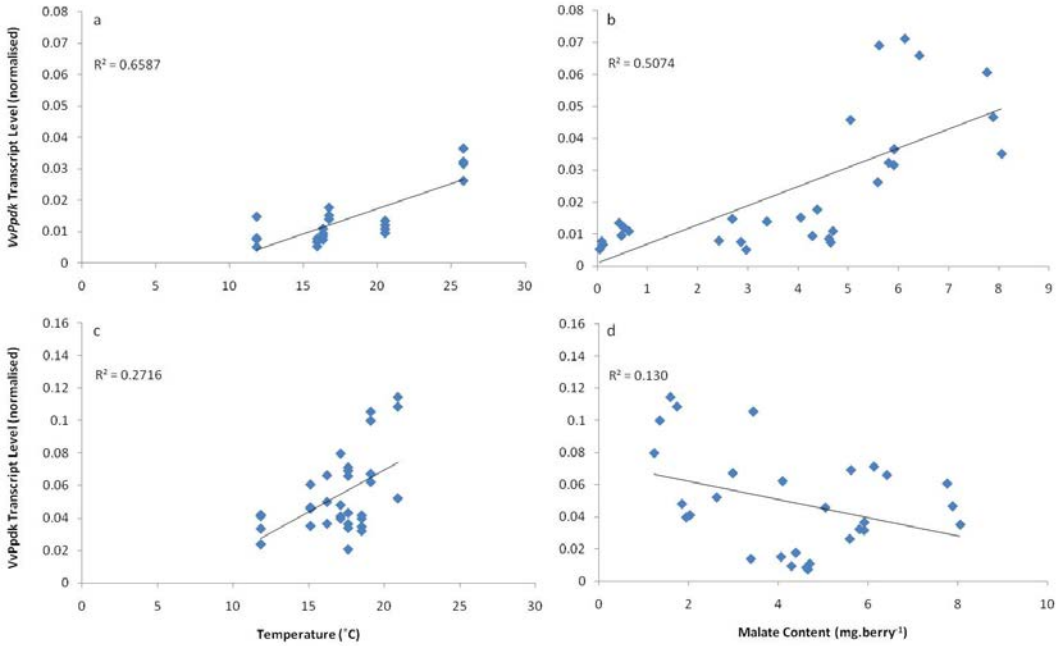


Figure A.4.9. PPDK transcript Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of PPDK gene transcript with temperature data (left; ABM, 2008) and malate content (right) of the developmental data series from pre-véraison (top) and post-véraison (bottom) stages (see Section 2). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

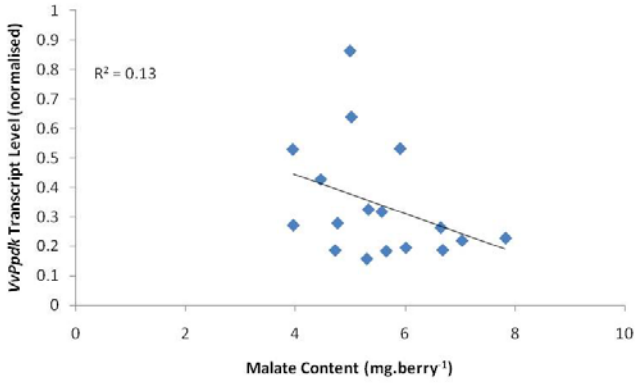


Figure A.4.10. PPDK transcript Vs malate content from heat treatments. Scatter plot comparison of individual measurements of PPDK gene transcript, with malate content of the veraison heat treatment data series (see Section 3). A trend line of linear regression was fitted, with R^2 value indicated in the top left corner.

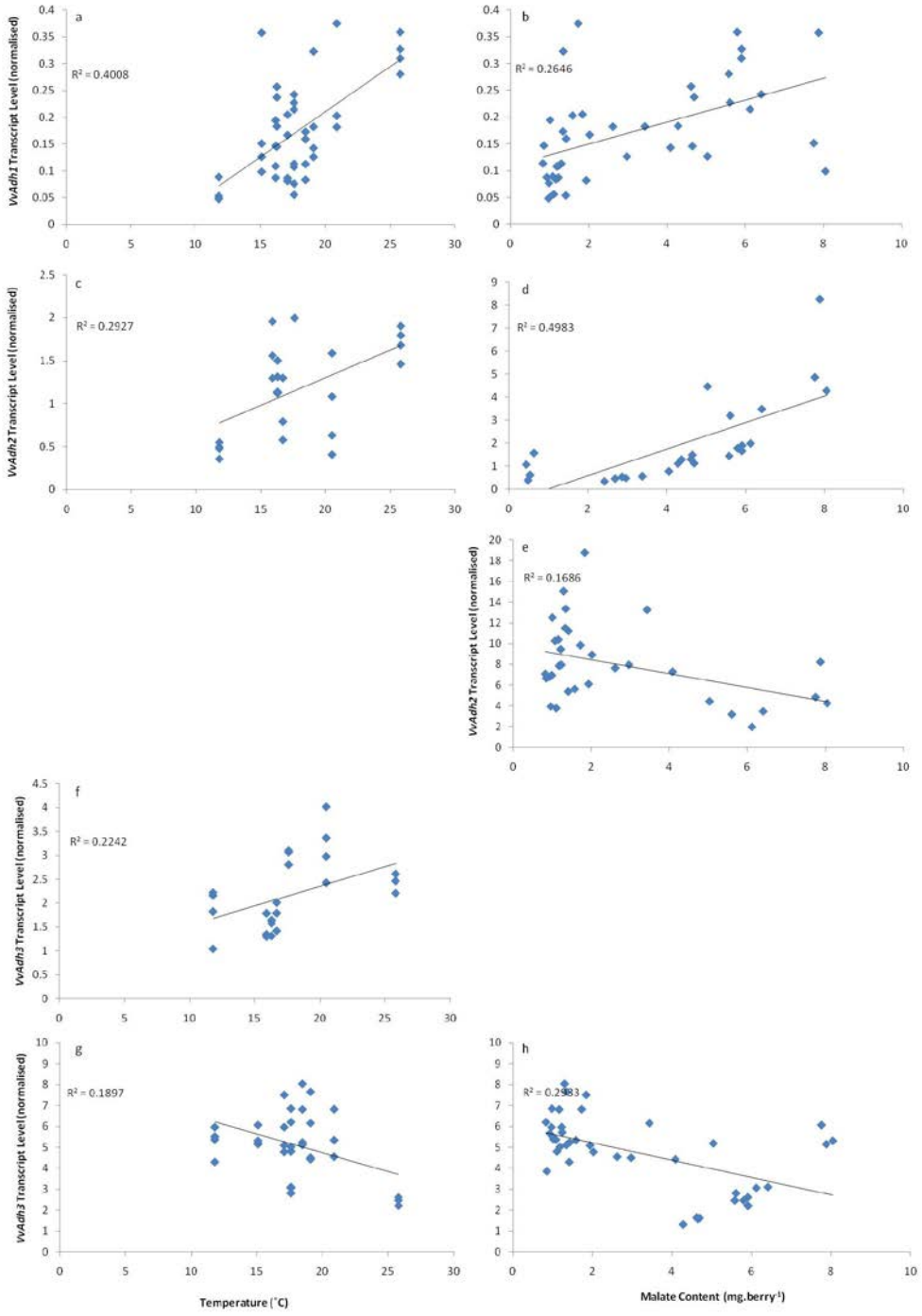


Figure A.4.11. ADH gene transcripts Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of ADH gene transcripts with temperature data (left; ABM, 2008) and malate content (right) of the developmental data series (see Section 2). Data were taken from pre-véraison (c,d,f) and post-véraison (a,b,e,e,g,h) stages separately. A trend line of linear regression was fitted, with R^2 values indicated in the top left corners of each plot.

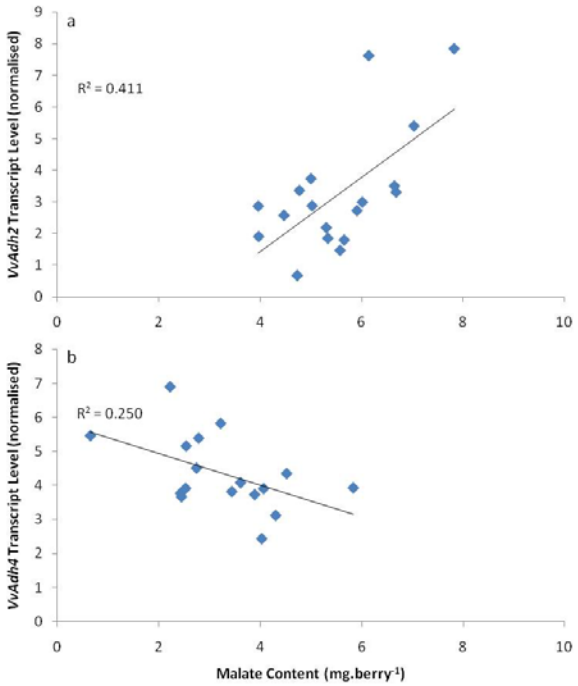


Figure A.4.12. ADH transcripts Vs malate content from heat treatments. Scatter plot comparison of individual measurements of ADH gene transcripts, with malate content of the heat treatment data series (see Section 3). Data were taken from post-set (b) and véraison (a) treatments separately. A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

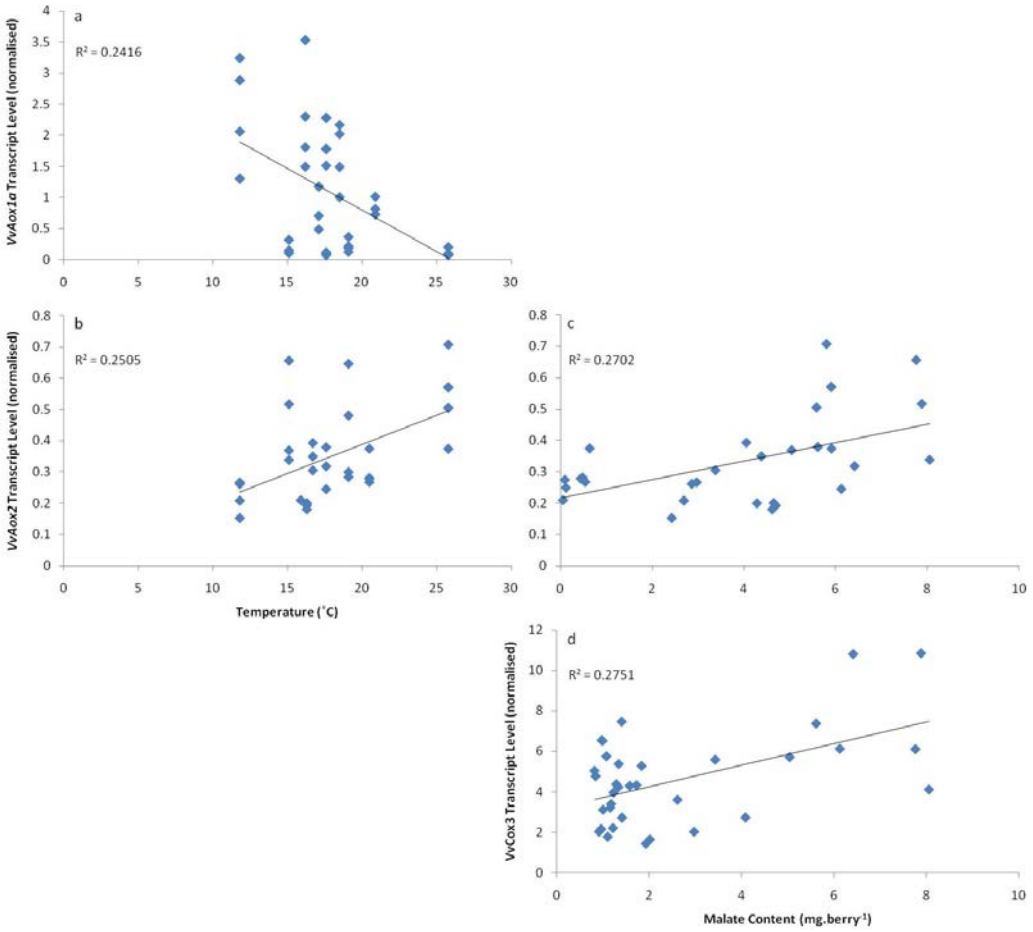


Figure A.4.13. AOX and COX transcripts Vs temperature and malate content from developmental series. Scatter plot comparison of individual measurements of AOX and COX gene transcripts with temperature data (left; ABM, 2008) and malate content (right) of the developmental data series (see Section 2). Data were taken from pre-véraison (b,c) and post-véraison (a,d) stages separately. A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

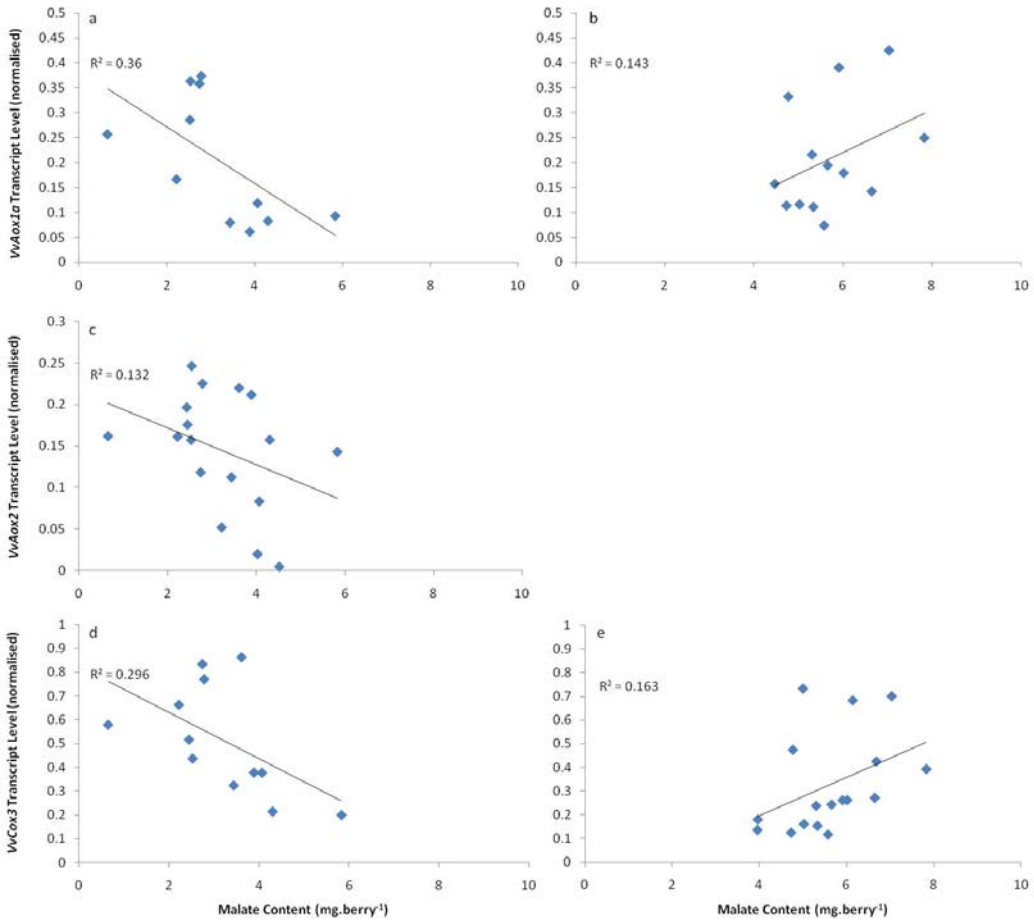


Figure A.4.14. AOX and COX transcripts Vs malate content from heat treatments. Scatter plot comparison of individual measurements of AOX and COX gene transcripts, with malate content of the heat treatment data series (see Section 3) from post-set (left) and véraison (right). A trend line of linear regression was fitted, with R^2 values indicated in the top left corner of each plot.

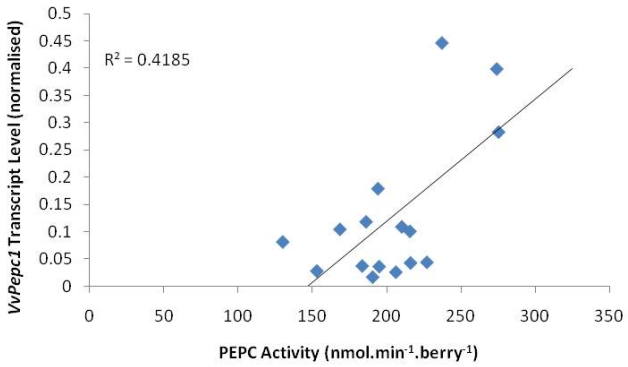


Figure A.4.15. PEPC transcript Vs PEPC activity from heat treatments. Scatter plot comparison of individual measurements of *VvPepc1* gene transcript, with PEPC activity from the veraison heat treatment data series (see Section 3). A trend line of linear regression was fitted, with R^2 value indicated in the top left corner.