DISSERTATION

# INFLUENCE OF PREHARVEST FACTORS ON PEACH FRUIT QUALITY AND METABOLISM

Submitted by

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ABSTRACT

INFLUENCE OF PREHARVEST FACTORS

ON PEACH FRUIT QUALITY AND METABOLISM

Worldwide peach consumption has been in steady decline throughout the past few decades due to poor fruit quality and consumer experiences. Fruit quality is developed in the orchard by optimizing preharvest factors and orchard practices. Several studies have been conducted to understand how these factors influence peach internal quality, but fail to control for confounding variables. One particular confounding variable that is influenced by preharvest factors and directly impacts fruit quality is maturation. Pomological experiments investigating the impact of preharvest factors on internal fruit quality must control for maturity. Historically, maturity control through destructive and subjective methods was not feasible nor efficient. The development of new technologies, such as visual radiation and near-infrared spectroscopy allowed the development of novel maturity indices (index of absorbance difference) that can be used for maturity control and quality assessment simultaneously in a single scan. The following literature review and experiments investigate three critical preharvest factors: training systems, canopy position and crop load (i.e., carbon supply), for their true impact on peach fruit quality development and metabolism, while controlling for maturity. The training system review demonstrates the progression of orchard design from three-dimensional, low-planting densities to planar, high-density plantings through the application of size-controlling rootstocks and vigor diffusion architecture. The canopy position trial revealed that the fruit's light environment is more influential in quality development and metabolic shifts than genotype or position alone. Canopies with

uniform light distribution generate fruit of uniform quality and metabolite profiles across distinct positions. Fruit under sufficient carbon supply (i.e., thinned fruit) will exhibit superior quality and phenotype when compared to carbon-starved fruits at harvest, even when assessed at equal maturity. Primary metabolite profile differences between distinct carbon supply conditions are minimal at harvest due to experimental maturity control and metabolic processes being heavily regulated by development and maturation. While differences in secondary metabolite profiles are more distinct at harvest between carbon supply treatments. Although, both the primary and secondary metabolism demonstrate vast profile differences between carbon supply treatments early, and may prime the quality phenotype at harvest. Flavonoids are consistently elevated in carbon sufficient fruit throughout development. Phenylpropanoids, such as catechin, along with benzenoids, sucrose and sorbitol demonstrate strong relationships with high-quality fruit throughout experiments, while lipids, amino acids, monosaccharides and organic acids showcase relationships with inferior quality fruit. Overall, maturity control is necessary in pomological experiments assessing the true impact of preharvest factors on fruit quality and metabolism.

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# DEDICATION

To the Lord, where my help comes from.

The Maker of heaven and earth.

To my Father, Michael:

To the man with more degrees than anyone in the world.

The biggest supporter, encourager and advocate of any of my pursuits.

To the man whose shoes I can only hope to fill one-tenth of, one day.

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CHAPTER ONE

# OPTIMIZING PEACH TREE CANOPY ARCHITECTURE FOR EFFICIENT LIGHT USE, INCREASED PRODUCTIVITY AND IMPROVED FRUIT QUALITY

## 1. Introduction

Peach (*Prunus persica* (L.) Batsch.) is ranked as the 6th most important tree fruit crop in the world. China is the number one producer of peaches worldwide at nearly 15 million metric tons (MMt) per year, with Spain (1.8 MMt), Italy (1.3 MMt), Greece (0.9 MMt) and the United States (US) (0.8 MMt) ranked as the 2nd–5th producers, respectively (FAO, 2019). Within the United States, the top seven producing states (in order) include: California, South Carolina, Georgia, New Jersey, Pennsylvania, Washington and Colorado (USDA-NASS, 2019). California produces over 500,000 tons per year for a total value of USD 350 million, while in Colorado, production is far less, at nearly 15,000 tons per year for a total value of USD 35 million. Global peach consumption per capita has experienced significant decreases over the past few decades. In the US alone, peach consumption per capita has been constantly decreasing to 1.3 kg/person, at present day (Minas et al., 2018) (Figure 1.1). Consumer surveys have revealed that the reason peach consumption has declined is due to poor quality fruit that are tasteless, not at optimum maturity (e.g., overripe, too green) or have abnormal texture due to postharvest disorders (Crisosto and Labavitch, 2002). The decreased demand for peaches in US is driving peach production down, as peach prices in the major producing state (CA) are low in a steady increasing cost of production (Figure 1.1).

Interestingly, there is still demand for high-quality peaches and consumers are still willing to pay additional money for them. For example, in CA, the top peach producer in US, the farmgate price for peaches is nearly USD 0.6 $kg^{-1}$ (USDA-NASS, 2019), while in CO, where the highest

farmgate price per kg in the country is recorded, the price is at USD 2.2 $kg^{-1}$ (USDA-NASS, 2019). This increased farmgate price is attributed as a "quality premium," due to the exceptional quality of Colorado peaches. Colorado's increased quality is a result of the climate (warm days/cool nights), frequent spring frosts leading to low crop loads and access to local markets, which enables harvesting at the "tree ripe" stage (fruit firmness between 30 and 36 N) (Minas et al., 2018). This example demonstrates the strong relationship between peach fruit quality and the profitability/sustainability of the peach industry.

To ensure a sustainable peach industry worldwide, reducing the cost of production, increasing yields and improving fruit quality must be the main focus for producers, researchers and breeders alike (Minas et al., 2018). Peach fruit quality can only be developed and enhanced in the orchard through the optimization of preharvest factors, while quality can only be maintained postharvest (Crisosto and Labavitch, 2002). Influential preharvest factors that impact fruit quality include: cultivar and rootstock selection, crop load management, fruit position in the canopy, irrigation, fertilization, pruning and training systems (Minas et al., 2018) (Figure 1.2).

Training systems manipulate the canopy architecture to achieve various goals of producers. In short, an ideal training system maintains optimal levels of light interception, uniform light distribution and facilitates high yields of high-quality fruit. This article provides a focus on the development and modernization of training systems in peach production, and how they are being optimized.

## 2. Fruit Quality

Fruit quality is defined by various properties, such as sensorial (e.g., appearance, texture, taste, aroma), mechanical (e.g., mass, volume, density, firmness) and nutritional value properties (e.g., vitamins, antioxidants, polyphenols) (Crisosto and Costa, 2008). A producer may define

quality by the characteristics that improve pack-out and profitability (e.g., fruit weight, size, free of defects, etc.), while a consumer may prioritize sensory, nutritional and safety aspects. Overall, to increase peach consumption, growers and researchers alike must be focused on improving the characteristics customers desire, such as taste, sweetness, color and aroma to encourage repeat sales (Crisosto and Labavitch, 2002).

Generally, peach quality is linked to the sugar content and sugar/acid ratio in the fruit, which is perceived as sweetness (Crisosto and Crisosto, 2005). Industry standards for optimal peach quality currently range between ~10 and 12% soluble solids concentration (SSC) (or Brix°), depending on the cultivar, along with titratable acidity (TA) levels below 0.7% (Minas et al., 2018; Crisosto and Crisosto, 2005; Testoni, 1995; Hilaire, 2003). Another critical parameter in respect to fruit quality is dry matter content (DMC, %) and is highly correlated with SSC (Minas et al., 2021). Previous studies in other tree fruit crops have demonstrated significant relationships between elevated DMC levels and increased consumer acceptance (Minas et al., 2018). Another critical component of postharvest and consumer performance is flesh firmness (FF) at harvest, which has historically characterized maturity status, along with ground color observations. In general, fruits exhibiting FF values between 45–54 N are classified as "well-mature" or noted as the "commercial harvest" stage, as they are less susceptible to bruising and can be stored/shipped longer. Fruits that are less firm (30–36 N) at harvest are known as "tree ripe," cannot be stored/shipped long and must be sold locally. However, FF values and their corresponding physiological maturity stage are influenced by the flesh texture typology, as some types such as "non-melting" or "stony hard," may delay softening or never become soft (Serra et al., 2020). However, one critical consideration in respect to quality and maturity, is that fruit that are more mature will have improved quality.

As a climacteric fruit, peach undergoes a spike of ethylene during ripening, accompanied by a peak in respiration, which translates into a cascade of highly regulated physicochemical changes (Giovannoni et al., 2017). Throughout maturation and ripening "on-tree," DMC and SSC increase, FF and TA decrease, pigments are accumulated, and aromatics are volatilized, all of which contribute to organoleptic attributes that consumers perceive and prefer (Crisosto and Costa, 2008). Several preharvest factors influence both maturity and quality at harvest (Minas et al., 2018) (Figure 1.2). Therefore, when conducting pomological experiments, it is critical to control for maturity in order to understand the true impact of pre- and postharvest treatments on fruit quality (Anthony et al., 2020a). However, until recently, controlling for maturity was extremely difficult to do, as destructive methods (e.g., FF assessment) are labor intensive, sample size limiting and not always indicative of the true physiological maturity status of the fruit (Minas et al., 2021). Further, ground color measurements to assess physiological maturity are subjective and cannot apply to cultivars that demonstrate a fully red overcolor early in the season, which has led to premature picks of immature/poor quality fruit in the past (Minas et al., 2021). With recent advancements in technology and the development of an index for maturity known as the index of absorbance difference ($I_{AD}$) (Ziosi et al., 2008; Costa et al., 2009), physiological maturity assessments and subsequent confounding variable control can be carried out rapidly, non-destructively and over a large sample size of fruit (Minas et al., 2021). This index can be integrated into open-source handled near-infrared spectroscopy (NIRS) instruments, along with additional parameters such as SSC and DMC, to evaluate both peach fruit maturity and quality rapidly and accurately in a single-scan (Minas et al., 2021).

Several experiments have been conducted to evaluate the role of preharvest factors such as rootstocks and training systems on peach fruit quality, but have failed to control for maturity. In

fact, these results are limited in application with a confounding variable present. Therefore, future studies should aim to control for maturity in their experimental design to better understand the true impact these preharvest factors have on fruit quality development (Anthony et al., 2020a).

**3. Rootstocks**

Before training systems can be discussed, an investigation into the pre-existing and new rootstock cultivars is imperative as tree size and vigor dictates the feasibility and optimization of any training system, canopy architecture and orchard design. Rootstocks provide a horticultural tool to control scion vigor, increase yields and yield efficiency and facilitate canopy architecture arrangements that can improve fruit quality (Reighard and Loreti, 2008). Typical challenges peach producers face in respect to soils, include high bulk density, high pH, nematodes, fungal pathogens, orchard replant disease syndrome, and texture issues (e.g., too clayey/too sandy) (Reighard and Loreti, 2008). These challenges promote objectives for rootstock breeding programs worldwide, so producers can ameliorate these difficulties through optimal selection of a particular rootstock genotype for specific pedoclimatic conditions.

Historically, peach has been planted into low-density plantings (LDPs), which are characterized by wide inter- and intra-row (i.e., between trees) spacings with vigorous peach seedling (*P. persica*) rootstocks. Although these peach seedling rootstocks have long been the mainstay of peach production globally, they are increasingly being criticized for their: inability to control tree vigor, withstand high soil pH and tolerate drought/waterlogging, nematodes, crown gall, fungal pathogens and replant disease (Reighard and Loreti, 2008; Layne, 1987). However, due to increased production costs, lack of pest control efficacy or resistances, and diminishing land available for fruit production, the development of interspecific *Prunus* rootstocks to overcome these abiotic, biotic and resource challenges has begun.

Several new genotypes are being bred all over the world in countries such as the US, Spain, Italy, Russia and France (Table 1.1, Figure 1.3). In particular, several interspecific *Prunus* hybrids, almond species and plum species are replacing the traditional peach seedling rootstocks in Europe and North America and are characterized by different vigor classifications (Reighard et al., 2015; Reighard et al., 2020). (Table 1.1, Figure 1.3). In particular, 'GF 667', a peach x almond hybrid, has found tremendous success in European peach production due to its ability to resist nematode infestation, replant disease, thrive in calcerous, poor and/or arid soils and be propagated easily in the nursery (Loreti and Massai, 2002). However, it maintains a vigorous classification and inhibits the potential of exploiting the advantages of higher-density plantings (Loreti and Massai, 2002). Characteristics now being considered in the breeding process for these new stocks include: propagation behavior, graft compatibility, vigor control, production efficiency and fruit quality (Reighard et al., 2015; Reighard et al., 2020; Loreti and Massai, 2006).

In the USA, the USDA, public universities, and private institutions have contributed to rootstock breeding. In the early 20th century, the various breeding programs goals were originally focused on identifying seedling genotypes with resistances to abiotic and biotic factors. In particular, nematode resistance was a large research priority, in which popular cultivars such as Nemaguard and Guardian® were developed and released. More recently, the University of California at Davis (UC-Davis) in the USA, was focused on developing peach-almond hybrids that can withstand calcareous soils with high pH, in which 'Hansen 536' was developed (Reighard and Loreti, 2008). Similarly, private entities such as Bright's Nursery, inc. released their Bright's Hybrid® series and Zaiger Genetics, inc. released other interspecific rootstocks such as 'Viking,' 'Citation' and 'Atlas' (Reighard and Loreti, 2008). In general, these rootstocks were developed to withstand issues with soil characteristics and pests, and not necessarily bred to control for vigor.

In the USA, a working group known as the North Central (NC) Regional Research Project 140 (NC-140) evaluates several new and existing rootstocks across the country through multi-state collaborative experimental trials (Reighard et al., 2015; Reighard et al., 2020; Perry et al., 2000). Among other important temperate tree fruit species NC-140 seeks to evaluate the compatibility and performance of *Prunus* rootstocks with peach cultivars across several states and years (Reighard et al., 2015; Reighard et al., 2020).

A primary focus of recent rootstock selection efforts is vigor control, as to facilitate higher density plantings, similar to the transition in apple and cherry (Autio et al., 2017; Autio et al., 2020; Musacchi et al., 2015; Lang, 2019; Robinson et al., 2013). Although dwarfing rootstocks have been bred, some genotypes negatively impact fruit size, as the vigor control mechanism in particular genotypes restricts xylem vessel diameter. Two series of *Prunus* interspecific hybrid rootstocks that have demonstrated promising size controlling genotypes include: the Controller™ series from UC-Davis, USA and the Rootpac® series from Agromillora, Spain (Table 1.1, Figure 1.3). Several of these genotypes are currently under evaluation in current NC-140 trials in the USA (Minas et al., 2020). In sum, dwarfing hybridized *Prunus* rootstocks, with various abiotic/biotic resistances, are becoming more and more available, which are facilitating the advancement of training system innovation and increased planting densities for peach production worldwide.

**4. Training Systems**

Peach training systems span from traditional, complex 3D canopy architectures, with multiple leaders per tree, to more modern high-density, simple/planar designs with single or multiple leaders per tree. The shift to modern orchard design is facilitated by genetic and horticultural manipulations that control vigor (e.g., low-vigor cultivars, dwarfing rootstocks, pruning/training, etc.) in order to increase planting density, production per hectare, light

interception, light distribution and fruit quality (Faust, 1989; Grossman and DeJong, 1998). Some of the main training systems for peach include: low to medium density multi-leader systems such as open vase, delayed vasette, Quad-V and Hex-V, along with higher density systems such as palmette/hedgerow, Y-shaped (e.g., Kearney Agriculture Center-V (KAC-V), bi-axis), and central leaders (e.g., Fusetto, Tall Spindle Axe (TSA), Slender Spindle Axe (SSA)) (Table 1.2, Figures 1.4-1.5) (Minas et al., 2018; Corelli-Grappadelli and Marini, 2008).

Traditional multi-leader 3D systems, such as the open vase and delayed vassette, can yield a higher amount of fruit per tree, given the larger canopy volume, but these systems produce less on a per land area basis given their lower densities (Grossman and DeJong, 1998). Additionally, these canopies may intercept a higher amount of light at the top/exterior portions of the tree, but often the bottom/internal portions are shaded. This is especially true for peach trees, as 80% of leaves are located in the top 40% of the tree in these 3D systems (Chalmers et al., 1983). Génard and Baret (1994) demonstrated the variability of light distribution in open vase systems, reporting that 30% of shoots received < 30% of available light, which is below the critical threshold for floral bud induction (Corelli-Grappadelli and Marini, 2008). The shade in these interior/basal portions of the canopy, especially in high vigor cultivars/rootstocks, leads to reduced tree performance, yields and fruit quality (reduced color and SSC) (Gullo et al., 2014). Subsequently, this can lead to lower crop loads in the lower/interior parts of the canopy and an excessive vegetative vigor response, which can only exacerbate the problem of poor light distribution, unless summer pruning (or other vigor control) interventions are used. Grossman and DeJong (1998) reported that open vase systems intercepted less light and produced less (Mt $ha^{-1}$) than higher density 2D cordon and KAC-V systems. Furthermore, these smaller tree, high-density planting (HDP) training systems facilitate better light distribution, so interior/basal portions of the canopy

do not decline in yields and can maintain higher crop loads (Robinson, 1997). Nuzzo et al. (2003) confirms this trend, reporting that when comparing a Y-system with an open vase system in peach, the Y-system resulted with higher levels of leaf area index (LAI, leaf area: ground area), light interception (LI, %) and yields. However, it is important to note that training system selection, along with its pruning/management, must be contextualized to cultivar/rootstock selection (Lauri and Corelli-Grappadelli, 2014).

*4.1. Low-Density Planting Systems*

Low-density plantings (LDP) generally include 200–550 trees per hectare, with heights ranging from 2.2–5.0 m, as inter-rows are typically wider (4.0–5.0 m) and can facilitate taller trees (Table 1.2). These systems are accompanied with more vigorous rootstocks and generate larger canopies, when compared to other medium-density planting (MDP) and HDP systems. The most notable peach training system, open vase, along with other free standing multi-leader systems, typically do not require trellising, as the permanent scaffolds and structure are strong enough to support the fruit load alone. This, paired with the reduced number of trees required at planting can minimize orchard establishment costs. However, their light interception remains low on a per hectare basis (< 50%), and their lack of uniform light distribution leads to yield and quality deficits (Grossman and DeJong, 1998).

4.1.1. Open Vase

The open vase system has been the traditional training system for peach production worldwide. In CA, it has been the dominant system for over a century and continues to be the most widely used in the state, as well as in most major peach producing countries around the world (20,37). The open vase typically consists of three to five primary scaffolds emanating from a short trunk and will split into secondary/tertiary scaffolds deriving from each primary scaffold (DeJong

et al., 2008). Open vase plantings include planting densities of 3.5–5.0 (intra-row) × 4.0–5.0 (inter-row) (Table 1.2, Figures 1.4-1.5) and can be arranged in either a square/rectangle, offset square/diamond or hexagonal/equilateral triangle design to manipulate land/light use efficiency (DeJong et al., 1989).

The open nature of this system can facilitate proper illumination to most areas in the canopy, but watersprouts and vigorous branches growing in the center of this 3D canopy can negatively impact fruit yields and quality. This canopy architecture does allow easy access to fruiting shoots for various management operations (e.g., pruning, thinning, harvesting), although typically requires ladders and can only facilitate low-density plantings of 600 trees $ha^{-1}$ or less (DeJong et al., 2008). Additional drawbacks to this system include the length of time needed to train these systems from planting to full production maturity (Figure 1.5), tree heights exceeding 4 m (hence the need for ladders) and the potential for excessive complex canopies (DeJong et al., 2008). For example, each open vase system maintains three to five primary scaffolds with two or three secondary scaffolds, and then an additional eight to 14 tertiary branches per each secondary scaffold, which can create a highly complex canopy (Figures 1.4-1.5) (Day et al., 2005). Increasing branches and/or growing points does not necessarily equal increased production, as light distribution needs to be considered for optimal yields and quality. Further, this complexity makes it difficult to increase labor efficiency, let alone the potential for mechanization of expensive orchard tasks (Day et al., 2005). However, when these systems are managed properly, they can produce large quantities of high-quality fruit (DeJong et al., 2008).

*4.2. Medium-Density Systems*

Medium-density plantings are typically planted around 3.0 × 4.5 m (600–1,000 trees $ha^{-1}$) and reach approximate heights of 3.0 m (Table 1.2, Figure 1.4). These orchard systems were

developed in an attempt to diffuse vigor, decrease tree height and reduce canopy complexity. As a result, alternative multi-leader systems were developed. These MDP multi-leader systems include the delayed vasette, Quad-V and Hex-V, while spindle systems such as the palmette represent single leader MDP systems. As a result of reduced tree heights and canopy complexity, trees can be planted at closer densities, achieving a larger number of trees and/or leaders per ha (Table 1.2). These MDP systems are a great compromise for growers who wish to maintain lower intensity management, while increasing land unit production without excessive orchard establishment costs.

4.2.1. Delayed Vasette

The delayed vasette is similar to an open vase system in terms of canopy architecture, but differs in how/when the scaffolds are developed. Similar to the open vase, the delayed vasette is developed with approximately four main scaffolds, originating at 50 cm above the ground. However, it differs in that the central leader is maintained in the center of the four main scaffolds for the first two to three years, in an attempt to distribute the vigor and encourage earlier fruiting production (Loreti and Massai, 2002). After two to three years, the central leader is completely removed, leaving behind a similar open vase structure, with four main scaffolds, with improved light penetration to the bottom portions of the canopy as the tree enters into maturity. This system is characterized by early bearing, high productivity and a mature architecture by year three or four (Neri et al., 2015). The delayed vasette is a MDP solution for growers who wish to increase their planting densities to approximately 600–800 trees $ha^{-1}$ (Table 1.2, Figure 1.4), and yet maintain a similar open vase style canopy architecture (Loreti and Massai, 2002).

4.2.2. Palmette

The palmette system is a very popular peach training system in central-northern Italy, where spring frosts are more frequent and require taller canopy architectures to minimize crop loss

due to temperature inversions (i.e., radiative frost). The palmette system consists of a central leader with six or more branches inserted into the main stem at approximately 45-degree angles, with canopies reaching heights of four to five meters (Loreti and Massai, 2002). Given the height of these trees, ladders or platforms are required for orchard management tasks. Planting densities for the palmette system include 2.0–3.5 × 4.0–4.5 m, achieving densities from 600–900 trees $ha^{-1}$ (Table 1.2, Figure 1.4). Although, some authors believe these systems can be pushed towards higher densities, reaching 700–1,100 trees $ha^{-1}$ in peach (Corelli-Grappadelli, 1998). The goal of the palmette system was to achieve a "true" fruiting wall, capable of inducing early yields and integrating the use of platforms for labor reduction and mechanization. The use of platforms for picking/pruning in the palmette system can simplify laborious tasks due to the planar and homogenous canopy architecture. If trees are planted too close, intra-tree shading can be a negative result, contributing to poor production and inferior peach fruit quality (Corelli-Grappadelli, 1998). Therefore, the palmette system should be paired with the use of plant growth regulators, available semi-dwarfing rootstocks and low-vigor growing conditions to ensure uniform productivity, maturation and quality within the tree/orchard (Corelli-Grappadelli, 1998).

4.2.3. Quad-V

The Quad-V system developed by Day et al. (2005; 1993) to further diffuse the vigor of higher-density two-leader systems such as the parallel and perpendicular V-systems (i.e., KAC-V) (Section 4.3.2), along with reducing orchard establishment costs by reducing the number of trees required to achieve the same number of leaders per acre (DeJong et al., 2008). This system mirrors the perpendicular V-system, but instead of a single pair of leaders extending over the inter-row, two sets of two-primary scaffolds are present (Figures 1.4-1.5). The Quad-V system ensured the same high yields, high light interception and canopy uniformity as the HDP system, KAC-V, while

reducing the number of trees required at planting. Further, the Quad-V simplified the architecture of an open vase system (LDP), promoting a compromise between the KAC-V and open vase systems (DeJong et al., 2008; Day et al., 2005). Typical planting densities for Quad-V systems range from 4.5 m in the inter-row to 2.5–3.0 in the intra-row (900–1,000 trees $ha^{-1}$; Table 1.2, Figure 1.4). Given the number of scaffolds diffusing the vigor of the tree, tree heights will typically be lower, especially if the angle of the scaffolds is bent more towards the horizontal. However, wider angles may produce increased watersprouts in the central portion of the canopy and require extensive summer pruning (Day et al., 2005). Overall, the Quad-V system became very popular in California and remains an optimal balance between LDP, open vase and HDP, KAC-V systems (Day et al., 2005).

4.2.4. Hex-V

The Hex-V is a further compromise for growers wishing to maintain lower-density plantings as a way to avoid expensive establishment costs, as Hex-V systems are typically planted at lower densities than the Quad-V. Hex-V orchards can maintain approximately 750 trees $ha^{-1}$ and are planted at densities approximately 3.0 m × 4.5 m (Table 1.2, Figure 1.4). The Hex-V system can either be developed with six primary scaffolds that are oriented with three scaffolds on either side of the alleyway, or with three primary scaffolds, which develop two secondary scaffolds each in a similar manner to an open vase system (Figures 1.4-1.5). The contrast between the open vase and the Hex-V is that the secondary scaffolds in the Hex-V do not continue to fork and promote tertiary branches. Only small fruiting shoots are developed along the scaffolds, allowing enhanced canopy uniformity, optimal light distribution and high light interception (DeJong et al., 2008). The benefit of shifting from the Quad-V (four scaffolds) to the Hex-V (six scaffolds) is that the increased number of leaders further help diffuse the tree/rootstock vigor to promote smaller

canopies (< 2.5 m in height). This results in trees that can be managed more easily and in some cases without ladders, given the further reduction in tree height (Table 1.2, Figures 1.4-1.5).

*4.3. High-Density Planting Systems*

High-density planting systems are typically planted around 1.5 × 4.0 m (1,000–2,000 trees $ha^{-1}$) and reach heights ranging 3.0–5.5 m (Table 1.2, Figure 1.4). Training systems used in HDPs, such as the central leader iterations (Fusetto, Tall Spindle Axe, Slender Spindle Axe) and Y-systems maximize land area production and light interception, while maintaining light distribution throughout the canopy. This allows for higher crop loads, which may reduce fruit size, but allows for improved SSC, DMC, color, and overall quality, due to improved light characteristics in the tree. The up-front cost for the higher number of trees and potential trellising may be a potential financial barrier of entry for growers, but these costs may be recouped quickly due to increased precocity and early yields obtained in these systems. Furthermore, HDPs may require more intensive management and horticultural knowledge, but the simple design may allow for the potential of mechanization, platforms and robotics to reduce labor time/costs. Some examples of intensive management and horticultural knowledge necessary in HDP systems include the use of plant growth regulators (PGRs) (e.g., gibberellin-inhibitors) and size-controlling rootstocks.

4.3.1. Central Leader Systems (Fusetto, Tall Spindle Axe, Slender Spindle Axe)

The Fusetto system is an Italian adaption of the slender spindle axe (SSA) system, which is widely popular in apple production. The Fusetto system is a central leader system, which develops seven to eight branches originating from the main axis (Loreti and Massai, 2002). On average, the Fusetto system is planted at spacings of 2.0 × 4.0 m (~1,250 trees $ha^{-1}$), although densities may vary based on soil, climate and growing conditions (Table 1.2). Trees are grown to heights of 2.8–3.5 m, ensuring not to exceed the inter-row spacing (Table 1.2, Figure 1.4). In

contrast, the Tall Spindle Axe (TSA) is typically grown to taller heights (3.0–3.7 m), although the TSA retains a similar canopy architecture to the Fusetto. Both the Fusetto and TSA are trained in a conical fashion, with larger, more dominant branches in the basal portion of the tree, while branches recede in size as they reach the apex portion of the canopy (Loreti and Massai, 2002). The shape of this system allows branches at the basal portion of the canopy to extend further into the inter-row in order to maintain high levels of light interception and subsequent production in the bottom of the canopy (Figures 1.4-1.5). However, these canopies can often be too large in respect to volume (i.e., high leaf area/density), leaving internal and/or bottom portions of the canopy with excessive shade and poor fruit quality and production. Therefore, the use of size-controlling rootstocks, PGRs, summer pruning, watersprout removal and reflective fabrics on these higher density systems may be advised to ensure optimal light interception, penetration and distribution values.

4.3.2. Y-Shaped Systems (Tatura Trellis, KAC-V, Bi-Axis)

The perpendicular-V or Y-shaped systems are characterized by two scaffolds that extend over the inter-row (i.e., tractor alleyway) with fruiting shoots grown on the exterior in a "herringbone" pattern (Figure 1.4), with shoot renewal occurring with each season (Loreti and Massai, 2002). The goal behind the development of these trees was to increase planting densities and increase light interception, by maximizing light in the interior of the canopy at solar noon. The original Y-system was the Tatura trellis, which was developed in Australia (Chalmers et al., 1978). In the following years, many iterations of these Y-shaped systems were developed, with different angles and densities utilized (Loreti and Massai, 2002). One of the most popular iterations was the KAC-V system (Figure 1.5), which was developed at UC Davis, in California, in 1982 as a hybrid of the traditional open vase system and the Tatura trellis (DeJong et al., 1994).

The criteria for the development of the KAC-V were to: increase production in the early years after planting, maintain yields similar to open vase systems, reduce the need for summer pruning, maintain similar row spacings for tractors, increase the labor use efficiency for various orchard tasks (e.g., pruning, thinning and harvesting) and increase light use efficiency (DeJong et al., 1994). The two leaders are selected in the first year, while the use of summer and dormant pruning to remove competing branches is conducted to ensure a simplified canopy (Figure 1.5). Long fruiting shoots are not to be perennial, but rather shoot renewal is conducted each year during the dormant season to adjust for an appropriate crop load by leaving the associated number of "hangers" left on the tree to fruit (1–2 fruit/hanger; i.e., fruiting shoot) (DeJong et al., 1994). One major benefit of the KAC-V system is the lack of trellis requirement, as these scaffolds are developed to be strong and free-standing. However, one major drawback of this system is the frequent need for summer pruning (two—three times a season) in the internal/basal portion of the canopy, where the development of watersprouts/suckers are highly prevalent (Figure 1.5) (Loreti and Massai, 2002). Therefore, the use of watersprout removal (WSR) is advised to ensure proper illumination in the dorsal/internal portions of the canopy. Strong heading cuts on these watersprouts (leaving 15 cm) should be made instead of complete thinning cuts to provide a small amount of leaf area/shade to prevent sunburn on the scaffolds (DeJong et al., 1994). These headed shoots can then be completely removed in the dormant season. Lastly, given the perpendicular orientation of the KAC-V, it is difficult to mechanize tasks parallel with the tractor row, as well as in the internal portions of the canopy. In sum, the KAC-V system reduces the complexity of the open vase system, arranges the canopy and fruiting shoots into manageable functional units to better manage crop load, intercepts high amounts of light and achieves higher densities of ~1,000 trees $ha^{-1}$ (DeJong et al., 1994).

The Bi-Axis is a similar Y-shaped system but it maintains two leaders in the parallel direction of the row and can therefore create a homogenous, continuous fruiting wall (Figure 1.5). The Bi-Axis is a combination of the KAC-V and the Fusetto. The Bi-Axis can be planted at high densities (> 2,000 trees $ha^{-1}$), but it can also achieve and/or increase the total number of leaders per hectare with less trees (Table 1.2, Figure 1.4). This is a major benefit for growers wishing to reduce upfront orchard establishment costs. The Bi-Axis systems, with high planting densities, achieve high light interception values, but also prioritizes uniform light distribution and high light penetration as these canopies are managed to be quite narrow (70–90 cm in depth). A primary advantage of all Y-shaped systems is the ability to diffuse tree vigor into two leaders, which can help minimize tree height and maximize labor efficiency, when compared to single-leader HDPs (Table 1.2, Figure 1.4). This is especially true with the Bi-Axis system, while the KAC-V is typically managed more like an open vase system achieving much taller tree heights to maximize production on the two leaders (Figure 1.5). In sum, the Bi-Axis orients the two primary scaffolds parallel to the row orientation in order to optimize light relations in the tree, promote the development of thin canopies and integrate the use of mechanization and/or robotics to reduce labor costs.

4.3.3. Cordon Systems

Cordon systems have been developed and implemented in several other tree fruit species, such as the cherry UFO (or Bi-UFO) and the apple Super-Vee (Lang, 2019; Long et al., 2015; Tustin et al., 2018; Zhang et al., 2015). Cordon systems have been developed for peach production systems as well to achieve uniform canopy shapes, induce high early yields and potentially reduce the need for ladders (DeJong et al., 2008; DeJong et al., 1999). Cordon systems are typically developed with one or two leaders that are bent towards the horizontal after the first growing

season. In trials, this system has been planted at a density of 2.4 × 4.0 m (919 trees $ha^{-1}$) and kept under 2.5 in height (DeJong et al., 1999) (Table 1.2). In respect to the Cordon (aka "Salter") system, which was initially developed in California for peach (Rogers, 1986), the trees are left to grow vigorously in the first year, until two dominant leaders can be selected for (DeJong et al., 1999). These leaders are then left in the horizontal position approximately 1.0–1.5 m above the ground and act as "cordons," in which upright growing fruiting shoots emanate from (DeJong et al., 1999; DeJong et al., 1997). However, it has been noted that trying to fruit on vigorous uprights is difficult in peach, unlike cherry, and so the system has been modified recently to develop short fruiting shoots on the semi-permanent upright scaffolds that originate from the cordon (DeJong et al., 1997). Several iterations of the cordon systems are now being developed in Spain, Greece and Colorado, USA (Minas Lab, Colorado State University), on vigorous and dwarfing rootstocks, experimenting with various numbers of uprights per cordon.

*4.4. Ultra High-Density Planting Systems*

The only UHDP training system evaluated includes the meadow orchard, which was initially developed for apple production. UHDPs were developed with the goal of inducing precocious early yields and enable complete mechanization of orchard tasks. Apart from mechanization, trees could be managed completely from the ground, not exceeding 2.2 m (Table 1.2). Meadow orchards, although interesting in theory, never fully took off in apple nor peach for various reasons, but it was primarily due to the excessive costs of orchard establishment given the large number of trees per hectare. UHDPs are typically planted around 0.5–1.0 × 1.5–3.0 m on average, and can range from 2,700 to greater than 10,000 trees $ha^{-1}$ (Table 1.2).

4.4.1. Meadow Orchards

Meadow orchards were initially developed in England for apple production with the goal of creating an early cropping system that maintained uniform fruiting structures, canopy light distribution and the ability to be fully mechanized (Hudson, 1971; Erez, 1976). Meadow orchards are very dense plantings that cover the entire field within the first year and are completely pedestrian, maintaining heights below 2.2 m (Erez, 1985). Meadow orchards can include plantings ranging from 2,700 to 19,000 trees per hectare, with planting densities of 0.4–1.0 × 1.3–4.8 m (Table 1.2). Meadow orchards are managed on a biennial cycle, cropping one portion of the orchard one year, while the other portion is completely pruned back and allowed to re-develop the canopy in the same year. These blocks would then alternate. Whichever portion was cropped in the first year is then pruned back, while the pruned portion would then be allowed to crop in the next year. However, the economics of a biennial system are not always desirable, so the use of early cultivars was suggested to condense this production system into one year (Erez, 1976). In a condensed one-year cycle, vigorous early cultivars could crop early and then be headed back immediately after harvest, which would allow the entire canopy to rebuild and grow in the same season to crop again in the subsequent year (Erez, 1976). Overall, the goal of this system is to maintain young one-year-old fruiting wood only and reduce the size and number of large permanent scaffolds, along with their potential to contribute to intra-tree shading (Erez, 1985). With a lack of dwarfing rootstocks, the use of intensive annual pruning could help control tree vigor. The systems' propensity to crop on one-year-old wood was why it was not largely adopted in apple, as only selected apple cultivars can bear on one-year old wood. In peach, this issue is ameliorated and therefore the meadow orchard system could be potentially more effective for peach producers (Erez, 1976). However, given the large number of trees planted at establishment,

the meadow orchard system failed to be fully commercially adopted in peach as well, due to excessive start-up costs (Erez, 1985).

## 5. Impact of Training Systems on Light Relations, Production and Fruit Quality

While several systems and various iterations of each exist, there are primary guiding principles and objectives each training system is trying to achieve. Generally, these include improving light relations (interception, distribution and penetration), increasing production, allowing easier thinning and harvesting, and enhancing fruit quality.

### *5.1. Training Systems and Light Relations*

A linear relationship exists between light interception and yield (Palmer et al., 2002). Light interception (LI) can be increased through two ways: (1) increasing the canopy leaf density, which limits light distribution within the tree, and (2) increasing the leaf area index (LAI; leaf area : ground area), which is accomplished by planting a higher number of smaller trees per hectare (i.e., planting density) (Corelli-Grappadelli and Marini, 2008). The second strategy is optimal to ensure uniformly illuminated canopies. However, LI is only linearly correlated with yields up to about 50–60% of light interception (Grossman and DeJong, 1998; Wünsche and Lakson, 2000). At this point, with increased LAIs, other factors must be considered, such as light distribution. Hence, shifting to a higher number of narrower/thinner canopies, facilitated by simple 2D training systems (grafted on size controlling rootstocks), helps encourage both high light interception and high light distribution throughout the entirety of the canopy. Both of these parameters are important, as increased light throughout the totality of the canopy allows for increased total flower bud initiation, yields and uniform high-quality fruit, which can reduce the number of picks and labor cost at harvest. However, if plantings are too dense, increased shading can cause vegetative imbalances within the tree and can negatively impact fruit quality and induce tree decline more rapidly

(Sansavini and Corelli-Grappadelli, 1997). These density thresholds for optimal light, yield and economics have been well established for apple (2,600–3,000 trees $ha^{-1}$) (Robinson et al., 2013; Sansavini and Corelli-Grappadelli, 1997; Lordan et al., 2018; 2019), but remain undetermined in peach.

While density plays a primary role in maximizing light interception, the training system and canopy architecture also influence how the incident light is intercepted and distributed. For example, open vase and other multi-leader or Y-shaped systems that share this similar shape (i.e., Hex-V, Quad-V, KAC-V, etc.), can maintain a higher level of light interception at solar noon, given their open and receptive shape. While planar systems such as the cordon or palmette systems have to heavily rely on the morning or late-afternoon light for photosynthesis, due to the lower amount of light intercepted at mid-day (Sansavini and Corelli-Grappadelli, 1997). In a training system trial comparing delayed vasette (DV), palmette (P) and Y-trellis (Y) in peach, DV and P intercepted a similar amount, while the Y-system intercepted the highest amount of light (Giuliani et al., 1998). Further, whole canopy photosynthesis was linearly related to light interception, with increased levels of photosynthesis occurring in the Y-system when compared to the DV and P system (Giuliani et al., 1998). Similarly, a cordon system along with a higher-density version of the KAC-V (HiD KAC-V), intercepted the highest amount of light when compared to an open vase system (Grossman and DeJong, 1998). This contributed to increased levels of fruit number per ground area and fruit dry mass per ground area in these HDP systems, when compared to the open vase (Grossman and DeJong, 1998).

When evaluating the impacts of density versus training system on light use efficiency in apple, Robinson et al. (1992) noted the highest amount of light-conversion efficiency (i.e., assimilates for the fruit) was observed not in the highest density planting, but rather in the system

that intercepted the optimal levels of light (69%), whereas in respect to maximum yield efficiency (kg $cm^{-2}$), the highest density system reached the highest levels, rather than the reduced planting densities with more optimal light thresholds (Robinson et al., 1992). In other words, if maximum yields are the goal, planting the highest number of trees is the best way to do it. Although, profitability and the cost of establishment/management must be considered, along with the quality of the fruit produced. This trend was true in a previous peach training systems trial evaluating open vase (299 trees $ha^{-1}$), cordon system (919 trees $ha^{-1}$), KAC-V (919 trees $ha^{-1}$) and HiD KAC-V (1,196 trees $ha^{-1}$), where yields were the highest in the HiD KAC-V and the lowest in the open vase (Grossman and DeJong, 1998). However, as mentioned previously, planting densities at excessive levels can negatively impact fruit quality and can deteriorate the life span of the orchard. Simply put, the highest quality potential for fruit in an orchard is reached well before maximum yields are met (Sansavini and Corelli-Grappadelli, 1997). Therefore, optimal densities and proper training system architecture is critical to ensure a balance of both high yields and high fruit quality.

The ideal combination for orchard design, training system selection and planting density is going to vary from farm to farm based on soil type, climate, cultivar and rootstock selections, management techniques and economics (Lauri and Corelli-Grappadelli, 2014). However, the guiding principle should be to reach light interception values of 60–70%, while maintaining uniform light distribution (vertically) and penetration (depth) through the canopy without increasing the vegetative growth potential (Grossman and DeJong, 1998; Wünsche and Lakso, 2000). In general, two main strategies have been adopted to achieve these objectives in a high-density context: (1) the planting of central axis conical trees (i.e., SSA, TSA, Fusetto, etc.) with narrow inter-row and intra-row spacings, and (2) the planting of Y-shaped, cylindrical Tatura trellis iterations, in close intra-row spacings to form slanted fruiting walls along the tractor

alleyways (Loreti and Massai, 2006). Overall, these orchard design systems have displayed increased photosynthetic, water use and yield efficiencies (Loreti and Massai, 2006).

*5.2. Training Systems and Productivity*

High density plantings increase light interception and subsequent yield potentials. Yield efficiency can increase with an increased number of trees per hectare (Robinson et al., 2013), but assimilation to fruit can diminish as shade avoidance strategies are induced, prioritizing vegetative growth and negatively influencing fruit quality. This is why Grossman and DeJong (1998) have suggested that training system selection is equally about optimizing light interception while reducing the vegetative growth potential of the trees. For example, although cordon systems facilitate increased densities, light interception and assimilate production, the way they are trained and heavily pruned to maintain upright scaffolds induces a lot of vegetative growth (Grossman and DeJong, 1998). As a result, although the cordon system intercepted a high amount of light in a previous study, it partitioned a high amount of photosynthates to vegetative sinks and resulted in the lowest harvest index (ratio of fruit dry mass : sum of fruit, leaf and stem dry mass) when compared to the KAC-V, HiD KAC-V and open vase systems (Grossman and DeJong, 1998). Overall, the cordon system was the least efficient system in respect to crop production in this trial, but it was still more economically efficient than the open vase, as this LDP system sustains minimal yields given the low number of trees per hectare.

In a recent study evaluating open vase (571 trees $ha^{-1}$), Y-shaped (1,333 trees $ha^{-1}$) and central leader (2,500 trees $ha^{-1}$) systems in Brazil, it was demonstrated that the central leader maintained the smallest canopy volume ($m^3$), yielded the least number of fruits per tree, but given the higher planting density, produced the most on a per hectare basis (Mt $ha^{-1}$) (Uberti et al., 2020). A similar trial revealed that after four years, a V-system (i.e., Tatura trellis, Y-shaped) yielded the

most on a cumulative basis (1,388 trees $ha^{-1}$, 80.1 Mt $ha^{-1}$), followed by a central leader/spindle (1388 trees $ha^{-1}$, 66.1 Mt $ha^{-1}$) and then a bush system (i.e., open vase) (606 trees $ha^{-1}$, 47.7 Mt $ha^{-1}$) (De Salvador and Fideghelli, 1993). The authors suggest that the diffusion of vigor in the V (two leaders) vs. the single leader encourages less vegetative growth and subsequent pruning, which maintains a better reproductive balance and increased yields over time (De Salvador and Fideghelli, 1993). However, this is only true for a mature V- or Y-system, as extensive pruning and training is required in the first two years to develop the scaffolds (Figure 1.5), while this is not required in the central leader systems (Rufato et al., 2004). Regardless, given the need for a trellis in the spindle system, the V was noted as a more cost-effective system given the increased production and reduced establishment/infrastructure costs (De Salvador and Fideghelli, 1993). In a contrasting study, a central leader system yielded more fruit than a Y-system, but the central leader demonstrated smaller fruit size (Rufato et al., 2004). Unfortunately, the planting densities and crop loads were not given, so it is difficult to ascertain whether these training system impacts on yield were an artifact of different plantings and/or crop loads (Rufato et al., 2004). In respect to Y-systems, it has been demonstrated that they can develop a larger percentage of fruiting shoots per plant dry weight than central leader trees (Giovannini et al., 1998). Overall, HDP systems tend to produce more on a per unit land-area basis than LDP/MDP systems, even while minor nuances may exist between specific HDP training systems in respect to production. Further, Y-systems have demonstrated an effective ability to produce high yields early on, helping to recoup high orchard establishment costs. However, these yield and profitability gaps between HDP and MDP systems may diminish over time (~12 years) as trees mature, maximum yield thresholds are met and less labor/costs are required in lower density plantings (Caruso et al., 1997).

Profitability in HDP systems can remain high, if high quality thresholds are met. That is to say that the economic returns of these systems are highly dependent on the farmgate price, or crop value, in a particular season (DeJong et al., 1997). This underscores the significance of prioritizing fruit quality in orchard design and training system selections. If quality and crop value is not properly developed in the orchard, nor maintained postharvest, the advantages of high-density systems will be negated. Therefore, to ensure maximum economic returns and HDP success, fruit quality must be prioritized.

*5.3. Training Systems and Fruit Quality*

Maximizing fruit quality begins in the field and can be achieved through the manipulation of canopy architecture to enhance light relations within the tree. Peach fruit size, overcolor, SSC and DMC positively correlate with increased light interception/availability (Corelli-Grappadelli and Marini, 2008; Palmer et al., 2002). Therefore, to elicit exceptional quality, the goal of a training system should be to optimize light interception and distribution.

Training systems influence the canopy shape, depth and size of the tree, which impacts the spatial distribution of fruit in the tree and their quality characteristics (Gullo et al., 2014; Farina et al., 2005). A study comparing a delayed vasette (DV) and a perpendicular-Y (Y) system demonstrated that the highest number of fruit and yield was in the middle portion of the canopy, regardless of the system, although the Y had a more uniform distribution of fruit across the tree (Farina et al., 2005). Fruit weight decreased from the top to the bottom in the DV, while in the Y, fruit weight remained stable across the canopy (Farina et al., 2005). This was perhaps due to the increased uniformity of light distribution across the Y-system and is also supported with more uniform red coloration in the Y, when compared to the DV as well (Farina et al., 2005). Similar to fruit weight trends, fruit SSC and coloration decreases from the top to the bottom of the canopy,

with decreasing light availability (Gullo et al., 2014; Caruso et al., 1997; Génard and Bruchou, 1992). An additional experiment evaluating a Y-system and a central leader revealed that the Y-system yielded more uniform fruit quality, pigmentation and size from the top to the bottom, when compared with the central leader (Caruso et al., 1997). It was hypothesized that the improved quality and yield efficiency of the Y-system in this study was due to the enhanced spatial distribution of the canopy and reduced leaf density, which contributed to increased light use efficiency (Caruso et al., 1997; Wagenmakers and Callesen, 1995). In sum, these experiments demonstrate that uniform canopies with even light distribution generate uniform fruit quality, and that fruit quality is more indicative of the environment the fruit experiences throughout development rather than the canopy position alone (Lewallen and Marini, 2003; Anthony et al., 2021). Therefore, training system selection and development should focus on creating thin canopies, whether planar or open, in an attempt to ensure proper illumination to all portions of the canopy.

As planting densities increase, the leaf area index on an orchard scale increases (LAI-orchard, $m^2\ m^{-2}$), as trees cover more of the orchard floor. However, it is critical that while LAI-orchard is increasing, LAI on a tree scale (LAI-tree, $m^2\ m^{-3}$) should remain stable or decrease (Anthony et al., 2020b), as tree size is reduced through the facilitation of dwarfing rootstocks (Loreti and Massai, 2006). Peach trees grafted on size-controlling rootstocks, with limited canopy leaf density (i.e., LAI-tree) will promote high fruit quality, in respect to size, color, SSC and DMC (Minas et al., 2021; Loreti and Massai, 2006), while inversely, if LAI-tree reaches excessive thresholds due to a lack of dwarfing rootstocks, highly fertile soils or improper planting densities, fruit quality and production will be impaired as shade develops. In peach, the photosynthetic activity of shaded leaves is less than 10% of leaves fully exposed to the sun (Testolin and Costa,

1991). As a result, shade negatively impacts the amount of photosynthates exported to the fruit for quality development, along with floral bud induction for production in the subsequent year. Therefore, training system selection can help re-arrange the canopy architecture of trees with vigorous rootstocks and/or soils to create thinner/more sun-exposed canopies.

To manage a high-vigor situation, training systems that diffuse vigor into multiple leaders may be selected (e.g., Y-shaped, KAC-V, Bi-Axis) or training systems that facilitate fruiting walls with taller tree heights (e.g., Palmette, TSA). Increased tree heights require increased inter-row spacings as to not project shade on the proximate rows in the morning and late afternoon. Additionally, summer pruning applications (or WSR) to control for vigor and maintain well-exposed canopies may also be required. Thus, planar training systems would be ideal to facilitate the mechanization of this task. Deficit irrigation may be another management practice that could be equipped to high-density plantings to reduce excessive vegetative growth and intra-tree shading for high fruit quality (Loreti and Massai, 2002). Smaller trees and planar training systems are more efficient in respect to light, water and yield efficiency, which allows for a more balanced tree and improved photosynthate resources for developing fruits to obtain higher quality at harvest. The selection of an ideal training system and orchard design will be largely contextualized to the environmental and economic conditions of the farm, but should seek to create thin, exposed and uniform canopies for consistent yields of high-quality fruit.

Lastly, when optimizing canopy architecture for optimal light and quality thresholds, fruit maturation must also be considered. Increased light advances peach maturation, which subsequently improves quality. So, any true evaluation on the impact of training systems on fruit quality, must control for maturity (Minas et al., 2018).

5.3.1. Controlling for Confounding Variables to Elicit the True Impact of Preharvest Factors on Peach Fruit Quality

Maturity control has been the focus of several recent pomological experiments investigating the role preharvest factors have on peach fruit quality and metabolism (Anthony et al., 2020a; Anthony et al., 2021). Maturity control can be achieved through the use of Vis-NIRS technology that can evaluate $I_{AD}$ and DMC simultaneously in a single scan (Minas et al., 2021). Trees planted in HDPs on dwarfing rootstocks tend to be highly precocious and set high crop loads, which can negatively impact fruit quality (Anthony et al., 2020a). In general, HDPs have demonstrated reduced fruit weight (perhaps due to unregulated high crop loads), but have compensated for this with improved coloration and internal quality parameters, such as SSC and DMC, due to increased light interception and photosynthetic efficiency (Minas et al., 2018). Therefore, crop load is another confounding variable such as maturity status that must also be controlled for (no. of fruit $cm^{-2}$ of TCSA, no. of fruit $m^{-3}$ of canopy volume, etc.) across training systems to ensure fair comparisons of their impact on quality.

In further respect to the role crop load has on peach fruit quality, it was determined that fruit from a thinned, carbon sufficient, treatment demonstrated increased fruit size, weight, SSC and DMC, when compared to an unthinned, carbon starved, treatment, even when controlling for maturity (Anthony et al., 2020a). This experiment demonstrated the true impact of carbon supply on peach fruit quality.

Further, this maturity control approach elicited the capacity to investigate the biological impacts of carbon supply, such as the metabolome. This study showcased that early metabolic shifts play a role in priming the quality phenotype at harvest (Anthony et al., 2020a). Metabolite profiles were widely distinct early in development, when phenotypes were similar between the

carbon starved and sufficient treatments. However, at harvest, metabolite profiles were fairly similar due to maturity control and the regulation of the primary metabolism by maturation, while quality phenotypes were widely distinct (Anthony et al., 2020a). In particular, catechin maintained significantly higher levels in the carbon sufficient treatment throughout development, when compared to the carbon starved treatment, and demonstrated strong linear relationships with DMC and SSC at harvest (Anthony et al., 2020a).

An additional experiment showcased that fruit quality differences across canopy positions are largely due to variable light environments (Anthony et al., 2021). Quality (i.e., DMC) was significantly different between canopy positions (e.g., top vs. bottom) in two cultivars of variable vigor (low vs. high), prior to maturity control (Anthony et al., 2021). However, after maturity was controlled for between positions in each cultivar, quality differences only remained in the high-vigor cultivar, where light distribution was non-uniform (Anthony et al., 2021). As a result, when investigating the metabolite profiles across these environments, metabolite profiles in the exocarp were highly variable in the high-vigor cultivar between positions, while profiles were similar in the low-vigor cultivar (Anthony et al., 2021). Further, two compounds serve as metabolic signatures of the light environment. Sorbitol and citric acid demonstrated similar abundances across canopy positions in the low-vigor cultivar (with uniform light), while in the high-vigor cultivar (non-uniform light) these compounds were significantly different across positions (Anthony et al., 2021). Sorbitol was up-accumulated in the top position and a signature of high-quality, while citric acid was elevated in the bottom position and an indicator of inferior fruit quality (70). In short, canopies with uniform light distribution will elicit uniform quality and metabolite profiles at harvest, when maturity is controlled for (Anthony et al., 2021).

Lastly, when evaluating the role of rootstock vigor on peach fruit quality, a recent study was conducted, controlling both for crop load and maturity status of the fruit (Minas et al., 2020). The results demonstrated a relationship between quality, vigor and light availability. With increasing rootstock vigor, light availability in the fruit zone (1.5 m above ground) decreased along with fruit DMC (Minas et al., 2020). Dwarfing rootstocks modified the canopy architecture to increase light availability within the canopy to enhance peach fruit quality at harvest (Minas et al., 2020). When confounding variables such as crop load and maturity status are controlled for, the true impact of these preharvest factors (e.g., crop load, canopy position and rootstock) can be determined. In sum, enhanced carbon supply improves quality, uniform canopies produce uniform quality and dwarfing rootstocks improve light in the canopy for increased DMC accumulation (Minas et al., 2020; Anthony et al., 2020a; 2021).

**6. Conclusion**

Peach production remains a significant industry in the global agricultural economy. However, peach consumption is in decline due to poor fruit quality, threatening the profitability and sustainability of the peach industry. Therefore, optimizing management for high fruit quality must be the focus of peach production, as consumers are willing to pay more for a superior product. Fruit quality can only be developed in the orchard through optimizing preharvest factors, such as orchard design and training systems. With the development of dwarfing rootstocks and vigor diffusion training systems comes the opportunity for peach production to transition, such as apple and cherry, to high-density planting systems to maximize land use efficiency, light, yields and fruit quality.

Several training systems have been developed over recent decades to improve economic, resource and labor characteristics of peach production. Historically, LDP training systems such as

the open vase has been and continues to be widely used given a lack of dwarfing rootstocks and its ease of management. However, LDP systems do not achieve maximum light, yield nor quality potentials in an orchard. Now, with the increasing availability of dwarfing rootstocks, higher-density plantings have been pursued. However, when size-controlling rootstocks are not available, multi-leader systems (or cordons with several uprights) can be used to diffuse vigor horticulturally. In the pursuit of increased densities, MDP systems have been developed, such as the delayed vasette, palmette, Quad-V and Hex-V. The goal of MDP systems include: increasing light interception, increasing yields and reducing tree heights. Unfortunately, the complex 3D architecture of these LDP and MDP systems limit light distribution and can promote intra-tree shading. HDPs such as the Fusetto, TSA and Tatura trellis (and its iterations) have been developed to further increase yields and quality across the totality of the canopy, as light distribution is a key objective with these systems. These HDP training systems can generate homogenous fruiting walls to promote the use of mechanization/robotics to reduce labor costs.

The ideal system will vary from farm to farm, but the overall goal of training system selection and orchard design should be: (1) to optimize light interception (60–70%) and yields, (2) promote thin canopies (70–90 cm) with reduced leaf density for high light distribution and enhanced/uniform fruit quality and (Minas et al., 2018) find a balance between maximum yield potential and maximum fruit quality potential.

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## Tables

**Table 1.1.** Peach rootstock genotypes and breeding programs around the world.

| Rootstock | Country Origin | Genetic Origin |
|---|---|---|
| Controller™5 (K146-43) | UC-Davis*, CA, USA | *P. salicina* × *P. persica* |
| Controller™6 (HBOK 27) | UC-Davis, CA, USA | *P. persica* × *P. persica* |
| Controller™7 (HBOK 32) | UC-Davis, CA, USA | *P. persica* × *P. persica* |
| Controller™8 (HBOK 10) | UC-Davis, CA, USA | *P. persica* × *P. persica* |
| MP-29 | University of Florida, FL, USA | *P. umbellata* × *P. persica* |
| *P. americana* | USA | *P. americana* |
| Lovell | G.W. Thissell, Winters, CA, USA | *P. persica* |
| Hansen 536 | UC-Davis, CA, USA | *P. amygdalus* × *P. persica* |
| KV 10123 | USDA Kearneysville, WV, USA | *P. persica* |
| KV 10127 | USDA Kearneysville, WV, USA | *P. persica* |
| Nemaguard | USDA, USA | *P. persica* × *P. davidiana* |
| Guardian® | USDA/Clemson, SC, USA | *P. persica* |
| Bright's Hybrid #5 | Brights Nursery, CA, USA | *P. dulcis* × *P. persica* |
| Viking | Zaiger Genetics, inc., CA, USA | *unknown interspecific cross* |
| Atlas | Zaiger Genetics, inc., CA, USA | *unknown interspecific cross* |
| Rootpac®R | Agromillora, Spain | *P. cerasifera* × *P. amygdalus* |
| Rootpac®70 | Agromillora, Spain | *P. persica* × (*P. amygdalus* × *P. persica)* |
| Rootpac®40 | Agromillora, Spain | *(P. dulcis* × *P. persica)* × *(P. dulcis* × *P. persica)* |
| Rootpac®20 | Agromillora, Spain | *P. besseyi* × *P. persica* |
| Microbac (Replantpac) | Agromillora, Spain | *P. domestica* |
| Fortuna | Russia | *P. cerasifera* × *P. persica* |
| Krymsk®1 | Krymsk Exp. Breeding Station, Russia | *P. tomentosa* × *P. cerasifera* |
| Krymsk®86 | Krymsk Exp. Breeding Station,Russia | *P. cerasifera* × *P. persica* |
| Empyrean®2 (Penta) | ISF, Italy | *P. domestica* |
| Empyrean®3 (Tetra) | ISF, Italy | *P. domestica* |
| Imperial California | Italy | *P. domestica* |
| GF677 | INRA, France | *P. amygdalus* × *P. persica* |

*UC-Davis=University of California, Davis; USDA=United States Department of Agriculture; ISF=Istituto Sperimentale per la Frutticoltura; INRA= Institut National de la Recherche Agronomique;

**Table 1.2.** Training systems and their associated orchard design characteristics.

| System | No. of Primary Leaders | Spacing (m) (Intra- × Inter-Row) | Trees ha$^{-1}$ | Tree Height (m) |
|---|---|---|---|---|
| Low-Density Planting (LDP) | | | | |
| Open Vase | 3 | 3.5–5.0 × 4.0–5.0 | 220–550 | 2.2–5.0 |
| Medium-Density Planting (MDP) | | | | |
| Delayed Vasette | 4 | 3.5 × 4.5 | 600–800 | 3.0–4.0 |
| Palmette | 1 | 2.0–3.5 × 4.0–4.5 | 600–900 | 3.5–4.5 |
| Hex-V | 6 | 3.0 × 4.5 | 750 | 2.0–2.5 |
| Quad-V | 4 | 2.5–3.0 × 4.5 | 900–1,000 | 2.5–3.0 |
| High-Density Planting (HDP) | | | | |
| Fusetto or Tall Spindle Axe (TSA) | 1 | 1.5–2.0 × 4.0 | 1,250–2,000 | 2.8–3.5 |
| Slender Spindle Axe (SSA) | 1 | 1.2–1.5 × 3.5–4.0 | 1,500–2,445 | 3.0–3.7 |
| Y-Shaped (Bi-Axis, KAC-V) | 2 | 1.5–2.0 × 4.0–4.5 | 900–2,000 | 3.0–5.5 |
| Cordon Systems | 1–2 | 2.4 × 4.0 | 900 | <2.5 |
| Ultra High-Density Planting (UHDP) | | | | |
| Meadow Orchard | 1–2 | 0.4–1.0 × 1.3–4.8 | 2,700–19,000 | 1.5–2.2 |

## Figures

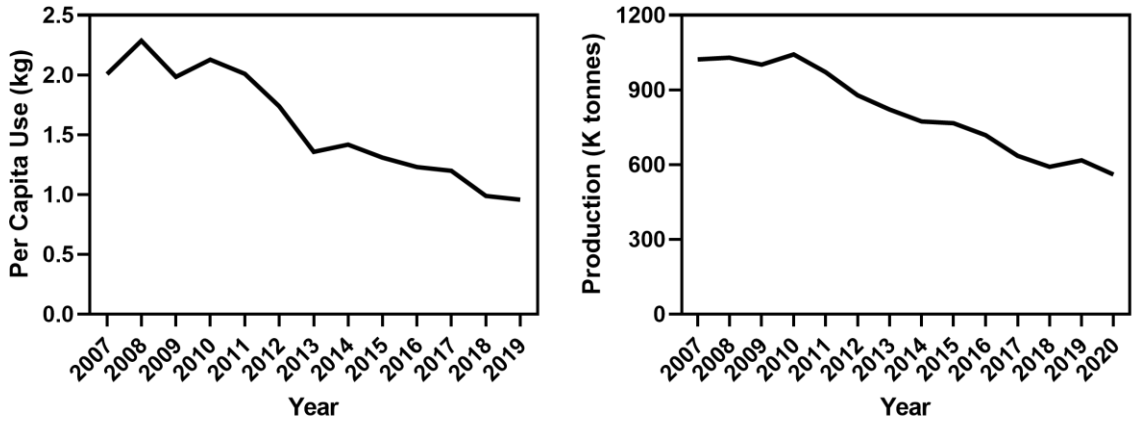



**Figure 1.1.** Peach production is declining in the United States, along with fresh peach/nectarine consumption per capita (USDA-NASS, 2021).

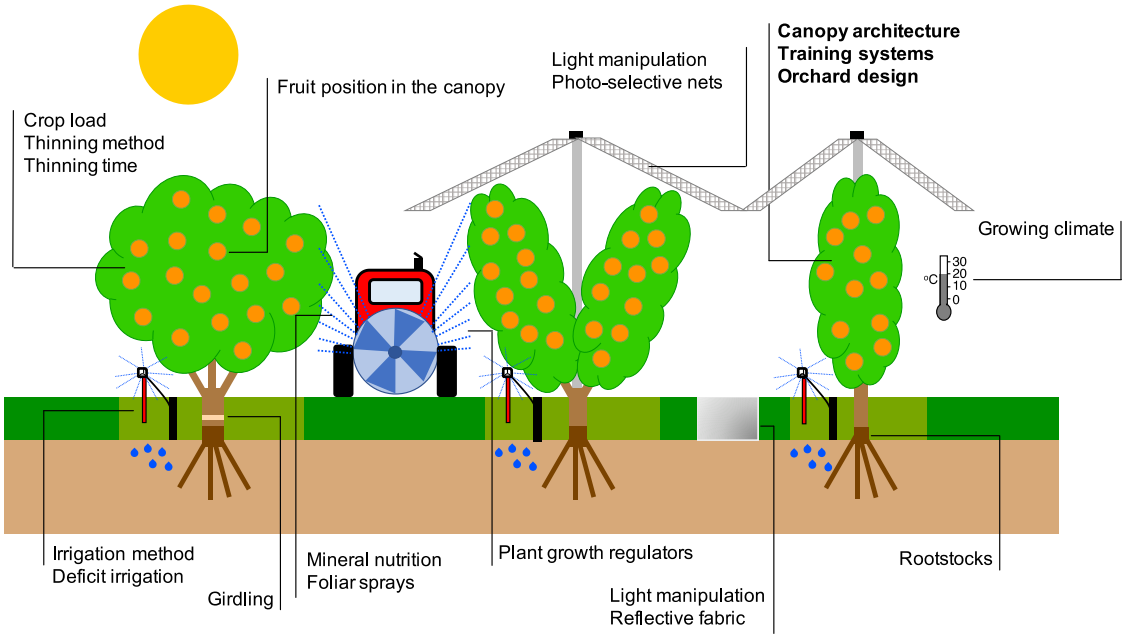
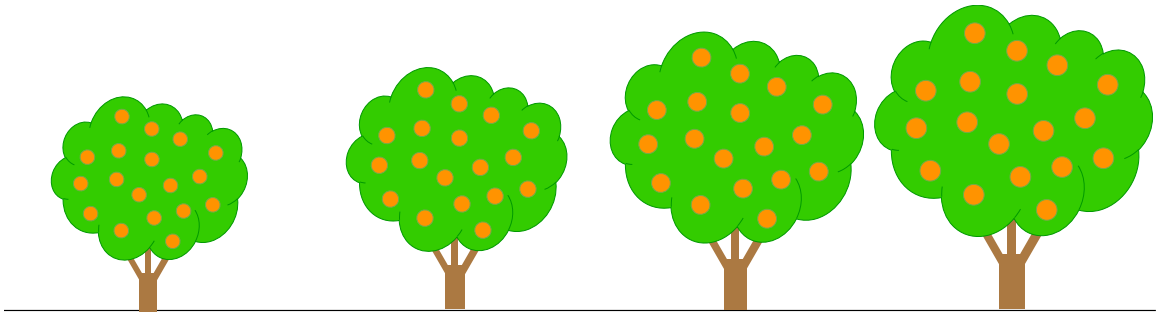



**Figure 1.2.** Overview of key orchard factors that influence preharvest peach tree and fruit physiology and affect harvest quality (Adapted from Minas et al., 2018).



| <60% | 60–90% | 90–110% | 110–120% |
|---|---|---|---|
| **Dwarf** | **Semi-dwarf** | **Standard** | **Vigorous** |
| Controller™5 | Controller™8 | Lovell | GF677 |
| Krymsk1 | Controller™7 | Krymsk®86 | Nemaguard |
| Rootpac®20 | Controller™6 | Imperial California | Atlas |
| MP-29 | Rootpac®40 | Hansen 536 | Bright's Hybrid #5 |
| | *P. americana* | KV 10127 | Viking |
| | Empyrean®2 (Penta) | KV 10123 | Guardian® |
| | Empyrean®3 (Tetra) | | Rootpac®R |
| | | | Rootpac®70 |
| | | | Microbac |

**Figure 1.3.** Peach rootstock genotypes and their vigor classification. Vigor classification is bracketed as follows: vigorous rootstocks are > 110% the size of Lovell with the size estimated by trunk cross-sectional area (TCSA); standard size rootstocks are 110–90% of Lovell size; semi-dwarfing rootstocks are 60–90% of Lovell and dwarfing rootstocks are < 60% the size of Lovell (vigor classification adopted from Reighard et al., 2015; 2020).

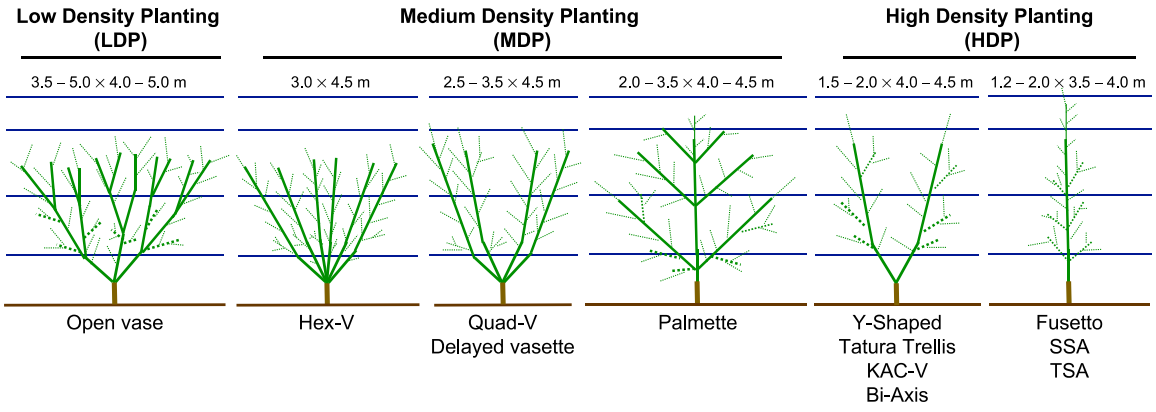



**Figure 1.4.** Canopy architectures of the most widely used training systems in peach and their planting densities. Spacings listed as: intra-row × inter-row.

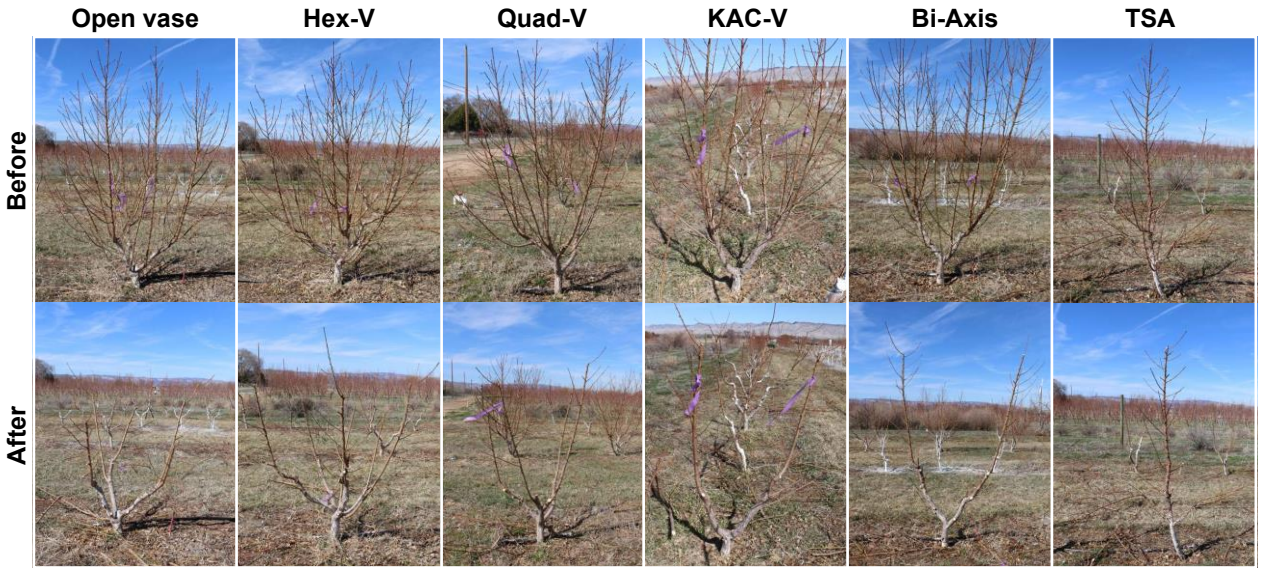



**Figure 1.5.** Orchard appearance of six canopy architectures of the most widely used training systems in 'O'Henry' peach trees (grafted on 'Krymsk®86' rootstock) before and after dormant pruning following the 3rd leaf from planting at the Colorado State University's Experimental Orchard at Western Colorado Research Center—Orchard Mesa (WCRC-OM) located in Grand Junction, CO, USA.

CHAPTER TWO

# METABOLIC SIGNATURES OF THE TRUE PHYSIOLOGICAL IMPACT OF CANOPY LIGHT ENVIRONMENT ON PEACH FRUIT QUALITY

## 1. Introduction

Several preharvest factors influence peach fruit quality (Minas et al., 2018). One particularly important preharvest factor that has been studied extensively in peach (*Prunus persica* (L.) Batsch), is the fruit canopy position and its impact on fruit development, maturation and quality (Minas et al., 2018). Light availability, distance from photosynthate sink/sources, shoot position, distance from mineral nutrition/water and hormonal signals comprise the fruit's "canopy position" (Corelli-Grappadelli and Coston, 1991; Marini and Sowers, 1994; Basile et al., 2007; Lewallen and Marini, 2003). All of which, contribute to significant influences on peach internal quality (Corelli-Grappadelli and Marini, 2008). Fruit positioned on the distal ends of shoots demonstrated inferior quality and reduced fruit size, especially when photosynthate competition was high (Wu et al., 2005; Corelli-Grappadelli and Coston, 1991). Several studies have demonstrated the impact of increased light availability and upper canopy positions accelerating peach fruit harvest time and enhancing size, overcolor, soluble solids concentration (SSC) and dry matter content (DMC) (Gullo et al., 2014; Alcobendas et al., 2013; Bible and Singha, 1993). However, it is not clear whether these fruit quality changes are a result of a shifting maturation rate (e.g., advanced maturity improving quality), or true quality enhancements as a result of increased light availability at the top of the canopy (Gullo et al., 2014).

Throughout "on-tree" maturation and ripening, peach, a climacteric fruit, will undergo several sensorial and textural changes that translate to quality enhancement (e.g., flesh softening, exocarp coloration, aroma volatilization, and increased SSC and DMC), which are regulated at the

genetic level (Giovannoni et al., 2017; Crisosto and Costa, 2008; Minas et al., 2018). Therefore, for effective assessments of preharvest factors on peach internal quality, it is critical to control for fruit maturity (Anthony et al., 2020). The majority of previous studies have not controlled for this confounding variable in their pre- and postharvest treatment assessments (Minas et al., 2018). Furthermore, given the molecular regulation of fruit maturation, any biological investigations into the fruit's genetic, proteomic and/or metabolic composition that have not controlled for maturity may have limited application (Anthony et al., 2020). Consequently, it is imperative for fruit of equal maturity to be compared between treatments to truly understand the physiological and biological impacts of preharvest factors on quality development.

Traditional destructive quality and maturity assessments are time-consuming and labor-intensive, limiting the ability to evaluate the wide variability of fruit quality and maturity on a single tree or across an entire orchard (Carlomagno et al., 2004). With the introduction of near-infrared spectroscopy (NIRS), non-destructive fruit quality and maturity assessments can be conducted rapidly and accurately in a single-scan while fruit are still on the tree (Minas et al., 2021). This technology enables the measurement of large numbers of fruit across both the tree and orchard scale to assess the impact of treatments on quality more comprehensively, while also controlling for fruit maturity. Historically, fruit maturity was determined through fruit size or ground color evaluations that were subjective and not relevant for cultivars that develop early full red overcolor. Standard maturity assessment by evaluation of fruit flesh firmness (FF) is limited due to its destructive nature and variable thresholds across different peach flesh textural typologies (Serra et al., 2020). In recent years, a new physiological maturity index, known as the index of absorbance difference ($I_{AD}$), has been developed and proven to be effective in non-destructively assessing peach fruit maturity (Ziosi et al., 2008; Costa et al., 2009; Anthony et al., 2020; Minas

et al., 2021). The $I_{AD}$ is measured by a handheld Vis-NIRS sensor (DA meter, Turoni) which measures (beneath the fruit skin's surface) the chlorophyll degradation that occurs during peach maturation and ripening (Ziosi et al., 2008; Costa et al., 2009). $I_{AD}$ does not correlate with direct FF values (Minas et al., 2021) in a given fruit population but only with clustered values (Minas et al., 2021; Ziosi et al., 2008), and may behave differently across specific cultivars or flesh typologies. On the other hand, $I_{AD}$ better described the impact of crop load and canopy position on peach physiological maturity that was differentially affected by carbon supply and the growing environment within the tree canopy compared to FF (Minas et al., 2021). Optimal $I_{AD}$ values vary per cultivar (Costa et al., 2009), with late ripening ones typically maintaining higher $I_{AD}$ values at commercial harvest stage (FF=~50 N) (Anthony et al., unpublished). Application of the $I_{AD}$ measurement facilitates rapid analysis in the field that enables researchers to control for fruit maturity, and results in more accurate assessments of preharvest factors on quality, as well as mechanistic studies of fruit metabolism (Minas et al., 2021).

Understanding metabolism is a critical target for improving fruit crop production and quality, as the metabolite composition contributes to the fruit's flavor, nutritional value and health benefits (Beauvoit et al., 2018). The fruit is the most metabolite-rich plant organ. Given the complexity of fruit maturation, ripening and quality development, global fruit metabolite evaluations can be extremely valuable for investigating how these mechanisms are impacted by preharvest factors (Monti et al., 2016; Anthony et al., 2020). Previous studies have utilized non-targeted metabolomics to evaluate how pre- and postharvest factors regulate quality development and biochemical composition in horticultural crops (Guy et al., 2008; Anthony et al., 2020). In the case of peach, preharvest factors are of utmost importance, as quality can only be built up in the orchard (Crisosto and Costa, 2008). Therefore, understanding how preharvest factors influence

metabolite concentrations in fruit tissues is critical for enhancing crop traits, such as phenotype and internal quality at harvest (Beauvoit et al., 2018). However, very few studies have evaluated the role of preharvest factors on peach fruit metabolism (Minas et al., 2018).

Recent peach preharvest -omics studies have investigated the impact of crop load (i.e., carbohydrate manipulation), cultivar selection, environmental conditions, and ripening/maturation on fruit metabolism (Anthony et al., 2020; Morandi et al., 2008; Monti et al., 2016; Karagiannis et al., 2016; Lombardo et al., 2011). Several studies have investigated the impact of canopy position on fruit metabolism in apple or pear, but limited information is available for peach (Li et al., 2013; Feng et al., 2014; Rudell et al., 2017; Serra et al., 2018; Minas et al., 2021). In addition, as discussed above, these previous canopy position investigations did not control for maturity. Therefore, it is difficult to determine whether the reported impacts on quality and/or metabolism are a result of positional treatments or if they are reflective of differences in fruit maturity (Serra et al., 2018; Minas et al., 2021). Variability in the impact of canopy position on fruit quality and metabolism can be further exacerbated by large, vigorous, non-uniform canopies that are characterized by poor light distribution (Zhang et al., 2016). A knowledge gap remains in our understanding of how these positional light microclimates influence peach fruit maturity, quality and metabolism.

Thus, the objective of this study was to characterize the impact of canopy position and light environment on fruit internal quality and metabolism across two cultivars of varying vigor and harvest time, while controlling for fruit maturity. Our results provide an important step towards the goal of optimizing orchard management and genotype (rootstock/cultivar) selection for exceptional quality and consumer performance.

## 2. Materials and methods

*2.1. Plant material and experimental approach*

In 2017, a study was conducted in two 11$^{th}$ leaf commercial orchards near Palisade, CO, USA. Each orchard contained a different peach cultivar: 'Sierra Rich,' which is an early ripening and low vigor cultivar (henceforth referred to as the Low Vigor or LV cultivar) and 'Cresthaven,' which is a late ripening and high vigor cultivar (henceforth referred to as the High Vigor, HV cultivar). Both cultivars were grafted on the same rootstock ('Lovell') and planted at a density of 516 trees per hectare (ha) (planting distances: 4 x 5 m). Trees were trained to an open vase system and managed according to commercial standards and practices. Three trees of uniform size and health were randomly selected for each cultivar in spring 2017 and thinned to a commercial crop load level. These trees were then subdivided into two canopy zones: bottom (0.3-1.2 m) and top (2.1-3.0 m) where fruit was assessed for physiological maturity, quality, and non-targeted metabolite profiling. In total, there were four treatments: LV – bottom (LVB) and top (LVT) and HV – bottom (HVB) and top (HVT) (Figure 2.1a-b).

Light availability was measured at the middle portion of each canopy zone (bottom = 0.6 m; top = 2.7 m) in each replicate tree using the Li-Cor LI-191R Line Quantum Sensor (Li-Cor Biotechnology, Lincoln, NE, USA). The methodology for measuring light availability used in this study is based on an adapted version from Lopez et al. (2008). Incident photosynthetically active radiation (PAR) was measured outside of the orchard using the Li-Cor 190R Quantum Sensor (Li-Cor Biotechnology). Data were logged with the Li-Cor LI-1500 Light Sensor Logger (Li-Cor Biotechnology). Light availability was calculated by the following equation:

$$\text{Light availability (\%)} = 100 \times \frac{\text{(average PAR transmitted at canopy position)}}{\text{(average incident PAR)}}$$

Light uniformity across the canopy was determined using the following equation:

$$\text{Light Distribution Index (LDI)} = \frac{\text{(Light availability \% at 2.8 m – Light availability \% at 0.6 m)}}{\text{(Light availability \% at 2.8 m + Light availability \% at 0.6 m)}}$$

Using this equation, the LDI is represented with a value ranging from 0.00 – 1.00. The classifications of uniformity based on LDI value are as follows: LDI = 0.9 – 1.00: strongly non-uniform, 0.50 – 0.89: non-uniform, 0.10 – 0.49: uniform, 0.09 – 0.00: strongly uniform. The LDI is an adapted index based on the crop fluctuation index from Hoblyn et al. (1936).

Prior to harvest, fruit physiological maturity ($I_{AD}$) and internal quality (DMC) were assessed non-destructively using near-infrared spectroscopy (NIRS) and were taken across the entire lot of fruit at each canopy zone using an "open-type" near-infrared spectrometer (F-750 Produce Quality Meter, Felix Instruments Inc., Camas, WA, USA) (F-750). The Vis-NIR wavelengths used for measuring $I_{AD}$ were 600-750 nm, while the wavelengths selected for measuring DMC were 729 – 975 nm (in 3 nm intervals). Dry matter content ($R^2$=0.96-0.98; root mean squared error of prediction (RMSEP)=0.38-0.41%) and $I_{AD}$ ($R^2$=0.94-0.96; RMSEP=0.07-0.08) were predicted with high accuracy and a single scan using cultivar specific models (for both cultivars tested in this study) that were created in Minas Lab following a Vis-NIRS calibration protocol (Minas et al. 2021; Minas et al., unpublished). Fruit was scanned on the sun-exposed cheek of the fruit, while on the tree. All fruit were also evaluated for size (mm) on-tree immediately prior to harvest.

Fruit of equal maturity ($I_{AD}$) were then selected to evaluate quality using both non-destructive methods on the tree as described above (n=all fruit in each position on the tree) and destructive methods in the laboratory (n=10). Fruit was selected based on previously determined cultivar specific calibrations for commercial harvest maturity (Anthony et al., 2020; Minas et al., 2021). Specifically, fruit from 'Sierra Rich' trees were selected in the range of 0.59-0.80 $I_{AD}$ and fruit from 'Cresthaven' were selected in the range of 0.90 – 1.19 $I_{AD}$.

For destructive measurements, fruit from 'Sierra Rich' were harvested on 28 July 2017, while fruit from 'Cresthaven' were harvested on 9 August 2017. These timings represent commercial harvest maturity determined by flesh firmness (FF=~50 N) and fruit quality analysis and tissue sampling was conducted on the day of harvest. The sun- and shade-exposed cheeks of each fruit were scanned using a factory calibrated Vis-NIRS spectrometer (DA Meter, T.R. Turoni, Sinteleia, Bologna, Italy) (Costa et al., 2009; Ziosi et al., 2008) and were immediately sorted by maturity ($I_{AD}$). Ten fruits of equal maturity (based on $I_{AD}$ ranges specified above) originating from each canopy zone x cultivar treatment were selected for destructive fruit quality analysis.

Destructive quality analysis was conducted according to the protocol laid out in Minas et al. (2021). The following parameters were evaluated during quality analyses: fresh fruit weight (FW, g), fruit diameter (mm), physiological maturity ($I_{AD}$), flesh firmness (FF, N), soluble solids concentration (SSC, %), DMC (%), titratable acidity (%, malic acid) and exocarp color lightness ($L^*$) and hue angle ($h^o$) from the sun- and shade-exposed cheeks from each fruit (Minas et al., 2021).

Mesocarp and exocarp (1-mm thick peel) samples were taken from ten individual fruit for each canopy zone x cultivar treatment. Exocarp samples were taken from both the shade- and sun-exposed cheek from each fruit. Three biological replicates were generated by combing ~ 2 g of mesocarp or exocarp tissue from each of 10 individual fruits (total of 10 g per biological replicate). The replicates were flash frozen with liquid nitrogen, freeze-dried using a lyophilizer (Freezone 4.5, Labconco, Kansas City, MO, USA) at -80 $^oC$ for 12 h, and then stored at -80 $^oC$.

*2.2. Non-targeted metabolite profiling using gas chromatography mass spectrometry (GC-MS)*

Biological replicates of freeze-dried exocarp and mesocarp were homogenized using a bead beater (Bullet Blender Storm, Next Advance, Troy, NY, USA) for five minutes. For each

biological replicate, three technical replicates were performed for a total of six samples per canopy position x cultivar treatment. Metabolite extraction was conducted according to Anthony et al. (2020). Briefly, 25 mg of homogenized freeze-dried sample was resuspended in one mL of HPLC grade 80% methanol (MeOH) in water solution in a two mL autosampler glass vial (VWR, Radnor, PA, USA). Samples were centrifuged at -4 ºC at a rate of 3500 rpm and the supernatant was then transferred into new glass vials. A pooled quality control (QC) was created by transferring 10 µL of each sample into a separate glass vial. Another 5 µL of the supernatant was transferred to a new vial for derivatization. All samples were centrifuged and dried under nitrogen gas. Derivatization was conducted immediately prior to analysis. Dried samples were resuspended in 50 µL of pyridine containing 25 mg/mL methoxyamine HCl (prewarmed to 60 ºC) and 50 µL of N-Methyl-M (trimethylsilyl) trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (ThermoFisher Scientific, Waltham, MA, USA) (Chaparro et al., 2018). Samples were loaded (~90 µL) into glass inserts within glass autosampler vials and centrifuged prior to analysis.

Samples were analyzed using the Clarus 690 GC coupled to a Clarus SQ 8S Mass Spectrometer (PerkinElmer, Waltham, MA, USA). Metabolites were separated with a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d. 0.25 µm film thickness). The GC program ran according to Anthony et al. (2020), with a slight modification of adjusting the split rate ratio to 1:6. Masses between 50-620 m/z were scanned at four scans/s after electron impact ionization. QC injections were analyzed after every $6^{th}$ sample and were used to monitor instrument performance and evaluate analytical variation.

Data processing was conducted according to Chaparro et al. (2018) and Anthony et al. (2020). GC-MS files were converted to .cdf format and processed by XCMS in R (Smith et al., 2006; Mahieu et al., 2016; R Core Team, 2015). Normalization was conducted on all samples to

the total ion current (TIC). Peak deconvolution into spectral clusters occurred in RAMClustR to facilitate metabolite annotation (Broeckling et al., 2014). Metabolites were matched in RAMSearch (Broeckling et al., 2016) by using retention time, retention index and matching mass spectra data with external databases including Golm Metabolome Database (Hummel et al., 2013; Hummel et al., 2007) and NISTv14 (http://www.nist.gov) (Broeckling et al., 2016).

*2.3. Statistical analysis*

All measurements were assessed for statistical differences between canopy positions in each cultivar using proc-GLM in SAS (SAS Inc., Cary, NC, USA). The effect of canopy position and vigor (cultivar) on physiological characteristics and mean peak area of metabolites were each assessed for significance by one-way ANOVA ($P \leq 0.05$). Tukey mean comparisons were used to assign different lettering groups where the model was significant at $P \leq 0.05$ across canopy positions. ANOVA was used to denote significance between cultivars (i.e., impact of vigor) on quality and annotated metabolites by Type III sum of squares at $P \leq 0.05$. Principal component analyses (PCA) were performed on metabolites detected in mesocarp and exocarp using SIMCA v15 (Umetrics, Umea, Sweden) and on fruit quality characteristics using JMP Pro v15 (SAS Inc., Cary, NC, USA). Metabolic heat maps were generated using the z-score of metabolite abundance across all four treatments (cultivar x canopy position) for each metabolite detected and annotated in mesocarp and exocarp. Heat maps, PCAs, and graphs were visualized using Prism v9.1.1 for Mac OS (Graph Pad Inc., San Diego, CA, USA).

## 3. Results

*3.1. Canopy position influence on peach fruit maturity and quality*

To evaluate the impact of canopy position on fruit quality and maturity, all fruit in each canopy zone were scanned non-destructively using an internally calibrated (Minas et al., 2021)

handheld Vis-NIRS sensor, immediately prior to harvest. On average, in both cultivars, fruit in the bottom position were less ripe (higher $I_{AD}$ values) than fruit at the top of the canopy (Figure 2.1a-c). The LV cultivar demonstrated fruit that had 25% higher $I_{AD}$ values at the bottom than at the top (Figure 2.1c). The HV cultivar revealed 31% higher $I_{AD}$ values at the bottom, when compared to the top (Figure 2.1c). The distribution of measured $I_{AD}$ values across all fruit for both cultivars is broad. The distribution for fruit at the top is clearly skewed towards lower $I_{AD}$ values (more ripe), while the distribution for fruit at the bottom skewed towards higher $I_{AD}$ values (less ripe), especially in the HV cultivar (Figure 2.2b, f). The distribution of $I_{AD}$ values in the bottom of the LV cultivar remain broader (Figure 2.2b). For dry matter content (DMC), both cultivars demonstrated significant differences ($P \leq 0.05$) across canopy positions when evaluating all the fruit in each position, prior to controlling for maturity by selecting fruit of uniform $I_{AD}$ values (Figure 2.1d). On average, there was a 1.5% and 2.0% difference in DMC (ΔDMC) between fruit from the top and bottom of the canopy position in the LV and HV cultivars, respectively (Figure 2.1d). In the LV cultivar 81% of fruit at the top and 51% of fruit at the bottom exceeded 12% DMC (Figure 2.2c), whereas in the HV cultivar 89% of fruit at the top (HVT) and only 27% of fruit at the bottom (HVB) exceeded 12% DMC (Figure 2.2g). Fruit size was not influenced by canopy position in LV, however in the HV cultivar fruit size was 10% higher in the top canopy position when compared to the bottom (data not shown). Distributions of fruit size across the canopy positions were similar in the LV cultivar (Figure 2.2a). Conversely, HVT contained a higher percentage of >70 mm fruit, when compared to the bottom (Figure 2.2e). In sum, these data demonstrate that fruit quality was more uniform across canopy positions in the LV cultivar, while quality was more differentiated across canopy positions in the HV cultivar.

The uniformity or variability in fruit quality across canopy positions may be a result of differences in light availability and distribution. In the LV cultivar, light availability was 97 and 47% in the top and bottom positions, respectively (Figure 2.1g). While in the HV cultivar, light availability was lower at 83 and 8% in the top and bottom positions, respectively (Figure 2.1g). When evaluating light distribution across the canopy, the LV cultivar demonstrated a more uniform canopy (LDI=0.35) than the HV cultivar (LDI=0.82), which yielded an LDI above the 0.50 uniformity threshold (Figure 2.1g). These differences in canopy vigor and light distribution help explain the lack of quality homogeneity in the HV cultivar. However, given that maturity was not controlled for in these initial scans, it cannot be determined whether these quality shifts are a direct result of the variable light environments in the canopy or a result of maturity advancement/delay.

*3.2. True canopy position influence on peach fruit quality*

To evaluate the true impact of canopy position on internal peach quality (i.e., DMC), maturity was controlled within each cultivar. As described above, the specific uniform maturity ranges selected for comparisons per cultivar included: SR=0.59 – 0.80 $I_{AD}$; CH=0.90 – 1.19 $I_{AD}$. When controlling for maturity, $I_{AD}$ values will be consistent across the canopy positions within each cultivar (Figure 2.1e). When maturity was not controlled, vast differences in quality (as measured by DMC) were observed (Figure 2.1d). However, after controlling for maturity, only the HV cultivar demonstrated a significant difference in DMC between canopy positions (ΔDMC of 2.1%), corresponding to the reduced light distribution and ununiform canopy light environments in this cultivar (Figure 2.1f-g). Importantly, in the HV cultivar the distribution in fruit quality between the top and bottom canopy positions remain distinct despite controlling for maturity.

Thus, this reveals a true and significant impact of light availability, distribution and canopy position on fruit quality in high vigor trees with non-uniform canopies (Figure 2.2f-h).

Destructive quality analyses reveal similar trends to the non-destructive Vis-NIRS predictions (Table 2.1). At equal maturity, minimal differences were detected across positions in the LV cultivar (Table 2.1). On average, only the exocarp parameters, lightness ($L^*$) and hue angle ($h^o$), resulted in significant differences between canopy positions in the LV cultivar, with darker and redder exocarp at the top when compared to the bottom (Table 2.1). In the HV cultivar, on average, when maturity was controlled, there were significant increases in fruit weight, diameter, SSC, and DMC at the top when compared to the bottom (Table 2.1). In particular, the HV cultivar exhibited a ΔDMC of 1.6%, while ΔDMC was reduced to 0.7% in the LV cultivar (Table 2.1). Exocarp parameters were not different between positions in the HV cultivar (Table 2.1).

Principal component analysis (PCA) was used to demonstrate the relationship between fruit quality and cultivar/canopy positions, in fruit of equal maturity (Figure 2.1h). PC1 and PC2 explain 98% of the variation (Figure 2.1h). The majority of the variation (PC1, 69.1% of the variation) appears to be explained by genotypic differences, pertaining to vigor classification, harvest date and/or phenotypic differences (e.g., fruit weight). PC2 (28.9%) is explaining variation that appears to be more related with the different positions in the canopy (Figure 2.1h). Canopy positions (top and bottom) for the LV cultivar are clustered closer together (circles) than for the HV cultivar (squares) (Figure 2.1h) indicating less variation in fruit quality for the LV cultivar. DMC and SSC appear to be correlated with the top canopy position (Figure 2.1h).

*3.3. Peach mesocarp metabolite profiling across canopy positions in two cultivars*

Thirty-five metabolites were annotated from the peach mesocarp GC-MS analysis and are presented and organized by chemical class in a heatmap (Figure 2.3a). Overall, metabolite

abundances appear to shift more dramatically across positions in the HV cultivar, than in the LV cultivar (Figure 2.3a and Table S2.1). At harvest, 12 and 14 annotated metabolites in the mesocarp were statistically different between canopy position and cultivar, respectively (Table S2.1). PCA demonstrated a large percentage of metabolic variation along PC1 (45.1%), attributed to differences in canopy position whereas cultivar variation appears to be explained along PC2 (21.7% of variation; Figure 2.4a) Interestingly, separation between canopy position within cultivar appears to be minimal and in agreement to that observed for the fruit quality parameters (Figure 2.1h), with greater differences in the HV cultivar (squares) than in the LV cultivar (circles) (Figure 2.4a). Overall, it appears that most of the metabolites (loadings, grey diamonds) positively correlate to the bottom canopy position (black symbols) in both cultivars (Figure 2.4a). The metabolites observed at increased abundance in the bottom canopy position include amino acids, organic acids, and monosaccharides, while sucrose and sugar alcohols (e.g., sorbitol and myo-inositol), appear to be increased in abundance in fruit from the top canopy position (red symbols) (Figures 2.3a-2.4a).

*3.4. Mesocarp metabolomes minimally influenced by canopy position and cultivar*

Several different hypotheses were assessed to determine how uniform light distribution, canopy position and cultivar influenced metabolite abundances. From these tested scenarios, four trends were identified based on changes in metabolite abundances (Figure 2.5), these include metabolite accumulation influenced by non-uniform light, canopy position and cultivar.

The only two metabolites that were influenced by non-uniform light distribution and elevated LDI across the peach tree canopy and highlighted in the HV cultivar include: sorbitol and citric acid (Figure 2.5a-b). These metabolites were similar across canopy positions in the LV cultivar (low LDI), but were significantly different across canopy positions in the HV cultivar

(high LDI) (Figure 2.1g). Sorbitol abundance was 31% greater in the top canopy position, while citric acid abundance was 28% greater in the bottom canopy position (Figure 2.5a-b). Other metabolites that were influenced by canopy position, but behaved similarly in both cultivars, include: shikimic acid, butanoic acid, asparagine, and threonine (Figure 2.5c-f). All four metabolites were higher in abundances in the bottom canopy position, when compared to the top (Figure 2.5c-f). Specifically, on average, shikimic acid and butanoic acid were 28% and 37% more abundant in the bottom canopy position, respectively (Figure 2.5c-d). On average, the amino acids (asparagine and threonine) were 70% and 76% more abundant in the bottom canopy position, respectively, but were not different across cultivars (Figure 2.5e-f). When analyzing for the influence of cultivar vigor, shikimic acid was significantly more abundant in the HV cultivar, while butanoic acid was significantly more abundant in the LV cultivar (Figure 2.5c-d).

Additional metabolites that were significantly different across cultivars included malic acid, tocopherol, quinic acid and catechin (Figure 2.5g-j). Malic acid and tocopherol were 36% and 52% more abundant in the LV cultivar, respectively, when canopy position was averaged (Figure 2.5g-h). The inverse was true for quinic acid and catechin, which were significantly more abundant in the HV cultivar (Figure 2.5i-j). Specifically, quinic acid was 56% more abundant in the HV cultivar (Figure 2.5i). The HV cultivar also displayed a 14-fold higher abundance of catechin as compared to the LV cultivar (Figure 2.5j). It is important to note that these four metabolites were not different across canopy positions within each cultivar (Figure 2.5g-j). Overall, in the mesocarp, only 10 metabolites fit into these four categories of metabolic trends between treatments (position x cultivar) (Figure 2.5). However, as presented below, a higher level of metabolic variation across treatments was observed in the exocarp.

*3.5. Peach exocarp metabolite profiling across canopy positions in two cultivars*

The 35 annotated metabolites and their trends across treatments (position x cultivar) in the exocarp are displayed in Figure 2.3b. At harvest, 14 and 23 annotated metabolites in the exocarp were statistically different between canopy position and cultivar, respectively, demonstrating a higher level of variation between treatments in the exocarp than in the mesocarp (Table S2.1). In contrast to the mesocarp heatmap (Figure 2.3a), there also appears to be stronger distinction and variation in the exocarp metabolite profiles across treatments (Figure 2.3b). Variation in the exocarp metabolites is also demonstrated in the PCA, where the LV canopy positions cluster closely together, whereas the HV canopy positions do not (Figure 2.4b). PC1 (51.5%) appears to be explained by genotypic differences (Figure 2.4b). While PC2 appears to explain variation according to canopy position (17.4%), but only in the HV cultivar (Figure 2.4b). Overall, the exocarp metabolite profiles demonstrate variation by canopy position only within the HV cultivar (Figure 2.4b).

Similar to the mesocarp, sugar alcohols and sucrose are higher in abundance in the top fruit compared to the bottom in the HV cultivar (Figures 2.6-2.7). Additionally, amino acids, organic acids and fatty acids were significantly more abundant in the bottom in both cultivars (Figures 2.3b, 2.6-2.7). Monosaccharides (fructose and sorbose) are only significantly more abundant in bottom canopy position in the HV cultivar, while they remain stable across positions in the LV cultivar (Figure 2.7).

*3.6. Exocarp metabolomes heavily influenced by canopy position and cultivar*

Exocarp metabolites that are influenced by the fruit's environment are displayed in Figures 2.6-2.7 and follow the similar categorical trends identified in the mesocarp. For example, sorbitol abundance was similar across canopy positions in the LV cultivar, while higher abundances were demonstrated in the top fruit when compared to the bottom fruit in the HV cultivar (Figure 2.6a).

Inversely, shikimic acid, butanoic acid and citric acid were elevated in the bottom fruit of the HV cultivar, but were similar across positions in the LV cultivar (Figure 2.6b-d). Amino acids demonstrated a uniform trend of decreased abundance in the top canopy fruit, similar to the abundance trends observed in the mesocarp (Figures 2.5-2.6, 2.8). Across cultivars (vigor classifications), only asparagine and glycine were significantly more abundant in the HV cultivar when compared to the LV cultivar (Figure 2.6e-f).

The abundance of sorbose, fructose, and sucrose was stable across canopy positions in the LV cultivar, but varied in the HV cultivar (Figure 2.7b-d). Specifically, in the HV cultivar, sorbose and fructose significantly decreased in abundance in the top canopy position compared to the bottom, while sucrose significantly increased (Figure 2.7b-d). The opposite trend was observed for malic acid and quinic acid, with no differences in abundance across canopy positions in the HV cultivar, but maintained significant variation in the LV cultivar (Figure 2.7a,f). Malic acid was 9% more abundant in the bottom canopy position, while quinic acid was 9% more abundant in the top canopy position in LV (Figure 2.7a,f). Catechin was 266% more abundant in the HV cultivar when compared to the LV cultivar (Figure 2.7e). Additionally, levels of catechin were 162% higher in the top canopy position when compared to the bottom in the LV cultivar (Figure 2.7e). However, there was no statistical differences detected across canopy positions in the HV cultivar in respect to catechin (Figure 2.7e). Lastly, three additional metabolites demonstrated significant differences between cultivars (positions averaged): glucose was 9% more abundant in the LV cultivar, while ursolic acid and oleanolic acid were 80% and 172% higher in the HV cultivar, respectively (Figure 2.7g-i).

## 4. Discussion

### *4.1. The true impact of canopy position and light environment on internal peach fruit quality*

As a preharvest factor, the fruit's canopy position has a significant impact on both maturity and fruit quality development (Minas et al., 2018). When evaluating the impact of canopy position on fruit maturity and quality in each cultivar, without controlling for maturity, the data clearly demonstrated superior quality and advanced maturity in the top canopy position as compared to the bottom (Figure 2.1c-d). Selected $I_{AD}$ values for a commercial pre-climacteric maturity for the LV cultivar ('Sierra Rich') and the HV cultivar ('Cresthaven') were 0.7 and 1.0, respectively. Industry standards for optimal peach consumer quality have established minimum SSC requirements for early/mid-season peach cultivars at ~ 11% and for late season at ~12% (Testoni, 1995; Hilaire, 2003; Minas et al., 2018). Based on these ranges and considering the high correlation of SSC with DMC (Minas et al., 2021), it can be determined that the fruit in the bottom canopy position for the HV cultivar are not reaching these standards and would be considered inferior on the market (Figure 2.1c, 2.2f-g). Conversely, for both the HV and LV cultivars, fruit positioned at the top of the canopy did achieve these quality thresholds and exhibited overall larger fruit size, although size differences across canopy positions were less pronounced in LV cultivar (Figure 2.1d, 2.2a, e).

Throughout literature, the effect of canopy position on peach fruit quality has demonstrated similar results, with fruit in the exterior, or upper portions of the canopy yielding higher values for fruit weight, size, overcolor, SSC and DMC (He et al., 2008; Lewallen and Marini, 2003). This is due to increased light availability at these positions in the canopy, which results in an increase in leaf nitrogen and photosynthetic efficiency and allows for increased photosynthate assimilation for the fruit in close proximity (Marini and Sowers, 1990). However, these light-rich microclimates in the canopy also facilitate advanced maturation (Lewallen and Marini, 2003). Non-uniform canopies will promote a lack of uniformity in peach fruit maturity across canopy positions (Bible

and Singha, 1993), which can lead to multiple picks and increased labor costs. Additionally, hastening/delaying maturity can directly influence the quality enhancement/detriment of fruit at these positions, as maturity impacts quality (Anthony et al., 2020).

When comparing fruit at equal maturity (controlling for maturity), the true impact of canopy position on internal peach fruit quality is revealed. The ability to identify the influence of these preharvest factors on quality, without the confounding variable of maturity, was not feasible in the past due to the lack of reliable non-destructive methods to measure fruit internal quality and maturity at the same time. With the development of accurate and reliable Vis-NIRS prediction models, rapid assessment of maturity and quality in a single scan has enabled the ability to elicit the true impacts of preharvest factors on internal fruit quality (Minas et al., 2021). However, quality differences are unique to each cultivar, as specific genotypes maintain different vigor classifications, canopy architectures, harvesting windows and fruit characteristics (Minas et al., 2018).

In this study, it was observed that in the LV cultivar differences in DMC between canopy positions are no longer significant (shrinking from a ΔDMC of 1.5% to 0.7%) when maturity is controlled for (Fig 2.1d, f). However, for the HV cultivar, the ΔDMC between canopy positions remains relatively stable at 2.0% before controlling for maturity and 2.1% after (Fig 2.1d, f). The distribution of DMC values measured for each cultivar across canopy position also demonstrates that the variability in DMC values decreased in the LV cultivar when controlling for maturity, while this was not observed in the HV cultivar (Figure 2.2c-d, g-h). In other words, the canopy position influence on fruit internal quality is more pronounced in the HV cultivar, as the microclimate conditions between the top and the bottom of the canopy are more distinct. The variation in canopy position microclimates in the HV cultivar is evidenced by vast differences in

available light (Figure 2.1g). Whereas, in the LV cultivar, the canopy maintains a more uniform shape, which allows for increased light availability at the bottom and a more uniform light distribution across the canopy (Figure 2.1g). The increased light availability and uniformity obtained in the LV cultivar ensures enhanced quality development and homogenous fruit quality across canopy positions at harvest. Simply put, the light environment available to the fruit is dictating the quality more than the position of the fruit in the canopy (Lewallen and Marini, 2003). Similar trends have been reported in literature, demonstrating more drastic differences in fruit quality across positions in a HV canopy when compared to a LV canopy (Gullo et al., 2014). In addition, they reported overall sensorial and metabolic characteristics like fruit size, SSC, overcolor and total phenolic concentration (TPC) were diminished in the HV canopy due to reduced light availability and non-homogeneous canopy development (i.e., poor light distribution) (Gullo et al., 2014). However, it is important to note that maturity was not controlled in this study, so these quality differences between cultivar and canopy positions may also be a result of variable ripening and maturity status (Gullo et al., 2014).

The destructive measurements obtained in this study further support the 'on-tree' measurements demonstrating a higher impact of canopy position on fruit quality in the HV cultivar (Table 2.1 and Figure 2.1h). For example, in the LV cultivar, only the exocarp hue angle ($h^o$) and lightness ($L^*$) are influenced by canopy position at equal maturity, revealing that at the top position, the fruits developed darker and redder coloration (Table 2.1). Darker, redder fruits at the upper canopy positions have also been demonstrated throughout literature (Bible and Singha, 1993). In the LV cultivar, all other destructive quality parameters were uniform across positions, while conversely in the HV cultivar there were numerous significant quality differences. Specifically, in the top canopy position, fruits were heavier and larger, with increased levels of SSC and DMC, all

parameters that correlate well with increased consumer acceptance (Minas et al., 2018). While at the bottom canopy position, the quality is notably inferior (Table 2.1). These differences within each cultivar were visualized and summarized in Figure 2.1h, with the PCA showing tighter clustering of the LV cultivar positions, while there is wider separation in the HV cultivar across positions, even when fruit were controlled for equal maturity.

Overall, an increase in quality traits in fruit from the HV cultivar in the top canopy position were due to both: 1) high light availability (~80%) and 2) longer tree residence, increasing its duration for carbohydrate assimilation (Figure 2.1g,h). The increased duration of time on the tree is critical for quality development, as most of the carbohydrates are assimilated into the fruit during the final stages of development (Chalmers et al., 1975; Minas et al., 2015). Therefore, late ripening cultivars may be able to obtain higher levels of photosynthates when compared to early ones, due to their extended periods of time throughout development, or given their relatively shorter period of carbon competition between actively growing vegetative – reproductive organs (DeJong et al., 1987; Grossman and DeJong, 1995; Minas et al., 2015). However, this does not negate the requirement of beneficial environmental conditions for quality development. Fruit from the HV cultivar in the bottom was negatively impacted by canopy position, as the uneven distribution and minimal amounts of light availability may have inhibited quality development (Figure 2.1).

These results underscore the need for equal maturity comparisons in pomological experiments, as many previous experiments demonstrate vast impacts of canopy position on peach internal quality, but do not effectively control for maturity (Gullo et al., 2014; Farina et al., 2005; Lewallen and Marini, 2003; Corelli-Grappadelli and Coston, 1991). However, as revealed in this experiment, the impact of canopy position is relative and dependent on the maturity status of the fruit, along with the microclimate they are developing in (e.g., canopy and light uniformity; Figure

2.1). Therefore, orchard management decisions should be optimized to ensure uniform canopies to facilitate uniform fruit maturity and quality at harvest. For example, summer pruning and watersprout removal has demonstrated beneficial effects on illuminating previously shaded portions of the canopy to enhance fruit quality development (Myers, 1993). Summer pruning, along with dwarfing rootstock selections and planar training systems may be ideal canopy management decisions for peach production to ensure uniform canopies that enable uniform quality development (Gullo et al., 2014; Robinson et al., 2013). This would allow for decreased pick numbers and labor costs, the opportunity for mechanization and better postharvest performance (Robinson et al., 2013; Minas et al., 2018).

*4.2. Canopy position-related primary metabolism responses were mainly observed in peach exocarp*

Maturity control allows for true physiological assessments of preharvest factors on peach fruit quality (Minas et al., 2018), but more than that, it enables accurate investigations into the biological characteristics of fruit (Anthony et al., 2020), as maturation is a heavily regulated process (Giovannoni et al., 2017). To better understand the influence of canopy position on peach fruit metabolism, non-targeted metabolite profiling was conducted on the mesocarp and exocarp of fruit of equal maturity across two canopy positions within two cultivars of variable vigor and canopy light environments. Metabolic variation across canopy positions appeared to be more pronounced in the late, HV cultivar, than in the early, LV cultivar (Figures 2.3-2.4). Metabolite variation in the mesocarp was minimal at harvest between positions, which may be a direct result of sampling fruits at equal maturity. A similar trend was noted in a previous experiment, where primary metabolite variation in the peach mesocarp across two extreme carbon supply treatments was very distinct early in development, but at harvest, their metabolomes were very similar even

with distinct phenotypes (Anthony et al., 2020). The primary metabolism, which is mainly captured through GC-MS, appears to be heavily regulated by maturation and development in the mesocarp (Anthony et al., 2020). This elicits similar profiles at harvest, when maturity is controlled, regardless of distinct preharvest factor treatments and phenotypes (Anthony et al., 2020). A lack of metabolic variation may also be a result of the mesocarp metabolism being protected by the exocarp. Metabolites act as response signals to physiological stimuli that connect the environment to the fruit's biology. Therefore, it is expected that the mesocarp metabolomes would not be heavily influenced by microclimate differences, as it does not interact with the environment directly, like the fruit exocarp. In a previous study, wide exocarp variation was detected amongst peaches grown at different elevations experiencing distinct microclimates (Karagiannis et al., 2016). This may explain, with respect to metabolite abundances/shifts, why wider variation was detected across positions in the exocarp rather than in the mesocarp. Overall, there were four primary trends in respect to mesocarp and exocarp metabolite abundances shifting according to cultivar differences, canopy position and light distribution (Figures 2.5-2.7).

*4.3. Genotype related metabolic shifts in peach mesocarp and exocarp*

Recent studies have demonstrated the vast differences in metabolite profiles across several peach genotypes (Wu et al., 2005; Monti et al., 2016). In our study, notable genotypic differences in metabolite abundances in the mesocarp include malic acid, quinic acid, tocopherol and catechin (Figure 2.5).

Malic acid has been associated with younger, less developed fruits (Wu et al., 2005). This may be why the earlier, LV cultivar has a higher abundance of malic acid at harvest than the later, HV cultivar (Figure 2.5). Early ripening peach cultivars have demonstrated higher levels of malic acid when compared to late ripening cultivars (Monti et al., 2016). Malic acid has also previously

been used as a metabolic indicator for quality (Chapman et al., 1991; Colaric et al., 2005). In particular, consumer preference and fruit quality can be correlated with a maximum malic:citric acid ratio (Chapman et al., 1991; Colaric et al., 2005). Malic:citric acid ratios appear to be stable across positions in the LV cultivar, although a slightly higher (16%) ratio is observed in fruit from HV cultivar in the top canopy position when compared to fruit from the bottom canopy position (data not shown). This metabolic ratio further supports the narrative that a lack of canopy light environment uniformity, especially in vigorous trees, can truly negatively impact fruit quality at harvest.

Quinic acid levels were stable across canopy positions within each cultivar, underscoring equal metabolic maturity (Figure 2.5). Although the late, HV cultivar maintains higher levels of quinic acid than the early, LV cultivar at harvest (Figure 2.5). Quinic acid is an important building block for phenolic compounds, such as flavanols and caffeoylquinic acids (CQA), which contribute significantly to antioxidant activity in fruits (Luo et al., 2008). In a previous study, elevated levels of quinic acid, catechin and tocopherol were associated with fruit that had been sufficiently supplied with carbon throughout development (Anthony et al., 2020). In this study, quinic acid and catechin remain higher in the late ripening HV cultivar in both the mesocarp and exocarp (Figures 2.5-2.6). These differences appear to be reflective of the diverse metabolomes that are characteristic of distinct cultivars (e.g., early vs. late; low vs. high vigor) (Monti et al., 2016). In Anthony et al. (2020), catechin was observed to be two-fold higher in abundance at harvest, in fruit exposed to a sufficient carbon supply treatment versus carbon starved. In this study, the late ripening HV cultivar maintains levels of catechin in the mesocarp that are 14-fold higher in abundance than the early ripening, LV cultivar (Figure 2.5). Such extreme differences in

catechin abundance between genotypes alludes to genotypic difference rather than resulting from preharvest treatment factors.

Tocopherol, a secondary metabolite related to antioxidant activity and vitamin E, was found in high abundance in the mesocarp of the early LV cultivar (Gramegna et al., 2018; Figure 2.5). A recent study demonstrated that increased light played a significant role in mediating phytochrome activities, which contributed to increased tocopherol and carotenoid biosynthesis (Gramegna et al., 2018). The increased levels of light availability and distribution across the canopy of the early LV cultivar (Figure 2.1g) may explain why tocopherol levels are significantly higher than in the HV cultivar (Figure 2.5). In fact, increased levels of tocopherol indicate enhanced plant stress tolerance, while reduced levels favor oxidative damage (Munné-Bosch, 2005). Ample and optimal levels of light availability may contribute to a plant's investment in these resilience-promoting compounds. However, this does not explain why tocopherol levels remain low in the mesocarp of HVT fruit, which experienced high light availability (Figure 2.1g). Therefore, mesocarp tocopherol levels may be better explained by other factors, such as ripening and maturation. In fact, tocopherol accumulation demonstrated a strong relationship with fruit development and $I_{AD}$ degradation in a previous peach study (Anthony et al., 2020). The uniform levels of tocopherol across positions within each cultivar appears to be a product of equal maturity sampling, while cultivar differences may be a product of genotypic variation.

The trends of minimal positional differences, the need for equal maturity sampling, and genotypic variation in metabolite abundance are further supported with oleanolic and ursolic acid levels in the exocarp (Figure 2.7). Both oleanolic and ursolic acids are triterpenoids that possess many pharmacological benefits such as anti-inflammatory, anticancer, and antioxidant activities (Pollier and Goossens, 2012). These triterpenoids are widely distributed in fruits belonging to the

Rosaceae family and are typically found in the epicuticular waxes of plants, which help prevent water loss and provide protection from abiotic and biotic stresses (Ludeña-Huaman, and Ramos-lnquiltupa, 2019; Pollier and Goossens, 2012). The increased levels of these triterpenoids in the exocarp versus the mesocarp (data not shown) is supported by literature, along with higher abundances of ursolic acid than oleanolic acid (Ludeña-Huaman, and Ramos-lnquiltupa, 2019; Belge et al., 2014; Figure 2.7). Cultivar differences in both ursolic and oleanolic acid abundances were detected between two late-maturing genotypes in Belge et al. (2014). Furthermore, Belge et al. (2014) suggests that triterpenoid abundances and the peach cuticle composition may be ethylene- and ripening-associated attributes. The data presented herein support this hypothesis with uniform levels of triterpenoids across canopy positions within each cultivar (Figure 2.7), which were sampled at equal maturity. Therefore, these data continue to support the need for equal maturity sampling from a metabolic standpoint, and that triterpenoid abundance variation may be more related to genotype and maturation than preharvest factors.

With respect to exocarp metabolites, increased solar radiation may also be influencing saccharide composition (Figure 2.7). Overall, the general trend showcases increased monosaccharides (sorbose, fructose (Fru) and glucose (Glc)) in the LV cultivar, while sucrose (Suc), a disaccharide, remains elevated in the HV cultivar (Figure 2.7). Increased solar radiation and light availability, especially in the LV cultivar, can increase fruit and leaf transpiration, which can potentially contribute to drought stress and water limitations. A recent study in apple demonstrated that water stress resulted in the accumulation of monosaccharides and the reduction of sucrose abundance (Yang et al., 2019). Fru and Glc are phosphorylated to glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) by hexokinase (HK) and fructokinase (FRK), respectively (Cirilli et al., 2016). Conversion from G6P to UDP-glucose (UDPG) is then facilitated by UDP-

glucose pyrophosphorylase (UDP) (Cirilli et al., 2016). Suc can then be synthesized from F6P via the glycolytic pathway with UDPG by sucrose phosphate synthase (SPS) (Cirilli et al., 2016). Under extreme transpirational losses, SPS activity decreases, resulting in reduced Suc and elevated hexose concentrations in apple and peach leaves (Yang et al., 2019; Escobar-Gutiérrez et al., 1998). Increased vigor can create intra-tree shade, reduce light availability and provide protection from solar radiation reducing transpirational losses (Green, 2003). Therefore, the HV cultivar may be able to maintain higher water potentials given reduced light availability into the canopy, and facilitate increased SPS activity, synthesizing higher levels of Suc in the exocarp, while reducing Fru and Glc abundances (Figure 2.7; Yang et al., 2019). Inversely, increased hexose and reduced disaccharide abundances in the exocarp of the LV cultivar may be indicating over-abundant light penetration and reduced water availability (Yang et al., 2019; Figure 7).

*4.4. Metabolic responses to non-uniform light environments and variable canopy positions in peach mesocarp and exocarp*

When considering canopy uniformity, the HV cultivar demonstrated more distinct light availability and internal fruit quality attributes between the top (HVT) and bottom (HVB) positions (Fig 2.1g-h). As a result, sorbitol and citric acid abundances appeared to be significantly affected by this lack of canopy light distribution and fruit quality uniformity in both the mesocarp and exocarp (Figures 2.5-2.6). Sorbitol is significantly higher in abundance in HVT, while citric acid is significantly higher in abundance in HVB in both tissue types (Figures 2.5-2.6). Sorbitol is one of the main sugars translocated throughout the phloem of peach trees and serves as an indicator for sufficient carbon supply and beneficial photosynthetic conditions (Anthony et al., 2020; Morandi et al., 2008). This may help explain why only HVB fruit demonstrated significantly lower sorbitol abundance than the other cultivar canopy position treatments (Figures 2.5-2.6), as the lack

of light availability in the bottom of a canopy (especially in the HV cultivar, <20%, Figure 2.1g) may inhibit leaf photosynthetic rates and photosynthate exportation to nearby fruits (Marini and Sowers, 1990).

Additionally, high abundance of citric acid, as experienced in the HVB, has been correlated with carbon starved fruits and a lack of sweetness (i.e., inferior quality) in peach (Anthony et al., 2020; Colaric et al., 2005). This lack of sweetness in HVB fruit may be supported with the differences in SSC across canopy positions in the HV cultivar (ΔSSC=1.6%) (Table 2.1). Previous literature has determined that for consumers to detect differences in fruit sweetness, ΔSSC has to be greater than 1% (or 1-degree $^{o}$Brix) (Harker et al., 2002). Elevated levels of citric acid in HVB fruit may also be a result of smaller fruit size, when compared to the HVT fruit (Figures 2.5-2.6 and Table 2.1), as citric acid concentrations are dissimilated throughout cell expansion (Famiani et al., 2020). A lack of light, especially during cell division, could negatively impact fruit size, which could lead to elevated concentrations of citric acid at harvest (Lakso and Corelli-Grappadelli, 1992). These differences in sorbitol, citric acid and SSC support the concept that light availability and canopy uniformity play a significant role in influencing peach fruit quality and metabolism, and are not solely influenced by canopy position alone (Lewallen and Marini, 2003).

In respect to titratable acidity (TA), there were no statistical differences between canopy positions (Table 2.1). The lack of differences in TA are an expected result of the equal maturity approach, which is supported metabolically with uniform mesocarp levels of quinic and malic acid (Figure 2.7), the other two major organic acids in peach flesh (Walker and Famiani, 2018). Quinic acid has been previously demonstrated to have an inverse relationship with sucrose and peach fruit development, decreasing in concentration as the fruit matures and synthesizes more complex carbohydrates (Anthony et al., 2020; Wu et al., 2005). Peach mesocarp levels of quinic and malic

acid have been used as metabolite indices of peak maturity (Chapman et al., 1991). Therefore, the observed similar levels of malic and quinic acid in both cultivars across canopy positions (Figure 2.7) supports that we successfully controlled for true metabolic maturity and further validates our Vis-NIRS approach for enabling equal maturity comparisons.

Additional metabolites that were influenced by canopy position and non-uniform light availability, regardless of cultivar and tissue type included the higher abundance of amino acids like threonine (Thr) and asparagine (Asn), shikimic acid and butanoic acid in fruit from the bottom canopy position (Figures 2.5-2.6). Asparagine is the major amino acid in deciduous tree species for short-term nitrogen transport and is the predominant source of nitrogen translocated via the xylem (Lea et al., 2007). Additional amino acids, such as glycine (Gly) and aspartic acid (Asp), were also in higher abundance in the exocarp of the fruit from the bottom canopy position, regardless of cultivar (Figure 2.6). Typically, amino acids appear to be in higher abundance in early cultivars (Monti et al., 2016) and/or in the mesocarp of underdeveloped fruits (Anthony et al., 2020). Amino acids have also demonstrated elevated levels in inner-canopy apple fruit (Feng et al., 2014). This particular trend is supported in our data as the amino acid levels were in higher abundance in both mesocarp and exocarp of fruit from the bottom canopy position (Figures 2.5-2.6). These elevated levels of amino acids may be a result of cooler temperatures in the bottom canopy position, which were less exposed to solar radiation (i.e., reduced light availability) (Figure 2.1g). Fruit experiencing cooler temperatures and lower light levels can maintain lower levels of metabolic functions, so amino acids may remain in higher levels in the bottom fruit, as less protein synthesis is occurring or may be required (Feng et al., 2014). Therefore, increased amino acid abundance may be an indirect response to a restriction in protein synthesis due to stress-limiting conditions (Lea et al., 2007). Apples with elevated levels of nitrogen and amino acids have also

indicated poor consumer eating quality and postharvest storage performance (Wang and Cheng, 2011). This is in support of the data presented herein, which demonstrates that bottom fruit maintain inferior quality and higher levels of amino acids, with these differences being more pronounced in the HV cultivar (Figures 2.5-2.6 and Table 2.1).

In the mesocarp, shikimic acid and butanoic acid demonstrated higher abundance in fruit from the bottom canopy position, regardless of cultivar (Figure 2.6). However, in the exocarp, which is more directly influenced by the lack of uniformity across canopy positions (i.e., growing environment), shikimic acid and butanoic acid are only higher in abundance in HVB, while abundances remain the same across canopy position in the LV cultivar (Figure 2.6). Previously, mesocarp levels of butanoic acid, a fatty acid, have demonstrated a negative correlation with peach fruit development, with levels decreasing towards fruit maturation and harvest (Anthony et al., 2020). Mesocarp and exocarp levels of butanoic acid were higher in the early ripening LV cultivar which agrees with the fatty acid's kinetics across fruit developmental stages (Anthony et al., 2020). Additionally, peach mesocarp butanoic acid has demonstrated an inverse relationship with SSC (Anthony et al., 2020), and may be a potential indicator for inferior quality. In the exocarp, butanoic acid levels are only different in the HV cultivar, which suggests this compound is influenced by the light environment, as the HVB fruit experience a significant lack of available light (Figures 2.1g, 6). Therefore, butanoic acid may be involved with light-dependent pathways in the peach exocarp as previously reported in strawberries (Campbell et al., 2020). In banana, this aromatic fatty acid has been associated with the onset of ripening and increase of aroma volatilization (Zhu et al., 2018). Lower levels of butanoic acid in the high-light-exposed fruit may be a result of increased aromatic volatilization.

In the mesocarp, shikimic acid abundance is reduced in the top canopy position in both cultivars (Figure 2.5), while in the exocarp, this trend is only noted in the HV cultivar (Figure 2.6). Shikimic acid is a cyclitol and an important metabolite that is used in the shikimate pathway. The shikimate pathway is critical in the connection between the primary metabolism and the synthesis of secondary metabolite classes such as aromatic amino acids, alkaloids, flavonoids, hydroxycinnamic acids and phenolic acids (Lara et al., 2020). Chlorogenic acids, a group of phenolic acids, are important precursors for the development of anthocyanins and several metabolites correlated with flavor, taste and nutrition (Clifford, 2000; Walker and Famiani, 2018). Reduced levels of shikimic acid in mesocarp and exocarp from fruit in the top canopy position may reflect increased utilization of shikimic acid for secondary metabolite synthesis. This underscores the hypothesis that preharvest factors may be more heavily impacting the fruit's secondary metabolite composition than the primary metabolism (Anthony et al., 2020).

Overall, the cultivars evaluated in this study create distinct canopy micro-environments due to their different vigor classifications. As a result, these canopies facilitate different growing conditions for the developing fruit, with respect to different levels of light availability, temperature and transpirational losses. These distinct cultivar x canopy position environments impact both the internal fruit quality and metabolism (Figure 2.8). This is noted with the increased abundances of sucrose, sorbitol and catechin in the top canopy positioned, superior quality fruit, and the increased abundance of amino acids (aspartic acid (Asp), asparagine (Asn), threonine (Thr)), citric acid, monosaccharides (sorbose and fructose), butanoic and shikimic acid in the bottom canopy positioned, inferior quality fruit (Figure 2.8). Furthermore, as the cultivars' harvest windows vary, this influences the amount of time fruit have to develop and appears to also influence the fruit metabolome. This may explain why larger, more complex, secondary metabolites such as ursolic

acid, oleanolic acid and catechin are higher in abundance in the late ripening, HV cultivar. When considering the impact of these variable microclimates on fruit metabolism, the exocarp appears to be more heavily influenced than the mesocarp given its more direct interaction with the environment. This is evidenced with the larger number of significantly different metabolites between cultivar x canopy positions treatments in the exocarp at harvest, when compared to the mesocarp (Table S2.1).

**5. Conclusion**

The true impact of canopy position, a highly influential preharvest factor, was determined on peach fruit quality and metabolism. Given the relationship between fruit maturation and internal fruit quality and the metabolome, a novel approach was conducted to control for maturity utilizing non-destructive Vis-NIRS technology. This approach facilitated comparisons of two canopy positions in two different cultivars of variable vigor, without the confounding variable of maturation. Physiological analyses revealed that the fruit's canopy position alone is not influencing quality, but rather it is the specific environment the fruit is developing in (e.g., light, temperature, water potential, etc.). In the LV cultivar, a more uniform canopy resulted in more uniform quality at harvest, when maturity was controlled. However, in the HV cultivar, quality was superior only at the top canopy position, even when controlling for maturity, as there was significantly more available light than in the bottom. Therefore, growers should aim to form uniform/planar canopies through proper training system/rootstock selections or the use of watersprout removal in summer to ensure uniform light distribution across canopies. Mesocarp metabolomes appeared to be uniform and minimally influenced by the environment, as it is protected by the exocarp, and the primary metabolism appears to be heavily regulated by maturation. Exocarp metabolome variation was more directly influenced by the environment and demonstrated vast distinction in the HV

cultivar, while there was minimal variation in the LV cultivar. Overall, sorbitol, sucrose and catechin correlate to quality and the top canopy position, while asparagine, threonine, citric acid, sorbose, fructose, butanoic and shikimic acid correlate to inferior quality and the bottom canopy position.

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## Tables

**Table 2.1. Effect of canopy position on peach fruit internal quality and maturity.** The impact of canopy position on fruit internal quality and maturity, assessed with destructive quality analyses across both positions in 'Sierra Rich' and 'Cresthaven' peach. Fruit of equal maturity from 'Sierra Rich' and 'Cresthaven' peach trees were harvested at two canopy position zones: bottom (1.2 m) and top (3.0 m) and were compared to each other within each cultivar. Peach fruits (n=10) were assessed and controlled for equal maturity (index of absorbance difference ($I_{AD}$)) non-destructively. Fruit was then measured for fruit weight (FW), fruit diameter, flesh firmness (FF), soluble solids concentration (SSC), dry matter content (DMC), titratable acidity (TA) and skin lightness and chroma.

| Cultivar | Canopy Position | Average fruit fresh weight (FW) | Fruit diameter (mm) | $I_{AD}$ | FF (N) | SSC (%) | DMC (%) | TA (malic acid, %) | Skin lightness ($L^*$) | Skin color ($h^o$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 'Sierra Rich' (SR) | 1.2 m (Bottom) | 145 ± 5.1 | 66 ± 0.8 | 0.76 ± 0.05 | 46.9 ± 2.9 | 10.5 ± 0.4 | 12.3 ± 0.4 | 0.84 ± 0.06 | 39.0 ± 1.3 a | 32.7 ± 2.1 a |
| Low Vigor | 3.0 m (Top) | 158 ± 5.0 | 67 ± 0.7 | 0.78 ± 0.05 | 51.9 ± 1.4 | 11.2 ± 0.2 | 12.9 ± 0.3 | 1.01 ± 0.12 | 34.7 ± 0.8 b | 26.8 ± 0.9 b |
| | Significance | ns | ns | ns | ns | ns | ns | ns | ** | * |
| 'Cresthaven' (CH) | 1.2 m (Bottom) | 170 ± 8.5 b | 69 ± 1.3 b | 1.06 ± 0.04 | 55.5 ± 4.5 | 10.3 ± 0.3 b | 10.5 ± 0.2 b | 0.61 ± 0.05 | 52.5 ± 1.7 | 50.5 ± 2.5 |
| High Vigor | 3.0 m (Top) | 197 ± 7.3 a | 73 ± 1.0 a | 0.96 ± 0.04 | 57.1 ± 1.3 | 11.9 ± 0.2 a | 12.4 ± 0.3 a | 0.58 ± 0.05 | 56.4 ± 2.3 | 56.0 ± 3.2 |
| | Significance | * | * | ns | ns | *** | *** | ns | ns | ns |

Mean values ± S.E. are displayed. One-way ANOVAs for each parameter are displayed indicating ns, *, **, *** for non-significance or significance at $P \leq 0.05$, $\leq 0.01$, $\leq 0.0001$, respectively. Means followed by the same letter are not statistically different according to Tukey's HSD test ($P \leq 0.05$).

## Figures

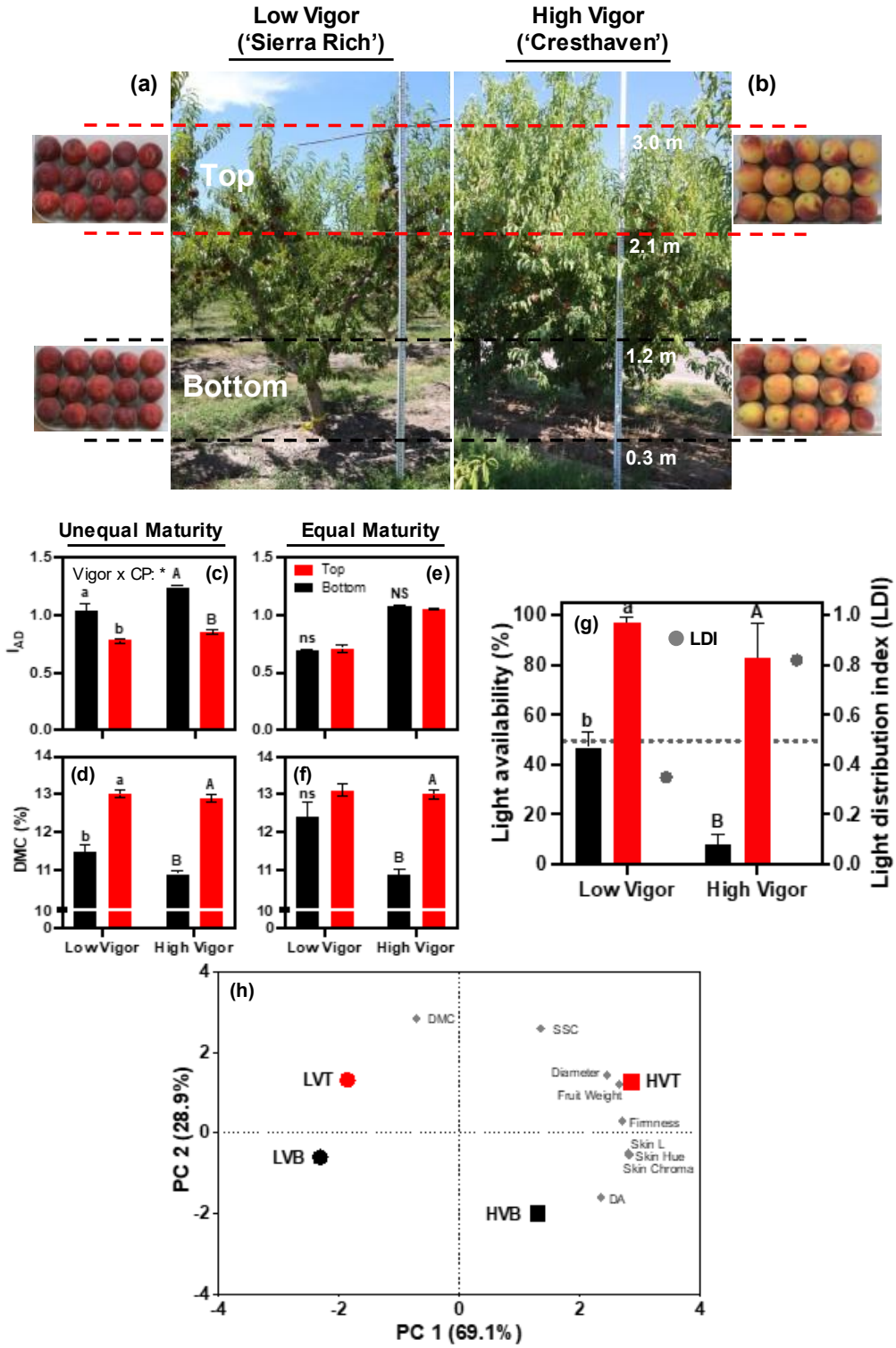
Low Vigor
('Sierra Rich')
High Vigor
('Cresthaven')
(a)
(b)
Top
Bottom
3.0 m
2.1 m
1.2 m
0.3 m
Unequal Maturity
Equal Maturity
Vigor x CP: * A
(c)
(e)
Top
Bottom
NS
ns
$I_{AD}$
(d)
(f)
DMC (%)
Low Vigor
High Vigor
(g)
LDI
Light availability (%)
Light distribution index (LDI)
(h)
DMC
SSC
Diameter
Fruit Weight
Firmness
Skin L
Skin Hue
Skin Chroma
DA
LVT
HVT
LVB
HVB
PC 2 (28.9%)
PC 1 (69.1%)

**Figure 2.1. Canopy position experiment with maturity control to assess the true impact of light availability on peach fruit quality and metabolism.** A canopy position study was conducted to understand the true impact of light availability on fruit quality, along with how mesocarp and exocarp metabolomes shift in response to different microclimates within the canopy. Two cultivars of variable vigor/ripening time were selected: 'Sierra Rich' (low vigor (LV), early) (a) and 'Cresthaven' (high vigor (HV), late) (b) and two canopy position zones were determined: bottom (0.3-1.2 m) and top (2.1 – 3.0 m). Fruit from each position in each cultivar were measured non-destructively for maturity ($I_{AD}$, c) and quality (dry matter content, DMC, d) to understand how canopy position influences these parameters. However, to understand the true impact of canopy position on fruit internal quality, maturity was then controlled for and quality was re-assessed on fruit of equal maturity ($I_{AD}$, e, f). Light availability was measured to demonstrate the influence of vigor on light uniformity across the positions (g). Light distribution index (LDI) was calculated and plotted on the secondary y-axis (g). An LDI value greater than 0.5 = non-uniform light conditions, whereas less than 0.5 = uniform light conditions. Mean values ± S.E. are displayed. Means with the same letter displayed above the bar are not statistically different according to Tukey's HSD test ($P \leq 0.05$) within each cultivar (low vigor=lower-case, high vigor=upper-case) (c-g). Two-way ANOVA in Figure 1c reveals a significant interaction between vigor x canopy position (CP). The fruit of equal maturity coming from these variable positions were also evaluated with destructive fruit quality analysis for fruit weight, diameter, firmness, soluble solids concentration (SSC), DMC, $I_{AD}$ (DA), and skin lightness ($L^*$), chroma ($C^*$) and hue angle ($h^o$). These quality parameters for each canopy position x cultivar treatment were plotted in a principal component analysis (PCA) with scores indicated for each treatment combination (LV-Top (LVT), LV-Bottom (LVB), HV-Top (HVT) and HV-Bottom (HVB)). Large symbols indicate the scores for the canopy position treatments (top (red) vs. bottom (black)) and cultivars (LV (circle) vs. HV (square)) and were scaled with the quality parameter loadings (grey diamonds) (h).

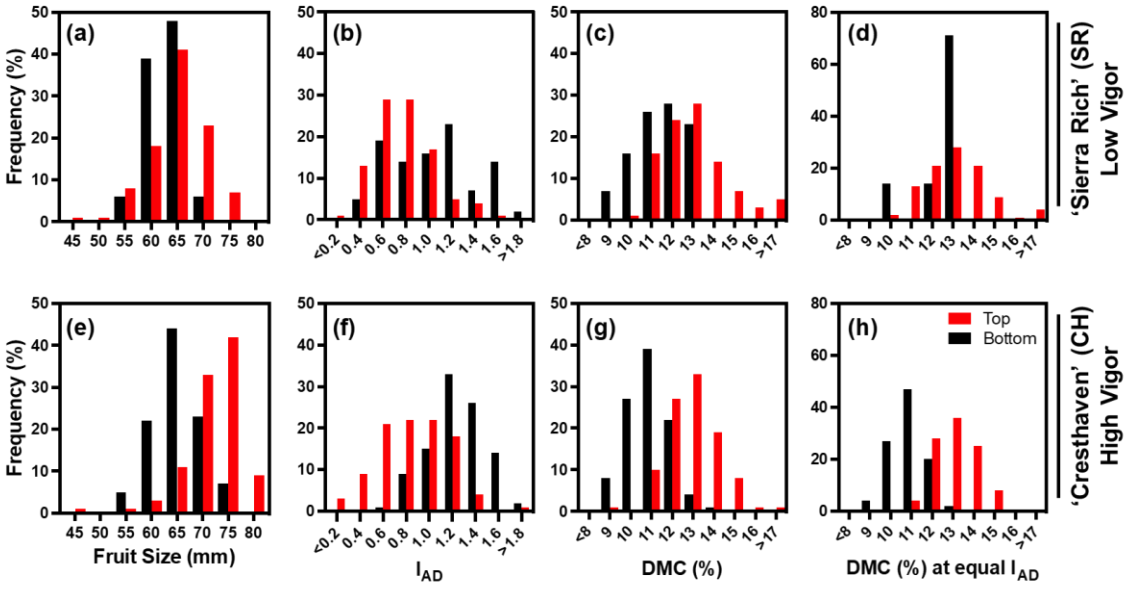


**Figure 2.2. Non-destructive distributions of quality parameters at harvest across canopy positions in two distinct cultivars of variable vigor and ripening window.** The impact of canopy position on 'Sierra Rich' (a-d) and 'Cresthaven' (e-h) fruit distributions across classes of

fruit size (a, e), $I_{AD}$ (b, f), DMC (c, g), and DMC on fruit of equal maturity ($I_{AD}$) only (d, h). Fruit was assessed using non-destructive Vis-NIRS prediction models for maturity and quality. Various canopy position treatments' frequencies for each parameter are visualized as: top (red) and bottom (black).

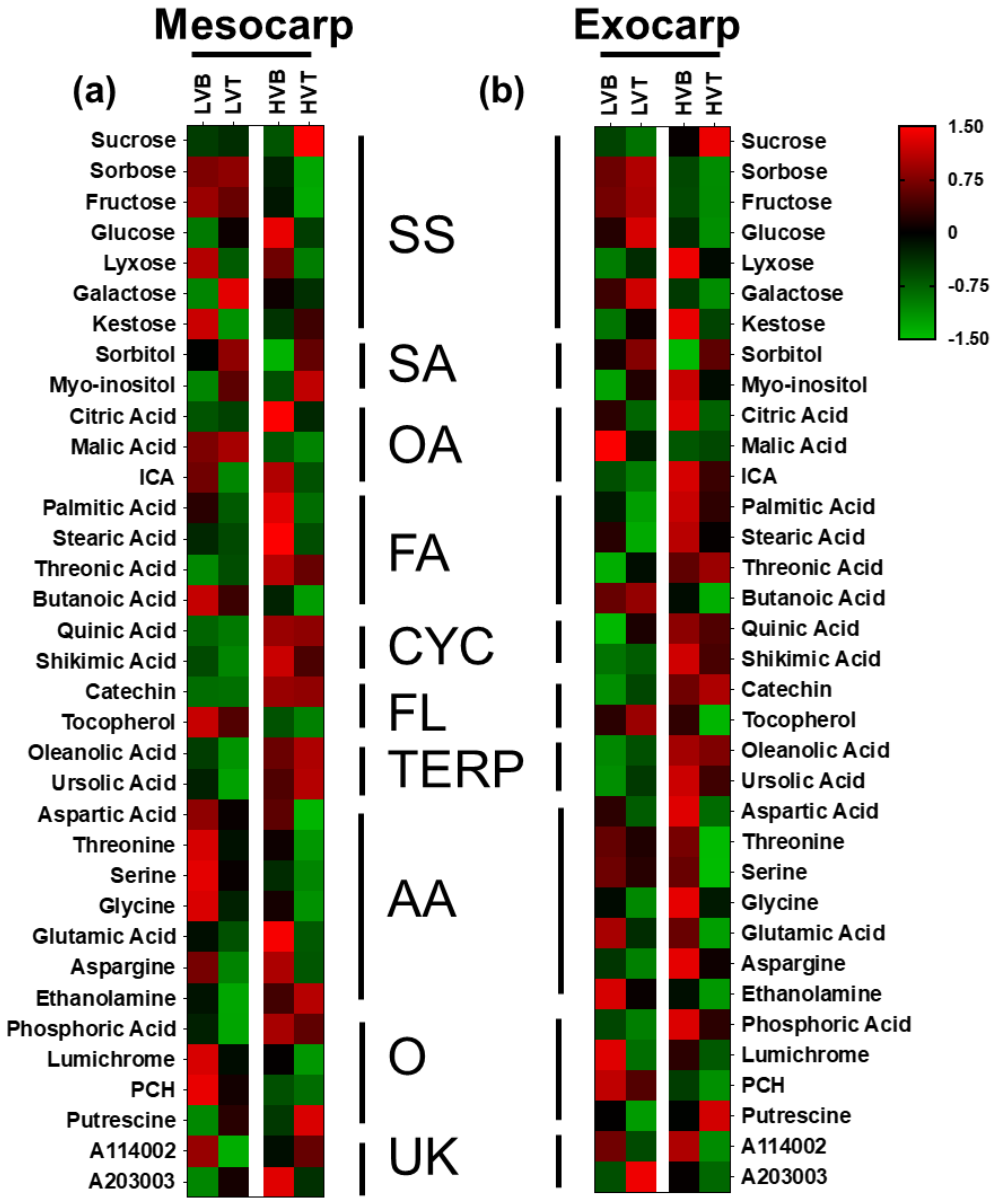


**Figure 2.3. Heat map of metabolite profiles across canopy positions in two distinct cultivars of variable vigor/ripening times in two different tissue types.** Profiles of metabolism changes across two canopy positions at harvest in peach fruit mesocarp and exocarp in 'Sierra Rich' (low vigor, LV) and 'Cresthaven' (high vigor, HV) fruit. Figure shows comparisons of the metabolite abundance across LV-Top (LVT), LV-Bottom (LVB), HV-Top (HVT) and HV-Bottom (HVB) in the mesocarp (a) and exocarp (b). Each of the 35 annotated metabolites were transformed z-scores and shown with the following color scale (green to red) according to Lombardo et al., (2011).

Fruits at each position were of equal maturity according to the $I_{AD}$ measured by the DA meter. Annotated metabolites are organized by chemical class: soluble sugars (SS), sugar alcohols (SA), organic acids (OA), fatty acids (FA), cyclitols (CYC), flavonoids (FL), triterpenoids (TERP), amino acids (AA), other (O) and classified unknowns (UK).

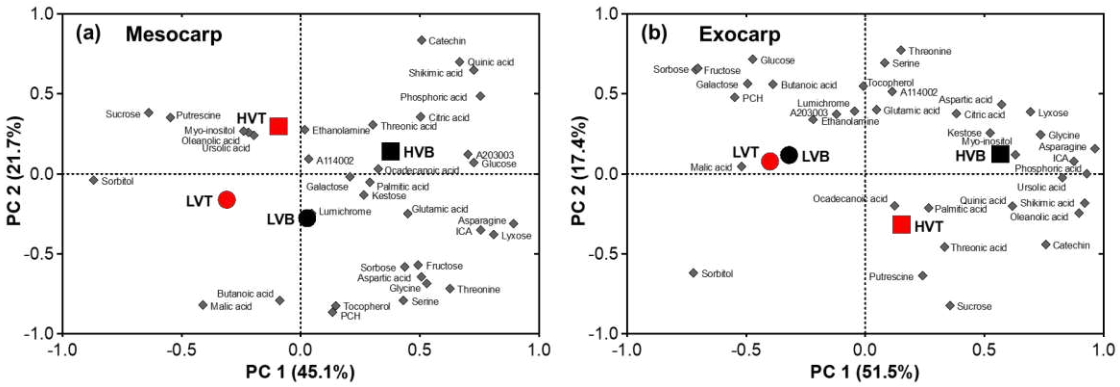


**Figure 2.4. Principal component analysis biplot of variable canopy positions on peach fruit metabolism in two cultivars across two tissue types.** Metabolite profiles across two canopy positions at harvest in peach fruit mesocarp and exocarp in 'Sierra Rich' (low vigor, LV) and 'Cresthaven' (high vigor, HV) fruit. Figure shows comparisons of the metabolite profiles across LV-Top (LVT), LV-Bottom (LVB), HV-Top (HVT) and HV-Bottom (HVB) in the mesocarp (a) and exocarp (b). Large symbols indicate the scores for the canopy position treatments (top (red) vs. bottom (black)) and cultivars (LV (circle) vs. HV (square)) and are pareto scaled (-1.0 – 1.0) with the 35 annotated metabolites detected in the peach mesocarp and exocarp (loadings, grey diamonds). Principal component analysis (PCA) of the six reps per each position x cultivar treatment were averaged in each biplot. The mesocarp PCA (b) demonstrate that both canopy position (PC1, 45.1%) and genotype (PC2, 21.7%) were major contributors for metabolome variation as indicated by their separation (a). In the exocarp PCA (b) variation between canopy positions were reduced in the LV treatment, while HV demonstrate wide separation between canopy positions (b). Cultivar differences appear to act as the major contributor for separation with PC1 accounting for 51.5% of the variation (b). PC2 appears to explain more of the variation attributed to canopy position with 17.4 explained (b). PCA shows that wide variation in the metabolome between canopy positions in the high vigor cultivar in the exocarp showcases that metabolites are significantly influenced by non-uniform environments.

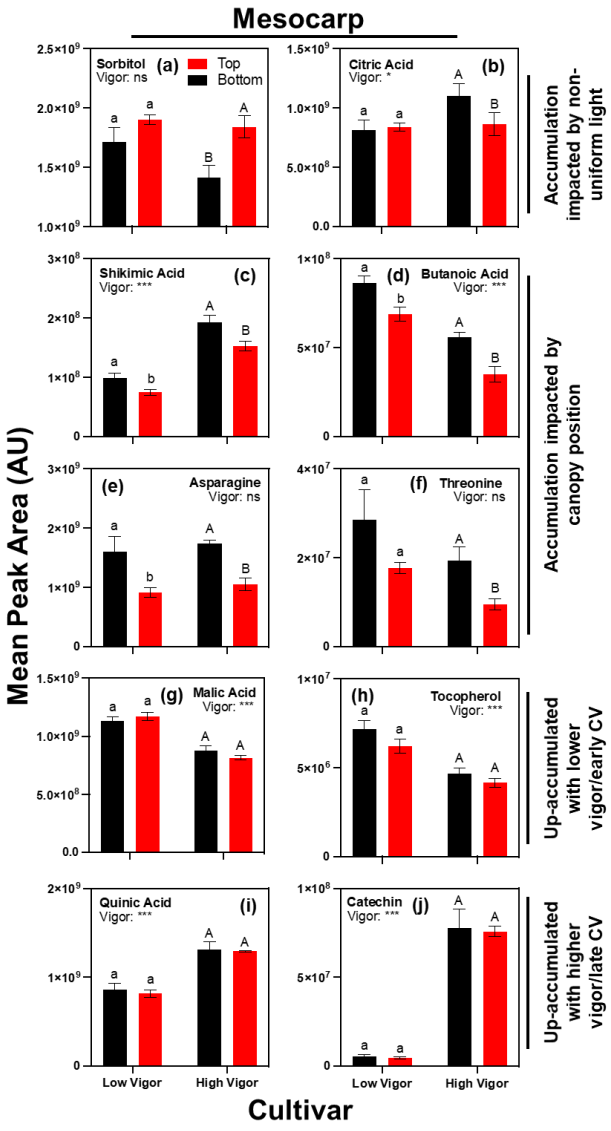


**Figure 2.5. Accumulation trends of metabolite abundances between canopy positions across two cultivars of variable vigor/ripening time in the peach mesocarp.** Figure showcases the mean peak area (AU) of selected metabolites that are influenced by non-uniform light: sorbitol (a) and citric acid (b); canopy position: shikimic acid (c), butanoic acid (d), asparagine (e), threonine (f); up-accumulated with the low vigor cultivar: malic acid (g) and tocopherol (h) or the high vigor cultivar: quinic acid (i) and catechin (j) in the peach mesocarp of 'Sierra Rich' (low vigor) and 'Cresthaven' (high vigor) fruit at harvest. The bars indicate canopy position treatments: bottom (black) and top (red). Samples were controlled for equal maturity ($I_{AD}$) at harvest and between canopy position treatments. Mean values ± S.E. are displayed with low vigor presented on the left of each graph, while the high vigor is displayed on the right. Means with the same letter displayed above the bar are not statistically significant according to Tukey's HSD test ($P \leq 0.05$) within each cultivar (low vigor=lower-case, high vigor=upper-case). Vigor: ns, *, *** indicates non-significance or a significant difference between cultivars at a $P \leq 0.05$, $\leq 0.0001$, respectively according to Tukey's HSD test.

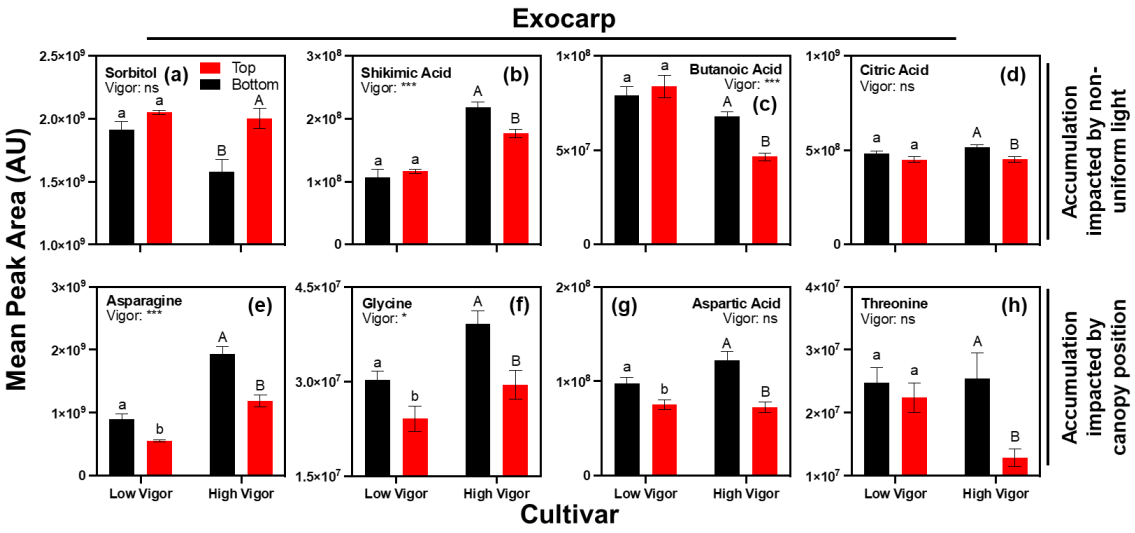


**Figure 2.6. Light and canopy position accumulation trends of metabolite abundances between canopy positions across two cultivars of variable vigor/ripening time in the peach exocarp.** Figure showcases the mean peak area (AU) of selected metabolites that are influenced by non-uniform light: sorbitol (a), shikimic acid (b), butanoic acid (c), citric acid (d); and canopy position: asparagine (e), glycine (f), aspartic acid (g) and threonine (h) in the peach exocarp of 'Sierra Rich' (low vigor) and 'Cresthaven' (high vigor) fruit at harvest. The bars indicate canopy position treatments: bottom (black) and top (red). Samples were controlled for equal maturity ($I_{AD}$) at harvest and between canopy position treatments. Mean values ± S.E. are displayed with the low vigor presented on the left of each graph, while the high vigor is displayed on the right. Means with the same letter displayed above the bar are not statistically different according to Tukey's HSD test ($P \leq 0.05$) within each cultivar (low vigor=lower-case, high vigor=upper-case). Vigor: ns, *, *** indicates non-significance or a significant difference between cultivars at a $P \leq 0.05$, $\leq 0.0001$, respectively according to Tukey's HSD test.

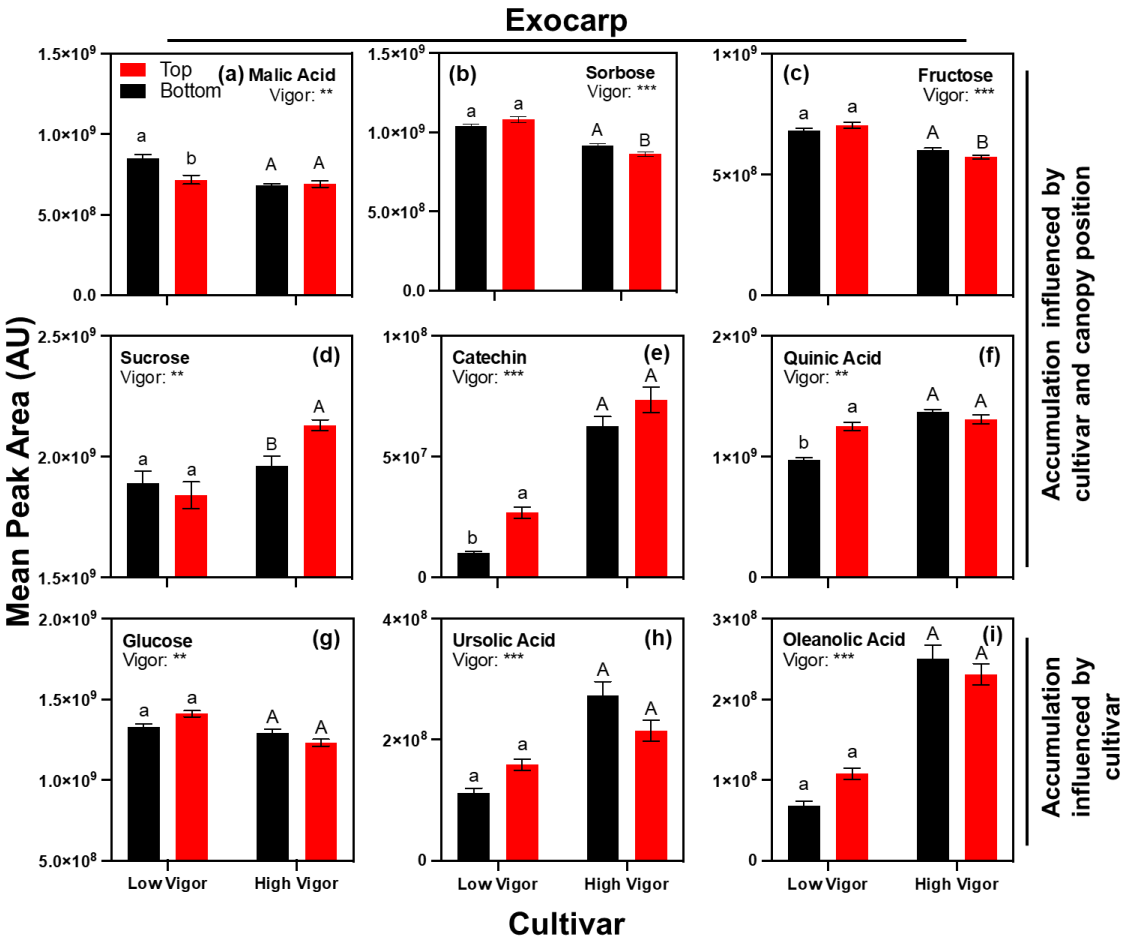


**Figure 2.7. Cultivar and vigor accumulation trends of metabolite abundances between canopy positions across two cultivars of variable vigor/ripening time in the peach exocarp.** Figure showcases the mean peak area (AU) of selected metabolites that are influenced cultivar and canopy position: malic acid (a), sorbose (b), fructose (c), sucrose (d), catechin (e), quinic acid (f); and cultivar alone: glucose (g), ursolic acid (h), oleanolic acid (i) in the peach exocarp of 'Sierra Rich' (low vigor) and 'Cresthaven' (high vigor) fruit at harvest. The bars indicate canopy position treatments: bottom (black) and top (red). Samples were controlled for equal maturity ($I_{AD}$) at harvest and between canopy position treatments. Mean values ± S.E. are displayed with the low vigor presented on the left of each graph, while the high vigor is displayed on the right. Means with the same letter displayed above the bar are not statistically different according to Tukey's HSD test ($P \leq 0.05$) within each cultivar (low vigor=lower-case, high vigor=upper-case). Vigor: ns, *, *** indicates non-significance or a significant difference between cultivars at a $P \leq 0.05$, $\leq 0.0001$, respectively according to Tukey's HSD test.

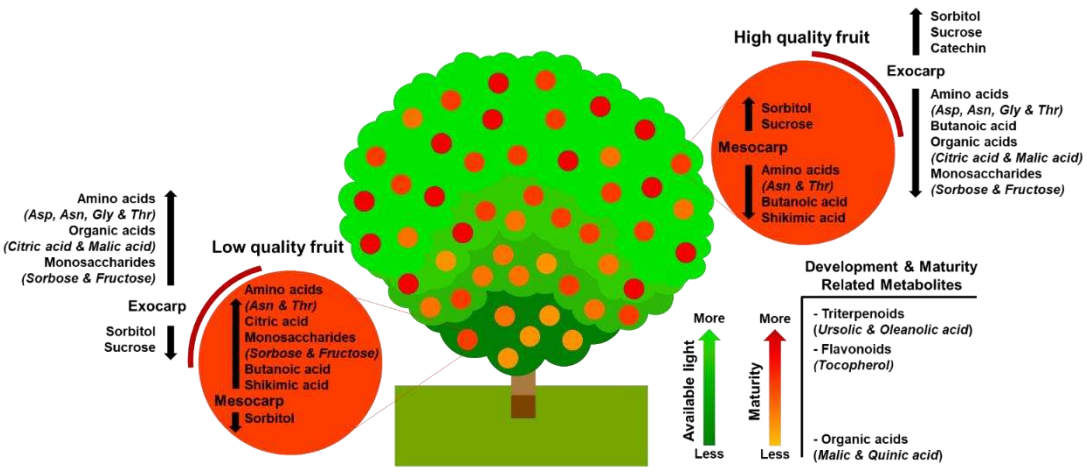


**Figure 2.8. The influence of light availability and canopy position on metabolite abundance changes in peach mesocarp and exocarp.** Up- and down-accumulation trends are presented for chemical classes and specific metabolites in peach mesocarp and exocarp as a result of variable light availability and canopy positions. Metabolites related to development and maturity are also displayed. A gradient of advanced maturity from the bottom of the canopy to the top is displayed, although metabolite profiling was conducted on fruit of equal maturity (note: same color fruit). Light availability generally increases as well, from the bottom of the canopy towards the top, especially in a canopy of higher vigor.

**Supplementary data**

**Table S2.1**. Relative abundances of the 35 annotated metabolites in peach mesocarp and exocarp from two cultivars of variable vigor and two canopy positions at harvest.

CHAPTER THREE

# EARLY METABOLIC PRIMING UNDER DIFFERING CARBON SUFFICIENCY CONDITIONS INFLUENCES PEACH FRUIT QUALITY DEVELOPMENT

## 1. Introduction

Improvement of peach (*Prunus persica* L. Batsch) fruit quality is of critical importance as peach consumption has been in steady decline in recent years due to poor performance with consumers (Minas et al., 2018). It is broadly accepted and supported by previous studies that fruit quality can only be improved at the preharvest stage in the orchard, and then maintained during postharvest handling (Crisosto et al., 1997; Minas et al., 2018). One of the most influential preharvest factors for peach fruit quality is crop load management (e.g. flower/fruitlet thinning) (Minas et al., 2018). Advantages of thinning include increased fruit weight and size, higher levels of dry matter content (DMC) and soluble solids concentration (SSC), and overcolor blush coverage (Marini, 2003; Crisosto and Costa, 2008; Minas et al., 2018, 2021). Thinning flowers or fruitlets balances the leaf-to-fruit ratio, or the source/sink relationship for photosynthates. This potential yield sacrifice is crucial to reduce competition for photosynthates between fruits, so that the remaining fruit have an adequate pool of carbohydrates at every stage of development enabling them to reach maximum growth and quality at harvest (Grossman and DeJong, 1995).

The main forms of carbon translocated from leaves to fruit are sorbitol and sucrose, via symplastic and apoplastic pathways, to support growth and development in peach (Zhang et al., 2004; Morandi et al., 2008). Plasma membrane-bound sorbitol transporters (SOTs) take up sorbitol into the cytosol of parenchyma cells where it is rapidly converted to fructose by sorbitol dehydrogenase (SDH) (Zhang et al., 2004). Conversely, sucrose, is either directly taken up by sucrose transporters (SUC; SUT) into the cytosol, or degraded to hexoses (e.g. glucose and

fructose) by cell wall invertases (CWINVs), which are then transported into the cytosol via hexose transporters (Bologa et al., 2003; Zhang et al., 2004). Cytosolic hexoses are phosphorylated through the glycolytic pathway and split into triose phosphates, which are finally oxidized to pyruvate (Li et al., 2018). Pyruvate can then be transported into the matrix of the mitochondria by a mitochondrial pyruvate carrier to fuel the tricarboxylic acid (TCA) cycle (Li et al., 2018; Boeckx et al., 2019). Unrestricted availability of these soluble sugars is of critical importance for the maintenance of the fruit's respiratory metabolic functions, which are vital to producing energy, and assembling carbon-based compounds for use in various biosynthetic processes in primary and secondary metabolism (Boeckx et al., 2019).

Similarly to carbon synthesis and utilization, maturation and ripening in climacteric fruit (e.g. peach) are highly regulated processes that initiate with an increase of respiration and release of ethylene (Giovannoni et al., 2017). Throughout "on-tree" maturation and ripening of peach, several sensorial and textural changes occur, such as increasing DMC and SSC, pigment accumulation, flesh softening, and aromatic volatilization; all parameters that improve organoleptic characteristics and consumer satisfaction (Crisosto and Costa, 2008; Minas et al., 2018). Preharvest factors that affect carbohydrate starvation/competition in the growing fruit (e.g. girdling, leaf-to-fruit ratio, crop load) can severely alter the regulation of the primary and secondary metabolism, influencing the maturation/ripening processes and internal fruit quality (Nardozza et al., 2019). Importantly, preharvest factor comparisons performed on fruit of unequal maturity are potentially confounded by the maturation status of the fruit. Therefore, to truly evaluate the impact of preharvest conditions on fruit physiology and metabolism it is critical that comparisons be made on fruit of equal maturity (Minas et al., 2018).

Understanding how preharvest factors impact fruit internal quality (DMC, SSC) and maturity (flesh firmness, FF) is essential, but can be very time-consuming and expensive with traditional destructive techniques (Minas et al., 2018). Furthermore, given the variability of fruit on a tree, or within an orchard, these narrow approaches fail to capture the totality of quality development in the field (Carlomagno et al., 2004). Near infrared spectroscopy (NIRS) is an alternative non-destructive technique that has been successfully utilized to accurately assess different quality attributes in peach (Minas et al., 2021). In addition, in recent years, a new index (index of absorbance difference, $I_{AD}$) has been developed to characterize physiological changes (e.g. chlorophyll degradation) that occur during peach maturation and ripening (Ziosi et al., 2008; Costa et al., 2009). This index effectively describes the physiological maturity of peach and has been integrated into open-source handheld Vis-NIRS sensors to enable simultaneous and rapid evaluation of peach fruit internal quality and physiological maturity (Minas et al., 2021). This technology can be paired with molecular tools to better understand regulation differences in fruit, as it can enable rapid maturity control assessments.

Given the complexity of the biological processes associated with fruit maturation, ripening, and quality development, the use of large-scale molecular tools ("omics") that allow for the global evaluation of metabolic pathways within the fruit is particularly important (Molassiotis et al., 2013). Non-targeted metabolomics is an approach that can be utilized in horticultural research to advance our understanding of the impact of preharvest factors on regulatory networks and biochemical interactions in plants (Guy et al., 2008). Fruit is the most metabolite-rich plant organ, and thus metabolomics offers an opportunity to investigate the metabolic processes involved in quality development (Monti et al., 2016). Thus, regulation of the central carbon metabolism through manipulation of preharvest factors is not only essential for fleshy fruit carbohydrate

accumulation (e.g. sugars and organic acids), growth, development, maturation, and ripening, but it is also key to fruit taste, flavor and quality (Cirili et al., 2016; Li et al., 2018; Nardozza et al., 2019). In addition, the accumulation of specific sugars, organic acids or secondary metabolites at different phases of fruit development as a response to stress or preharvest conditions might be related to signaling or priming procedures (Tohge et al., 2014).

Previous studies that have been conducted to better understand metabolic regulation in peach have primarily evaluated postharvest factors and cold storage disorders (Lauxmann et al., 2014; Bustamante et al., 2016; Tanou et al., 2017; Santin et al., 2018; 2019; Monti et al., 2019; Lillo-Carmona et al., 2020; Wang et al., 2018), along with maturation/ripening physiology, environmental conditions and pest tolerance (Lombardo et al., 2011; Capitani et al., 2012; Monti et al., 2016; Karagiannis et al., 2016). Characterizing fruit metabolic shifts in response to preharvest factors is key to fruit quality improvement, as several of these compounds contribute to organoleptic characteristics that correlate with consumer preference. Many studies have reported effects of various preharvest and orchard factors on tree fruit quality (Minas et al., 2018, Musacchi and Serra, 2018). However, despite the enormous advances in "omics" sciences, few experiments have addressed the influence of these preharvest factors on fruit quality at the metabolic level (Monti et al., 2016; Serra et al., 2018; Michailidis et al., 2020). Furthermore, it is important to highlight that these previous studies did not control for equal maturity when analyzing fruit coming from various preharvest conditions. Therefore, to truly understand how these factors influence both fruit quality and metabolism, comprehensive experimental approaches that control for maturity amongst comparisons are critical pre-requisites (Minas et al., 2021).

The goal of this study was to evaluate the true impact of crop load management, a major preharvest factor, on peach quality using phenotypic and metabolomic assessments of fruit of equal

maturity. Our experimental approach enabled a detailed physiological characterization of photosynthate availability reflective of variable fruit-to-fruit competition conditions (i.e. thinning severities) during peach fruit growth and development. Using non-destructive NIRS technology (to control for fruit maturity) and non-targeted metabolite profiling to evaluate the impact of carbon supply, we identified metabolic shifts associated with fruit quality priming/suppression under carbon sufficiency/starvation.

## 2. Materials and Methods

### *2.1. Plant material and experimental approach*

Fifteen peach (*Prunus persica* L. Batsch.) trees of a late-ripening cultivar ('Cresthaven') and of uniform size and health were selected from Colorado State University's experimental orchard at the Western Colorado Research Center-Orchard Mesa, Grand Junction, CO (39°02'31.3"N, 108°27'56.8"W). The trees were nine years old, grafted on 'Lovell' rootstock, and trained to an open vase system at a planting density of 509 trees $ha^{-1}$ (4 × 5 m spacing). Fifty-two days after full bloom (DAFB: April 9, 2016) five thinning treatments were administered to shift the photosynthate source:sink ratio and create distinct carbon supply levels. Each treatment was performed on three replicate trees. The treatments were designed to achieve varying levels of photosynthate sufficiency for the growing fruits and included leaving trees unthinned (control) or by thinning and spacing the remaining fruit every 5, 10, 15 and 30 cm from each other on the fruiting shoots (Figure 3.1a). Trees were thinned after "June drop" during early stage 2 (S2) of peach fruit development to ensure no further natural fruit abscission would occur and to maintain the spacing and predetermined levels of carbon competition amongst the remaining fruit. The trees were managed according to industry standards and practices.

Trunk cross sectional area (TCSA) was measured in the spring to enable the selection of uniform trees, and again in the fall (postharvest) to assess tree growth responses to treatments throughout the growing season and to calculate crop load (fruit · $cm^{-2}$ TCSA) (Table 3.1). Fruit growth (diameter, mm) was monitored in 10 fruit per experimental tree the day prior to thinning, immediately after thinning and every week the rest of the growing season until harvest, using a fruit lasso. Peach volume ($cm^3$) was calculated as was previously described (Minas et al., 2015; Figure 3.1b, e).

Yield data were collected at the conclusion of the experiment (August 25) when fruit were harvested at the pre-climacteric commercial harvest maturity (stage 4I, referred to as S4). The fruit was counted and then weighed to determine yield (kg · $tree^{-1}$), while the average fruit fresh weight (FW, g) was calculated. Crop load was calculated and expressed as fruit no. · $cm^{-2}$ of TCSA (Table 1). Fifteen fruit from each experimental tree (n=45 per treatment) were assessed destructively and non-destructively to evaluate the impact of crop load on peach maturity and internal quality at harvest (Table 3.2; Figure 3.1c).

To determine potential implications of carbon competition on tree mineral status 100 leaves were sampled mid-season (July 20) from the middle portion of several one-year-old shoots from each experimental tree (Gavlak et al., 2003). These leaves were then washed, dried, and shipped to Ward Laboratories (Kearney, NE, USA) for elemental analyses. Macro- and micro-nutrients assessed included: N %, P %, K %, S %, Ca %, Mg %, Zn ppm, Fe ppm, Mn ppm, Cu ppm, B ppm and Mo ppm (Table S3.1). To assess the impact of carbon supply on return bloom next season, five shoots per experimental tree, the number of flowers, and their length were measured at full bloom in spring (March 22, 2017). The fertility index was then calculated by dividing the number

of flower buds by the length of the shoot (cm). Later in spring (May 9), the fruit were counted, and fruit set (%) was calculated (Table 3.1).

To characterize the true impact of carbon supply on peach quality development without the confounding impact of maturation status, two crop load treatments were selected to be studied in detail during peach growth and development on fruit of equal maturity (Figure 3.1e, f). Specifically, the unthinned treatment which represents a carbon "starved" condition due to the high demand and competition between fruits, and the 15 cm fruit-to-fruit spacing (thinned) treatment, which represents an adequate and sufficient carbon supply throughout development due to reduced photosynthate competition were chosen. Five fruit of equal maturity (determined non-destructively using NIRS) per experimental tree (n=15 per treatment) were evaluated for quality (non-destructively and destructively as described below) at three development stages: stage 2 (S2, 72 DAFB), stage 3 (S3, 109 DAFB) and stage 4 (S4, 138 DAFB) (Figure 3.1e-f). Three biological replicates consisting of five homogenized fruit mesocarp samples of equal maturity from these two treatments at the three developmental stages were sampled, flash frozen (quenched) with liquid nitrogen and stored at -80 $^{o}$C (Figure 3.1f) until analysis by inductively coupled plasma mass spectrometry (ICP-MS) and gas chromatography mass spectrometry (GC-MS). Prior to analysis, peach mesocarp samples were freeze dried with the lyophilizer (Freezone 4.5, Labconco, Kansas City, MO, USA) at -40 $^{o}$C for 12 h. Finally, samples were pulverized into a powder with a bead beater (Bullet Blender Storm, Next Advance, Troy, NY, USA) for five minutes. These samples were then kept in -20 $^{o}$C until digestions and/or extractions could take place.

*2.2. Fruit quality analyses*

Fruit were assessed for quality, non-destructively, using an "open-type" near-infrared handheld spectrometer (F-750 Produce Quality Meter, Felix Instruments Inc., Camas, WA, USA).

The F-750 produce meter was calibrated to non-destructively estimate 'Cresthaven' fruit quality based on dry matter content (DMC, %) and soluble solids concentration (SSC, %) (Minas et al., unpublished) and physiological maturity based on the index of absorbance difference ($I_{AD}$) with a single scan as previously described (Minas et al., 2021). This approach allows for rapid assessment of fruit quality and maturity "on-tree," to assure that comparisons among treatments and sampling for large-scale analytical methodologies are based on fruit of equal maturity. The non-destructive estimations were validated at each stage with actual destructive data, by scanning and measuring for each parameter from the same side of the fruit as described for field validations (Minas et al., 2021; Fig 3.1).

Physiological maturity measurements were validated using a factory calibrated Vis-NIR spectrometer (DA meter, Sinteleia SRL, Bologna, Italy), which uses the non-destructive metric, $I_{AD}$. The DA meter assesses the absorbance difference ($I_{AD}$=A670 nm-A720 nm) at the specific wavelengths that chlorophyll is absorbing light beneath the fruit surface, which provides an estimate of fruit physiological maturity (i.e. background color) (Ziosi et al., 2008; Costa et al., 2009; Spadoni et al., 2016). Fruit were scanned with the F-750 and DA meter on the sun exposed and shaded side of the fruit, along the equatorial region, and these values were averaged.

Dry matter content measurements were validated destructively by cutting out 25-mm diameter peach mesocarp cylinders with the skin removed using a cork borer. Peach mesocarp cylinders were weighed for fresh weight (FW) using a digital scale (TC-204, Denver instruments, Arvada, CO, USA) and then moved into a forced-air oven (VWR Oven F Air 104 L, VWR, Radnor, PA) at 65 $^{o}$C to dehydrate, until samples reached a stable weight (~three days). Dry weight (DW) of the dehydrated peach samples were then measured. Dry matter content was then determined by the mass difference between the FW and DW of each peach sample, as previously described (Minas

et al., 2021). For SSC (%) measurement validations, similar peach mesocarp cylinders (that were cut from the opposite sides of DMC samples) were juiced through a garlic press into a digital refractometer (PR-32α, Atago, Tokyo, Japan) as previously described (Minas et al., 2021).

Additional quality analyses were conducted at each developmental stage, which included parameters such as: skin color (hue angle, $h^o$), overcolor blush (%), fruit size (mm), individual fruit fresh weight (FW, g), flesh firmness (FF, N) and titratable acidity (malic acid, %) as previously described (Minas et al., 2021).

*2.3. Ionomic analysis using inductively coupled plasma mass spectrometry (ICP-MS)*

The lyophilized, pulverized, and homogenized samples were randomized into standard 75 mL microwave vessels (PerkinElmer, Waltham, MA, USA). Each vessel received 125 mg of peach mesocarp tissue placed inside of a teflon weighing cup. After the peach tissue was placed into the vessel, 8 mL of doubly distilled nitric acid ($HNO_3$, 70% by volume solution spiked with an Indium (In) internal standard) was added and the vessels were left to react for 15 min. An additional 2 mL of ultra-trace grade hydrogen peroxide ($H_2O_2$, 30% by volume solution) was added to each vessel and left to react for an additional 15 min. The seals and rupture discs were then placed onto the vessels, the caps were screwed on and they were loaded into the microwave (Titan MPS Microwave Sample Preparation System, PerkinElmer, Waltham, MA, USA). Samples were processed in random batches of 15 plus a blank (capacity of the Titan MPS system). The preset method (provided by the manufacturer) for "dried fruit" was utilized (Table S2). After the completion of the digestion procedure, the vessels were removed and placed into a refrigerator (4 $^oC$) for 30 min to cool. Once cooled, the contents from each vessel was poured into a 50 mL conical falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) and the volume was raised to 15 mL total with 18.2 MΩ ultrapure water (Milli Q Direct, Millipore Sigma, Bedford, MA, USA). This

first dilution was then followed by a second dilution, in which 1 mL of the diluted and digested sample from each 50 mL conical falcon tube was then pipetted into a 15 mL conical falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) and again raised to a total volume of 15 mL with Milli-Q water. The final solution contained an internal standard of 20 nL · $L^{-1}$ of In and 2.5% nitric acid.

ICP-MS was performed using the NexION 350D (PerkinElmer, Waltham, MA, USA). In each sample, elemental concentrations of Arsenic (As), Aluminum (Al), Barium (Ba), Boron (B), Cadmium (Cd), Calcium (Ca), Chromium (Cr), Cobalt (Co), Copper (Cu), Iron (Fe), Lead (Pb), Lithium (Li), Magnesium (Mg), Manganese (Mn), Molybdenum (Mo), Nickel (Ni), Phosphorous (P), Potassium (K), Selenium (Se), Sodium (Na), Strontium (Sr), Sulfur (S), Vanadium (V), Tungsten (W), and Zinc (Zn) were measured. Digested samples were injected using a prepFAST SC-2 (Elemental Scientific, Omaha, Nebraska) autosampler. Samples were nebulized using a PFA-ST (Elemental Scientific) nebulizer into a quartz cyclonic spray chamber that was kept at 4 $^{o}$C via a PC3 peltier cooler (Elemental Scientific). Li, Be, B, Na, P, S, Mg, K, Ca, W, As, and Pb were measured in standard mode. Cd, Se, and As were measured in dynamic reaction (DRC) mode using oxygen as the reactive gas. Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, and Ba were measured in DRC mode using ammonia as the reactive gas. Prior to analysis, the ICP-MS underwent a daily tune, ensuring that the torch was aligned for maximum In signal. The nebulizer gas flow was also optimized for maximum In signal intensity while also ensuring that oxide formation ($CeO^+$:$Ce^+$) was kept below 0.02. Additionally, the quadrupole ion deflector (QID) was optimized for maximum signal across the mass range by monitoring Li, Mg, In, Ce, Pb, and U. Furthermore, the formation of doubly charged species was limited by ensuring that $Ce^{++}$:$Ce^+$ ratio was below 0.03. A calibration curve was created by evaluating 7 dilutions of a multi-element stock solution, created

by a mixture of single element stock standards (Inorganic Ventures, Christiansburg, VA, USA) (Chaparro et al., 2018). Calibration curve was matrix matched to the samples, 2.5% $HNO_3$ and 20 ppb In. For instrument drift correction, internal standard solutions consisting of $^6$Li, Rh and Ir was added to each sample via the autosampler. Quality control samples were generated by pipetting 1 mL from each sample into a pooled QC and analyzed after every 6 samples.

Ionomic data processing was conducted as previously (Chaparro et al., 2018). Data was processed using Excel (Microsoft, Redmond, WA, USA). Each element analyzed was corrected with an internal standard and subsequently underwent drift correction and minimizing the coefficient of variation (CV) of the QC samples identified how each element should be corrected. Samples were then corrected for the appropriate dilution factor. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve respectively. Final element concentrations are reported as µg/g of freeze-dried peach mesocarp.

*2.4. Non-targeted metabolite profiling using gas chromatography mass spectrometry (GC-MS)*

Metabolite extraction was conducted by weighing out 25 mg of each freeze-dried peach mesocarp sample and placing them into clean 2 mL autosampler glass vials (VWR, Radnor, PA, USA). One mL of 80% by volume LC-MS grade methanol (MeOH) in water solution was then added to each vial and vortexed on a plate shaker (Fisherbrand™ Analog Multitube Vortexer, ThermoFisher Scientific, Waltham, MA, USA) at 4 °C at max speed (2500 rpm) for 2 hours (h). Samples were then held at -80 °C for 1 h followed by centrifugation for 25 min at 3500 rpm at 4 °C. The supernatant was extracted (~800 µL) without disturbing the pellet and pipetted into new a 2 mL autosampler vial. A pooled quality control (QC) solution was created by transferring 10 µL of each sample into a separate glass vial. Fifteen µL of each sample was transferred to another set

of glass vials, centrifuged for 2 min at 3500 rpm and then dried under $N_2$ (g) for 30 min. Dried samples were stored at -80 $^oC$ until derivatization. Derivatization (methoximation and silylation) took place immediately prior to running the samples. Dried down samples were allowed to warm to room temperature and then re-suspended in 50 µL of methoxyamine HCl (prewarmed to 60 $^oC$) and centrifuged for 30 sec. Samples were then incubated at 60 $^oC$ for 45 min, followed by a brief vortex, sonication for 10 min and an additional incubation at 60 $^oC$ for 45 min. Following this, the samples were centrifuged before receiving 50 µL of N-Methyl-M (trimethylsilyl) trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (ThermoFisher Scientific, Waltham, MA, USA), briefly vortexed and incubated at 60 $^oC$ for 40 min, as described previously (Chaparro et al., 2018). Samples were loaded (~ 80 µL) into glass inserts within glass autosampler vials and centrifuged for 30 sec prior to GC-MS analysis.

GC-MS analysis was performed using the Clarus 690 GC coupled to a Clarus SQ 8S Mass Spectrometer (PerkinElmer, Waltham, MA, USA). Metabolites were separated with a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d. 0.25 µm film thickness). The GC program began at 80 $^oC$ for 0.5 min and ramped to 330 $^oC$ at a rate of 15 $^oC$ per minute and ended with an 8 min hold at a 1 $mL \cdot min^{-1}$ helium gas flow rate. The inlet temperature was held at 285 $^oC$ and the transfer line was held at 260 $^oC$. Masses between 50-620 m/z were scanned at four scans/sec after electron impact ionization. QC injections were analyzed after every $6^{th}$ sample and were used to control for and detect analytical variation.

Metabolomic data processing was conducted as previously described (Chaparro et al., 2018). GC-MS files were converted to .cdf format and processed by XCMS in R (Smith et al., 2006; R Core Team, 2015; Mahieu et al., 2016). All samples were normalized to the total ion current (TIC). RAMClustR was used to deconvolute peaks into spectral clusters for metabolite annotation

(Broeckling et al., 2014). RAMSearch (Broeckling et al., 2016) was used to match metabolites using retention time, retention index and matching mass spectra data with external databases including Golm Metabolome Database (Hummel et al., 2007; Hummel et al., 2013) and NIST (Broeckling et al., 2016).

*2.5. Statistical analyses*

All parameters were assessed for statistical differences between thinning treatments using proc-GLM in SAS (SAS Inc., Cary, NC, USA). The effect of thinning and developmental stage on physiological characteristics, elemental concentrations and mean peak area of metabolites were assessed for significance with either an one-way ANOVA (thinning or developmental stage) or a two-way ANOVA (thinning x developmental stage) ($P \leq 0.05$). Tukey mean comparisons were used to assign different lettering groups where the model was significant at $P \leq 0.05$. Principal component analyses (PCA) was performed on physiological, ionomic, and metabolomic data using SIMCA (Umetrics, Umea, Sweden). Ions, metabolites and fruit physiological characteristics were assessed in R to conduct correlations (using spearman's rank) and hierarchical clustering using cor, aov, and hclust functions and to develop heat maps using the corrplot package (Wei et al., 2010; R Core Team, 2015; Turner et al., 2016). Heat maps, PCAs, and graphs were visualized using Prism v8.2.1 (Graph Pad Inc., San Diego, CA, USA).

## 3. Results

*3.1. Crop load affected fruit size, quality and maturity at harvest*

The various thinning treatments provided a significant impact on the number of fruit per tree, crop load (fruit · $cm^{-2}$ of TCSA) and yield (kg · $tree^{-1}$), although these differences were minimized between the 10 and 30 cm fruit spacing treatments. Final crop loads on trees of similar size, as expressed by TCSA, ranged from 1.6 to 11.8 fruits · $cm^{-2}$ of TCSA (Table 3.1). Average fruit

diameter increased linearly with increasing thinning severity ($R^2$=0.99; data not shown), with a 40% increase, from the unthinned control to extreme thinning (30 cm fruit spacing) (Table 3.1; Figure S3.1). A large percentage of fruit (96%) in the extreme crop load (unthinned) were smaller than 70 mm in diameter, whereas the 15 and 30 cm treatments yielded 94 and 93% of their fruit with a diameter greater than 70 mm at harvest (Figure S3.1). Fruit FW and volume also increased with reduced crop load and competition for photosynthates, with a two-fold increase in both FW and volume from the unthinned when compared to the extreme thinning (30 cm) treatment (Table 3.1; Figure 3.1). Average fruit FW only exceeded 200 g with the 15 cm and 30 cm thinning treatments (Table 3.1). Return bloom and fruit set increased with reduced carbohydrate competition during flower bud initiation in the previous season amongst all thinning severities, when compared to the unthinned control (Table 3.1). However, return fruit set was maximized with the 15 cm thinning treatment (Table 3.1). Minimal impacts were seen in the leaf elemental content across crop loads. Specifically, significant differences were observed only for K, with elevated levels in leaves from the 15 cm and 30 cm spacing treatments compared to the unthinned control and other treatments (Table S1).

Non-destructive simultaneous internal quality (DMC, SSC) and maturity ($I_{AD}$) analyses (Minas et al., 2021) using an accurately calibrated (high $R^2$ values and low RMSEP in Figure 2A, B, C) handheld NIRS sensor for 'Cresthaven' peach (Minas et al., unpublished) demonstrated significant differences across all parameters assessed (Table 3.2). Dry matter content and SSC increased significantly by 25 and 27%, respectively, from the heaviest to the lowest crop load (corresponding to a decrease in carbon competition) (Table 3.2; Figure 3.2d-e). However, the degree of differences diminished between the 10 and 30 cm fruit spacing treatments (Table 3.2). Interestingly, 99 and 98% of fruit from the 15 and 30 cm fruit spacing exceeded a DMC level of

11%, while only 31% of fruit surpassed this level in the unthinned treatment (Figure 3.2d). Similarly, fruit from same thinning treatments had 87% of its fruit surpassing an SSC level of 11%, a minimum consumer quality standard (Hilaire, 2003), while the unthinned treatment had only 8% of its fruit surpassing this threshold (Figure 3.2e). Maturity ($I_{AD}$) advanced (lower $I_{AD}$ values) with increased thinning severity, with fruit that were coming from unthinned and heavily cropped (5 cm fruit spacing) trees being less ripe than the fruit coming from the remaining thinning (10, 15 and 30 cm) treatments at harvest (Table 3.2). At harvest, 69% of fruit were classified as immature (> 0.60 $I_{AD}$) in the unthinned control, while the 15 cm treatment gave less immature fruit (57% > 0.60 $I_{AD}$) (Figure 3.2f). Flesh firmness (FF) exhibited a weak relationship between the NIRS estimation and actual destructive values (Minas et al., 2021; Minas et al., unpublished), so only the destructive values are presented (Table 3.2). Minimal differences were detected in FF across the five crop load treatments, with only fruit from the 15 cm treatment being significantly softer than fruit from the 5 cm treatment (Table 3.2). Overall, these data demonstrate that increased thinning severity (corresponding to increased carbon availability) resulted in improved fruit quality characteristics and advanced maturity at harvest. However, it is important to note that these data do not confirm if these observed quality shifts are direct results of carbon availability due to the differing fruit-to-fruit competition and/or the advancement/delay in fruit maturation.

### *3.2. Phenotyping fruit of equal maturity demonstrates superior quality in thinned fruit, especially at S4*

In order to determine the true impact of carbon supply on fruit quality development, fruit of equal maturity from one thinning treatment (15 cm, carbon sufficient) were compared with the unthinned (carbon starved) control (Figure 3.1e-f, Figure 3.3) during peach growth and development. As described above, a handheld non-destructive NIRS sensor was utilized to sort for

fruit of equal maturity at each developmental stage (S2, S3 and S4) between the carbon starved (unthinned) control and carbon sufficient (thinned at 15 cm fruit spacing referred from this point forward as "thinned") treatments (Figure 3.3f). Sorting for equal maturity allows for comparisons of preharvest treatments without the confounding influence of variable maturity. Maturity control was confirmed by ensuring that $I_{AD}$ values did not differ between the crop load treatments at each developmental stage (Figure 3.3f). Selected fruit from the thinned treatment and the unthinned control exhibited $I_{AD}$ values that decreased by 66 and 63% over time from S2 to S4, respectively (Figure 3.3f). In addition, FF decreased relatively similarly across the developmental stages, with an 81 and 73% reduction in firmness from S2 to S4 in the thinned treatment and unthinned control, respectively (Figure 3.3e).

The PCA demonstrates that developmental stage was a major contributor for quality variation as indicated by separation on PC 1 (~ 71%). Additional quality variation due to the thinning treatment and other factors are visualized on PC 2 (~ 27%). PCA shows minimal difference in fruit phenotype at S2, while at S4, phenotypes between thinning treatments (Figure 3.1e) are highly separated.

In general, fruit quality parameters were not significantly different between the thinned treatment and unthinned control at S2, but improved fruit quality in the thinned treatment was observed at S3 and S4 (Figures 3.1d-e, 3.3a-d). For example, fruit FW and diameter increased faster and to heavier/larger levels (1.5-fold heavier and 37% larger) at harvest (S4) than the unthinned control (Figures 3.1d-e, 3.3a-b). For both fruit FW and diameter, fruit from the unthinned control at S4 were equivalent to fruit from the thinned treatment at S3 (Figure 3.3a-b). Similar trends were observed for DMC and SSC. Specifically, a 28% increase in both DMC and SSC were observed in fruit from the thinned treatment when compared to the unthinned control at

S4 in fruit of equal maturity (Figure 3.3c-d). Importantly, both of these carbohydrate-based quality parameters (DMC and SSC) increased and remained higher in the fruit from the thinned treatment representing a condition of low fruit-to-fruit competition. Conversely, in the unthinned control, these parameters decreased as fruit reached commercial harvest maturity (between S3 and S4), reflecting the magnitude of the carbon starvation condition (Figure 3.3c-d). These results suggest that the thinning treatment improved carbon supply to the fruit, resulting in enhanced fruit quality.

Global changes in fruit quality can be visualized using principal component analysis (PCA, Figure 3.3g) further demonstrating the trends described above. At S2 (triangles, Figure 3.3g) fruit from the thinned treatment and unthinned controls are highly similar (Figure 3.1e). However, at S3 and S4 (squares and circles, Figure 3.3g) it is clear that the overall fruit quality between the thinned treatment and the unthinned control diverges (Figure 3.1e). Evaluation of the influence of the loadings (presented as orange diamonds in the PCA biplot in Figure 3.3g) indicates increased values in quality parameters (DMC, SSC, FW, and fruit diameter) in fruit from the thinned treatment at S3 and S4.

### *3.3. Elemental composition in peach fruit mesocarp is affected by maturation and early carbon sufficiency/starvation*

Ionomic analysis was conducted to evaluate the impact of carbon limitation/availability across developmental stages on elemental composition of equal maturity peach mesocarp. The analysis screened for a panel of 23 elements, of which, 3 (Li, Pb, and W) were below the assay LOQ (Table S3). Principal component analysis demonstrates a large percentage of the variation being attributed to PC1 (66.6%) representing developmental stage, with an additional portion of the variation explained with PC2 (13.1%) representing carbon supply and likely other unknown factors (Figure S3.2). The concentration of detected elements decreased in both treatments with the advancement

of peach fruit growth, development and maturation (Figure S3.3). At S2, 10 elements (Al, As, Ba, Ca, Cd, Fe, K, Mg, Sr, V and Zn) had significantly higher concentrations in the unthinned control compared to the thinned treatment (Table S3.3). At harvest (S4), only 4 elements (Al, As, Sr and V) remained significantly higher in the unthinned controls (Table S3.3). Taken together, these results demonstrate that there is an overall trend in decreasing elemental concentration with developmental stage advancement, and that most of the significant differences due to the carbon availability that were observed early in development, were no longer relevant at harvest.

### *3.4. Vast metabolic differences shift early on during fruit growth and development as a response to carbon sufficiency/starvation*

In total, 189 metabolites were detected in peach mesocarp samples (Figure 3.4). Of those, 36 metabolites were confidently annotated (orange diamonds, Figure 3.4). Principal component analysis revealed that a large percentage of the metabolic variation is attributed to PC1 (34.1%) representing fruit developmental stage, with additional variation explained by PC2 (14.7%) representing carbon supply manipulation (Figure 3.4). Importantly, the largest metabolic separation was observed between the thinned treatment (red symbols) and unthinned control (black symbols) at S2 (triangles). This separation is reduced at S3 (squares) and further at S4 (circles). These early shifts in the metabolome resulting from variable carbon supply levels due to thinning treatment are in direct contrast to the observed trends in fruit phenotype, which were greatest at the last developmental stage (Figures 3.1d, 3.3). In other words, this early dramatic shift in the metabolome (Figure 3.4) is in direct contrast to phenotypic shifts, where PCA separation at S2 in Figure 3.3g is minimal. Overall, shifts in the metabolome (Figure 3.4) appear to behave inversely with shifts in fruit quality and maturity attributes (Figure 3.3g).

General shifts in the peach metabolome across developmental stages include a decreasing abundance of various amino and organic acids (Figure 3.5). Some hexoses decreased with fruit development (e.g. glucose, fructose and galactose), while more complex saccharides increased with development (e.g. sucrose and kestose; Figure 3.6). Additional metabolites showing this trend include valeric acid and tocopherol (Figure 3.6). Metabolites including myo-inositol, proline and butanoic acid showed the inverse trend, decreasing significantly throughout development (Figures 3.5, 3.6, S3.4). Fructose, sorbose, myo-inositol and threonic acid were all significantly more abundant in the carbon starved, unthinned control at S2, by 75, 73, 79 and 67%, respectively (Figures 3.6, S3.5). Interestingly, these differences became less significant as fruit development progressed (Figure 3.6). Conversely, sorbitol, quinic acid and tocopherol were significantly more abundant by 46, 50 and 47%, respectively, in the thinned treatment at S2 (Figure 3.6).

### *3.5. Metabolite accumulation and degradation throughout development correlate with fruit physiological attributes*

Of the 36 annotated metabolites, 25 strongly correlated ($r_s$>0.70 or <-0.70) with at least one fruit physiological characteristic (fruit FW, fruit diameter, DMC, SSC, FF and $I_{AD}$) throughout development in fruit from the thinned treatment (15 cm fruit spacing; Figure 3.7), which is a commonly used commercial thinning practice. Sucrose demonstrated extremely strong positive correlations with fruit FW ($r_s$=0.97), diameter ($r_s$=0.95) and SSC ($r_s$=0.71) (Figure 3.7). Kestose (saccharide), along with tocopherol (i.e. antioxidant; vitamin E) exhibited a strong positive correlation with fruit FW ($r_s$=0.74 and 0.79, respectively). Glucose-6-phosphate was the only metabolite to have a strong correlation ($r_s$<-0.70) with DMC across fruit development (Figure 3.7). Many strong negative correlations were observed for SSC including several monosaccharide hexoses (lyxose, altrose, galactose, and glucose; Figure 3.7). Other metabolites showing strong

negative correlations with SSC include organic and fatty acids such as glutamic acid, butanoic acid, threonic acid, pyroglutamic acid, and quinic acid ($r_s$=-0.90). Many positive correlations between physiological maturity and metabolite abundance were also observed. For example, as $I_{AD}$ values decreased (maturity advancement), the abundance of several amino acids and monosaccharide hexoses (e.g. glucose, fructose and galactose) decreased, while the abundance of di- and polysaccharides increased (e.g. sucrose and kestose; Figures 3.5-3.7). Strong positive correlations with $I_{AD}$ was observed for multiple amino acids including alanine, glycine, and proline ($r_s$=0.93). A strong negative correlation with $I_{AD}$ was observed for sucrose ($r_s$=-0.97), however positive correlations were observed for other monosaccharides including glucose, altrose, galactose ($r_s$=0.89). The strongest metabolite-metabolite relationships (positive/negative) include altrose with quinic acid ($r_s$=0.99) and sorbitol with malic acid ($r_s$=-0.95).

### *3.6. Catechin and a classified unknown metabolite represent different relationships with fruit quality parameters at harvest*

At the S2 developmental stage, 16 of the 36 annotated metabolites were significantly different between the unthinned control (carbon starvation), and the sufficiently carbon supplied, thinned treatment ($P \leq 0.05$; Table S3.4). Whereas only two of these metabolites remained significantly different at S4 (Table S3.4), catechin and a classified unknown, A244002 (Figures 3.5, 3.8a, d). Catechin remained highly elevated in the thinned treatment throughout development, but the magnitude was greatest at S2 (47-fold; Figure 3.8a). At S3 and S4, catechin was still higher in the carbon sufficient condition when compared to the starved, but to a lesser degree (7-fold and 4-fold, respectively; Figure 3.8a). The opposite trend was observed for A244002, with a greater abundance (2-fold) observed throughout fruit development in the carbon starved condition compared to carbon sufficiency (Figure 3.8d).

At harvest (S4), these two compounds were assessed for their relationship with the physiological internal quality parameters related to photosynthates (SSC and DMC), in both the carbon sufficient (thinned) and the carbon starved (unthinned control. Catechin exhibits positive and significant ($P \leq 0.05$) correlations at harvest with both DMC ($R^2$=0.73) and SSC ($R^2$=0.74), indicating superior fruit quality (Figure 8B, C). Alternatively, A244002 showed strong negative and significant correlations ($P \leq 0.05$) with DMC ($R^2$=-0.81) and SSC ($R^2$=-0.79) at harvest, signaling inferior quality (Figure 3.8e-f).

## 4. Discussion

### *4.1. Increased thinning severity enhances fruit quality, but is it a result of crop load management or advanced maturation?*

Reducing the sink number on trees (via fruit thinning) allows for increased photosynthates for the remaining fruit, which enhances quality and advances maturity (Minas et al., 2021). Determining an optimal crop load for a particular cultivar in a growing region must balance fruit production and quality standards. In our study, increased thinning severity reduced yields, but enhanced fruit quality and advanced fruit maturity (Tables 3.1 and 3.2). Among the different crop load management strategies studied the 15 cm fruit spacing (2.6 fruit · $cm^{-2}$ of TCSA) provided an acceptable yield (~40 kg $tree^{-1}$ or 20 MT · $ha^{-1}$) for mid-density late-ripening peaches in Colorado growing conditions (USDA, 2019) with commercially acceptable fruit FW, size and quality to secure grower profitability (Minas et al., 2018). At the same time, 15 cm fruit spacing maximized return fruit set and had higher return bloom numbers when compared to the rest of the crop load treatments (Table 3.1). This demonstrates evidence that a sufficient carbon supply was achieved, allowing for optimum fruit quality development, balanced vegetative growth (Grossman and DeJong, 1995) and adequate floral bud differentiation and fruit set for the next season.

Collectively, these production, quality and reproductive capacity indicators make 2.6 fruit · $cm^{-2}$ of TCSA (i.e., 15 cm spacing in this experiment) a potential target for consistent high yields of high-quality late-ripening peach fruit.

This trade-off of yield for quality has been documented in several peach crop load studies, where reduced crop loads improve internal fruit quality (Inglese et al., 2002; Marini, 2003; Alcobendas et al., 2012). However, the reported quality enhancements may be unintentionally confounded by maturity advancement in the lower crop loads and thus not truly linked to the preharvest factor alone (Minas et al., 2018). Therefore, our study sought to evaluate fruit of equal maturity, sorted by a handheld NIRS sensor, to understand the actual impact of thinning on fruit internal quality development. With the selection of the 15 cm fruit spacing thinning treatment as the optimal crop load management strategy, comparisons were made between this treatment and the unthinned control for fruit quality parameters, assessed at equal maturity, as confirmed with $I_{AD}$ (Figure 3.3f).

At S2, there were minimal, although statistically significant, differences in physiological parameters between the thinned treatment and unthinned control (Figure 3.3a-f), while the phenotype of these fruits at this stage were very similar (Figure 3.1e). However, initiating at S3, both phenotype and fruit quality characteristics begin to diverge (Figures 3.1e, 3.3g). At S4, fruit phenotypes between the thinned treatment and unthinned control were drastically different, in respect to size, weight and overcolor (Figures 3.1d-e, 3.3a-f, S3.1, and Table 3.1). Dry matter content and SSC increased throughout development in thinned fruit but begin to decrease between S3-S4 in unthinned control (Figure 3.3c-d). A high number of fruit during the critical stages of fruit growth and development, could generate a sink demand that can be too high and fruits can become source (carbon) limited (Grossman and DeJong, 1995). This leads to deleterious effects

on fruit quality in the unthinned trees as a result of diluted photosynthate availability. Multiple studies have demonstrated that increased crop load reduces DMC and SSC accumulation (Berman and DeJong, 1996; Crisosto et al., 1997; Cirilli et al., 2016; Minas et al., 2021), however these may be attributed to maturation delay. Here, by controlling for fruit of equal maturity we demonstrate that these reductions in internal fruit quality are directly related to crop load and carbon availability during fruit growth and development.

### *4.2. Ionome variation linked mainly with peach fruit development and maturation, and less with carbon availability*

Overall, the ionome appears to shift with peach fruit development and maturation (Figure S3.2). The concentrations of nearly all elements decreased over time, with little variation in elemental composition between the thinned treatment and unthinned control (Figure S3.3). Elemental differences are more abundant in S2 than at harvest (S4, Table S3.3). Specifically, at S2, 10 elements were statistically different ($P \leq 0.05$), while only 4 elements were different at harvest (S4) (increased concentrations of Al, As, Sr and V in the unthinned control) (Table S3.3). All 10 elemental differences at S2 indicate higher concentrations in the unthinned treatment as well, including key nutrients such as Ca, Fe, K, Mg, and Zn. This may be a stress response of the tree, to flood the fruitlets early with critical nutrients to support the heavy fruit load due to high competition conditions or to reduce fruit expansion rates (Wünsche and Ferguson, 2005). However, as the tree's nutrients get exhausted, or as nutritional support may be more related to maturation, less differences in elemental composition appear. Previous studies in apple have observed more significant variation in elemental composition with crop load treatment (Wünsche and Ferguson, 2005), however, this result is likely confounded by fruit maturity. The results

presented here from fruit of equal maturity suggest that carbon availability has minimal impact on elemental composition at harvest.

*4.3. Peach fruit metabolites are related to fruit growth, development and maturation processes*

Fleshy fruit development, maturation and ripening are intricately regulated by physiological and molecular processes (Giovannoni et al., 2017). Therefore, to understand any preharvest factor effect on peach fruit metabolism, samples must be evaluated at equal maturity to eliminate this confounding variable (Minas et al., 2018). The novel methodological approach of this study enabled the opportunity to investigate the true impact of carbon manipulation on peach fruit metabolism and to generate a baseline understanding of metabolites that are involved in fruit growth and development. Importantly, most of the metabolome variation observed is reflective of fruit developmental changes rather than carbon manipulation (Figures 3.4-3.5). Thus, our data suggest that metabolic shifts are intricately connected to the highly coordinated processes of fruit maturation and ripening (Giovannoni et al., 2017).

Since maturity control was validated using $I_{AD}$ (Figure 3.3f), abundances of specific compounds could be linked with developmental stage and maturation. Previously, it has been suggested that physiological maturity could be determined metabolically based on the abundances of sucrose and/or quinic acid in peach fruit mesocarp and seed (Chapman et al., 1991). Sucrose continues to accumulate throughout peach development, reaching maximum levels at harvest, while quinic acid behaves inversely, starting high and depreciating throughout maturation (Chapman et al., 1991; Wu et al., 2005). Our data confirm these trends and reveal no differences in sucrose nor quinic acid across carbon supply treatments at harvest (S3.4; Figure 3.6d, k). Furthermore, there are minimal differences (only two compounds) in the metabolome between carbon sufficiency and carbon starvation treatments at S4 (Figure 3.5 and Table S3.4). This result

underscores the strong connection between the primary metabolism and fruit growth and development, as these compounds are fundamental for essential fruit functions. In other words, the lack of observed differences in metabolites reflective of the primary metabolism between carbon sufficiency/starvation conditions at harvest, is likely a result of controlling for maturity.

Additional metabolites that strongly correlate with fruit maturation are presented in Figure 3.6. Similar to previous research, our results show that glucose, fructose and galactose, along with various organic acids, are highly abundant in immature fruit and decrease in abundance throughout peach fruit development, while sucrose steadily increases in abundance until ripening, where it rapidly increases and becomes the most abundant saccharide at harvest (Figure 3.6; Chapman et al., 1991; Wu et al., 2005; Lombardo et al., 2011; Bae et al., 2014). These saccharide conversions from mono- to polysaccharides across maturation are supported in the literature (Cirilli et al., 2016), which demonstrates the conversion of simple hexoses into more complex sugars throughout development (Figures 3.5-3.6). Sorbitol has also been linked to fruit development, showing a peak in sorbitol abundance at the S2/S3 transition followed by a decline towards S4, which is supported with our results (Cirili et al., 2016; Figures 3.5-3.6).

Several monosaccharides and sugar alcohols detected in this study, under sufficient carbon supply (thinned treatment), show inverse correlations with FW and SSC and decreased with maturation (Figure 3.7). For example, galactose and glucose abundances are positively correlated with $I_{AD}$ ($r_s$=0.89 and 0.73, respectively) and firmness ($r_s$=0.96 and 0.91, respectively), meaning their abundances decrease with maturity advancement and flesh softening. Textural changes and flesh softening in pome and stone fruit has been attributed to the solubilization of cell wall components and pectic substances, such as saccharides (Nara et al., 2001; Brummell et al., 2004). Galactose, found in cell walls, has been documented to decrease with flesh softening, as it is

solubilized throughout maturation due to the activation of polygalacturonase (Brummell et al., 2004). Complex carbon compounds, such as sucrose and kestose, demonstrate opposite trends, increasing with maturation ($r_s$=-0.97 and -0.78, respectively; Figure 6), FW ($r_s$=0.97 and 0.74, respectively) and SSC ($r_s$=0.71 and 0.58, respectively; Figure 3.7).

The most significant changes observed for amino acids across maturation were for proline, which was not significantly impacted by carbon availability (Figure 3.7). Proline during fruit growth and development was also highly positively correlated with $I_{AD}$ ($r_s$=0.93). Other amino acids including glycine, threonine and alanine were positively correlated to firmness and maturity ($I_{AD}$), and negatively correlated with weight and SSC (Figure 3.7). These results are consistent with previous studies showing that amino acids are typically elevated in immature fruit/early and decrease throughout development (i.e. when firmness and $I_{AD}$ values drop, and weight and SSC increase) (Monti et al., 2016).

Malic acid showed a positive correlation with FW and a negative correlation with maturity advancement (i.e., $I_{AD}$ decrease) (Figures 3.6-3.7). This result supports the use of malic acid abundance as a maturity index and it has also been shown to correlate well with consumer preference (Chapman et al., 1991; Colaric et al., 2005). Organic acids typically accumulate in immature fruit and decrease with fruit development (Bae et al., 2014). Valeric acid, a fatty acid, does not follow this general trend, reaching a maximum abundance in S4. This suggests that it may be involved in fruit ripening and aroma volatilization. Butanoic acid, another fatty acid, which has been shown to be a predominant aromatic compound associated with the onset of ripening in banana (Zhu et al., 2018), was found to decrease with peach maturation (Figure S3.4).

4.4. *Early sufficient carbon supply allows for metabolic investments that are priming fruit quality potential*

There are apparent differences in the metabolite profiles between carbon supply treatments, although this variation appears be more significant early on, immediately after thinning in S2 (Figures 3.4-3.5). Upon S4, few compounds were significantly different between the carbon sufficient (thinned) and starved treatments (unthinned) (Table S3.4, Figures 3.6, 3.8a, d), underscoring the high level of metabolite regulation throughout maturation and justifying our methodology of sampling fruit of equal maturity. It is significant to note that at S2, there is a high level of metabolic variation, while there are very few differences in quality characteristics and phenotype (Figures 3.1, 3.3-3.5). At S4, most metabolic differences appear to dissipate, while there are extreme differences expressed in quality and phenotypic characteristics (Figures 3.1, 3.3-3.5). The extreme differences in inter-fruit carbon competition conditions appears to create an early and dramatic metabolic shift that primes quality development.

With sufficient carbon supply (adequate fruit thinning), the priming appears to lead to quality enhancement, while the carbon stressed unthinned control (high carbon competition), leads to quality detriment. The importance of early action for crop load management to maximize carbon resources for the developing fruit is strongly recognized (Grossman and DeJong 1995). This is because under sufficient carbon supply (via thinning), fruit can achieve their maximum fruit growth potential (Grossman and DeJong, 1995). Similarly, our results further suggest that this early carbon supply adjustment initiates vast metabolic changes in the developing fruits, which allows them to achieve maximum fruit quality potential as well.

Fruit from unthinned trees had a high abundance of several organic acids at S2, such as citric acid, malic acid and threonic acid (Table S3.4), which have been previously correlated with a lack of sweetness in peach fruit (Colaric et al., 2005). Furthermore, threonic acid had a negative correlation with SSC in the carbon sufficient treatment throughout development ($r_s$=-0.79), which

also supports the lack of quality at harvest in the unthinned controls (e.g. reduced SSC and DMC) (Figures 3.3, 3.6-3.7). Additionally, the accumulation of particular organic acids has been shown to be a response to abiotic stresses (e.g. salt, soil acidity, drought) in other species (Timpa et al., 1986; Fougere et al., 1991; Zeng et al., 2008). Thus, our results suggest that carbon limitation, resulting from a high crop load (i.e. unthinned control), may induce a similar abiotic stress response, as reflected with an elevated abundance of organic acids at S2 (Table S3.4).

Multiple carbohydrate-related metabolites such as myo-inositol, fructose and sorbose also exhibited higher abundance at S2 in the unthinned control (Figure 3.6). These compounds are known to accumulate in the vacuoles of cells within the fruit (Bae et al., 2014). The increased accumulation of these solutes in the cell's vacuole could be an attempt to increase the osmotic concentration and influx of water supply, as water transport to fruit cells is critically important for early fruit development (Shiratake and Martinoia, 2007).

At S2, the unthinned control demonstrated lower sorbitol and higher fructose abundance than in fruit from the thinned treatment (Figure 3.6). Sorbitol is the main sugar transported throughout the phloem in peach and is readily converted to fructose by SDH (Zhang et al., 2004; Morandi et al., 2008). Under heavy crop load conditions, fruit are in high competition for photosynthates (Grossman and DeJong, 1995). This limited carbon condition may explain why lower levels of sorbitol and higher levels of fructose are present in the fruit from the unthinned controls at S2 (Figure 3.6), as fruit are perhaps rapidly converting sugar-alcohols into saccharides to be used for metabolic processes integral for survival under high carbon stress conditions.

Quinic acid is significantly higher in abundance in fruit from the carbon sufficient treatment at S2 and S3 (Figure 3.6k), although levels drop before harvest (S4) in both the carbon sufficient and starved treatments, agreeing with previous research (Wu et al., 2005). Quinic acid is a product

of the shikimic pathway, which initiates with imported carbohydrates (Walker and Famiani, 2018). The pentose phosphate pathway (PPP) converts available carbohydrates to glucose-6-phosphate (G6P), which are converted to erythose-4-phosphate (E4P). Phosphoenolpyruvate and E4P are then synthesized to form 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP), which is then converted to dehydroquinic acid (DHQ). Quinate dehydrogenase subsequently converts DHQ to quinic acid, which can be used for the synthesis of chlorogenic acids (conjugates of quinic and cinnaminic acids; Guo et al., 2014). Chlorogenic acids are a group of phenolic acids (Clifford, 2000), which are important precursors for the development of anthocyanins and numerous other metabolites associated with quality attributes such as flavor, color, taste and nutrition (Walker and Famiani, 2018). This pathway is also responsible for the connection between the primary metabolism and the biosynthesis of various secondary metabolites, such as aromatic amino acids, folates, quinones, phytohormones, alkaloids, indole glucosinolates, flavonoids, hydroxycinnamic acids and lignins (Lara et al., 2020).

In fruits, the phenolic content is mostly comprised of phenolic acids and flavonoids (Häkkinen et al., 1999). Similar to the elevated levels of quinic acid (an intermediate for phenolic acids), levels of flavonoids such as catechin and tocopherol, were also higher in the carbon sufficient treatment (Figures 3.6, 3.8). Thus, our data suggest that when carbon is in sufficient supply (achieved via thinning), fruit metabolism is able to prioritize the synthesis of secondary metabolites that help protect the fruit against abiotic/biotic stresses, pathogens and increase its' quality attributes (Ullah et al., 2017). This prioritization of secondary metabolism under sufficient carbon supply has previously been shown in kiwifruit (Nardozza et al. 2019), where a sufficient carbon supply resulted in a higher abundance of anthocyanins in fruit flesh, when compared to a carbon starved (i.e., girdled) treatment. Few differences were observed in metabolite abundances

between the thinned treatment and the unthinned control at harvest, when controlling for equal maturity. This may be due to the high level of priority given to the primary metabolism, even under stressed conditions (i.e., high fruit-to-fruit competition for photosynthates). When secondary metabolites and pathways are considered, the carbon sufficient treatment (thinned) shows elevated abundances (especially at S2), as a potential "luxury" investment, given the increased carbohydrate availability. Under these conditions, increased carbon can fuel the primary metabolism, which contributes to the synthesis of intermediates that act as precursors in the secondary metabolism to develop increased secondary metabolite content that results in enhance quality characteristics at harvest (Pott et al., 2019). Therefore, we hypothesize that metabolic differences observed as a response to carbon starvation/sufficiency may be more pronounced in the fruit's secondary metabolism than in the central carbon (i.e., primary) metabolism at harvest when controlling for maturity, this should be further investigated in future studies.

### *4.5. Catechin and a classified unknown metabolite may act as primers for superior or inferior fruit quality*

When evaluating the metabolite profiles between the carbon sufficient and starved treatments at harvest (S4), only two compounds exhibited significant differences (Table S3.4). These included a classified unknown, A244002 (Hummel et al., 2007), which was elevated under carbon starvation, and catechin, a flavonoid that was elevated under carbon sufficiency (Figure 3.8a, d).

The classified unknown, A244002 has yet to be fully annotated, but it has been found previously in two legume plant metabolomes, alfalfa (*Medicago sativa*) and *Lotus japonicus* (Fester et al., 2011; 2014). In both occurrences, it appears that A244002 was associated with stress, elevated in plants forming mycorrhizal associations in alfalfa and increased in *Lotus japonicus* under depreciated soil nitrogen levels (Fester et al., 2011; 2014). There may be a potential link

between this compound and a stress response in peach as well, as it remains significantly higher during carbon stress throughout development (Figure 3.8d). Furthermore, when assessing how this compound relates to quality parameters at harvest, it demonstrates a significant negative correlation with both DMC and SSC (Figure 3.8e-f).

Catechin is a phenolic compound that is found throughout several plant species, including many tree fruits like apricots, apples and cherries (Bernatoniene and Kopustinskiene, 2018) and has been reported as the most abundant flavan-3-ol in peach, especially in the peel (Chang et al., 2000). Phenolic compounds contribute to color, pigment synthesis, flavor and demonstrate several health benefits (Pietta et al., 1998). Catechin, like many antioxidants, helps to provide protection against plant pathogen pressure and abiotic stresses (Zhang et al., 2016). Higher levels of catechin were reported in peach exocarp when fruit was grown at higher altitudes, and were linked with superior quality (Karagiannis et al., 2016). Additionally, in a cultivar trial the highest levels of catechin were observed in peaches that also had the highest levels of SSC (Saidani et al., 2017). Therefore, our results suggest that under sufficient carbon supply, the tree is investing more resources for the synthesis of compounds for reproductive organs, which result in enhanced quality. This is supported by the significantly higher abundance of catechin observed in the carbon sufficient treatment (thinned) throughout development and at harvest (Figure 3.8a). Furthermore, catechin has a strong positive correlation with both DMC and SSC at harvest, indicating enhanced quality (Figure 3.8b-c).

Additionally, while significant differences in abundances for each of these compounds were observed across the developmental stages, the high abundance of both compounds at S2 could have a significant impact on fruit quality at harvest (i.e., DMC and SSC; Figure 3.8). Therefore,

these two compounds may act as respective primers early in development, providing a potential link between primary/secondary metabolism and fruit quality characteristics in peach.

## 5. Conclusion

This study sought to determine the true impact of crop load, a critical preharvest factor, on peach fruit quality and metabolism. As quality and metabolism are heavily influenced by maturation, the use of Vis-NIRS technology allowed for sampling of equal maturity fruit. This novel approach enabled comparisons between differing carbon competition conditions (i.e. thinning severities), without the confounding influence of maturation. Physiological analyses showed that 2.6 fruit · $cm^{-2}$ of TCSA could be a potential optimal crop load for 'Cresthaven,' to maximize quality without sacrificing excessive yields. Furthermore, when fruit of equal maturity coming from thinned trees (15 cm fruit spacing) were compared to the unthinned control, superior quality enhancements were noted, underscoring the true impact of crop load on fruit internal quality. Minimal differences in the fruit ionome were detected between treatments, although concentrations appeared to decrease with fruit development. Similarly, peach metabolite profiles appear to be heavily regulated by development and maturation. Several significant differences in metabolites were detected early on, with vast metabolic shifts in S2, when phenotypic differences are absent. Inversely, at S4, minimal differences in the fruit metabolome were observed, while vast differences in fruit quality and phenotype were prevalent. This contrasting trend between the metabolome and fruit quality indicate a potential metabolite priming effect for fruit quality development, as a result of variable carbon conditions. When photosynthates are not limited due to competition, fruit appear to begin prioritizing the synthesis of secondary metabolites related to internal fruit quality attributes at harvest. Only two metabolites were significantly different between treatments and strongly correlate to fruit quality (e.g., DMC and SSC) at harvest. Catechin

and A244002 may act as potential primers in the peach fruit metabolome, providing a link between metabolite abundances and fruit quality enhancement or detriment, respectively.

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## Tables

**Table 3.1. Effect of thinning severity on peach tree physiology and fruit external quality attributes.** The impact of thinning severity on trunk cross sectional area (TCSA) ($cm^2$), number of fruit per tree, crop load (fruit per $cm^2$ of TCSA), yield (kg $tree^{-1}$), average (avg.) fruit weight (g), fruit diameter (mm), overcolor (%), number of flowers/branch, and fruit set (%) in 'Cresthaven' peach. 'Cresthaven' peach trees were thinned to various levels (5, 10, 15 and 30 cm) and compared with unthinned control trees. Mean values ± S.E. are displayed. Means followed by the same superscript letter are not statistically different according to Tukey's HSD test ($P \leq 0.05$).

| Thinning treatment | TCSA[†] ($cm^2$) | No. of fruit per tree | Crop load (no. of fruit/$cm^2$ of TCSA) | Yield (kg/tree) | Production (MT/ha) | Avg. fruit weight (g) | Diameter (mm) | Overcolor (%) | Return bloom (no. of flowers per branch[#]) | Return fruit set[‡] (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Unthinned | 62.0 ± 4.1 | 731 ± 51$^{a}$ | 11.8 ± 0.27$^{a}$ | 61.5 ± 6.9$^{a}$ | 31.2 ± 3.5$^{a}$ | 84.6 ± 9.3$^{c}$ | 57 ± 0.4$^{e}$ | 64 ± 1.0$^{b}$ | 9 ± 1$^{b}$ | 10 ± 5$^{c}$ |
| 5 cm | 60.9 ± 0.3 | 346 ± 43$^{b}$ | 5.7 ± 0.72$^{b}$ | 53.6 ± 3.6$^{a}$ | 27.2 ± 1.8$^{a}$ | 157.4 ± 10.4$^{b}$ | 69 ± 0.3$^{d}$ | 59 ± 1.0$^{c}$ | 18 ± 1$^{a}$ | 23 ± 5$^{bc}$ |
| 10 cm | 61.0 ± 5.1 | 220 ± 45$^{bc}$ | 3.6 ± 0.62$^{c}$ | 40.5 ± 9.7$^{ab}$ | 20.6 ± 4.9$^{ab}$ | 181.5 ± 6.5$^{b}$ | 75 ± 0.3$^{c}$ | 67 ± 0.8$^{b}$ | 20 ± 1$^{a}$ | 30 ± 5$^{b}$ |
| 15 cm | 63.8 ± 3.3 | 169 ± 21$^{c}$ | 2.6 ± 0.27$^{cd}$ | 38.5 ± 4.0$^{ab}$ | 19.5 ± 2.0$^{ab}$ | 228.6 ± 7.6$^{a}$ | 78 ± 0.3$^{b}$ | 68 ± 0.9$^{ab}$ | 20 ± 1$^{a}$ | 56 ± 5$^{a}$ |
| 30 cm | 71.9 ± 10.0 | 111 ± 5$^{c}$ | 1.6 ± 0.18$^{cd}$ | 27.8 ± 1.6$^{b}$ | 14.1 ± 0.8$^{b}$ | 249.9 ± 13.7$^{a}$ | 80 ± 0.4$^{a}$ | 71 ± 0.8$^{a}$ | 21 ± 1$^{a}$ | 34 ± 6$^{b}$ |
| Significance | ns | *** | *** | ** | | *** | *** | *** | *** | *** |

ns, *, **, *** indicate no significance or significant at p-values of ≤0.05, 0.01, or 0.0001; [†]trunk cross sectional area; [#]n=3 branches; quantified at full bloom on 22 March 2017; [‡]fruit set calculated as: number of fruit/number of flowers x 100 on 9 May 2017

**Table 3.2. Effect of thinning severity peach fruit internal quality and maturity.** The impact of thinning severity on fruit internal quality and maturity, assessed with a non-destructive NIRS sensor across all treatments, in 'Cresthaven' peach. 'Cresthaven' peach trees were thinned to various levels (5, 10, 15 and 30 cm) and compared with unthinned control trees. Peach fruits (n=45) were assessed non-destructively for internal quality and maturity by measuring for dry matter content (DMC) and soluble solids concentration (SSC) along with flesh firmness (FF) and index of absorbance difference ($I_{AD}$). Due to a lack of accuracy in non-destructive estimation of FF, destructive values (n=15) are displayed. Performance of the non-destructive prediction models (DMC, SSC and $I_{AD}$) is presented in Figure 2A, B, and C. Mean values ± S.E. are displayed. Means followed by the same letter are not statistically different according to Tukey's HSD test ($P \leq 0.05$).

| Thinning treatment | Sample size (n) | DMC (%) | SSC (%) | FF (N) | $I_{AD}$ |
|---|---|---|---|---|---|
| Unthinned | 45 | $11.0 \pm 0.2^{d}$ | $10.1 \pm 0.2^{d}$ | $47.9 \pm 2.5^{ab}$ | $1.03 \pm 0.05^{a}$ |
| 5 cm | 45 | $12.8 \pm 0.1^{c}$ | $11.5 \pm 0.1^{c}$ | $49.7 \pm 3.0^{a}$ | $1.09 \pm 0.05^{a}$ |
| 10 cm | 45 | $13.3 \pm 0.2^{b}$ | $12.1 \pm 0.1^{b}$ | $40.5 \pm 4.6^{ab}$ | $0.78 \pm 0.05^{b}$ |
| 15 cm | 45 | $13.7 \pm 0.2^{ab}$ | $12.5 \pm 0.2^{ab}$ | $33.1 \pm 3.2^{b}$ | $0.75 \pm 0.05^{b}$ |
| 30 cm | 45 | $14.1 \pm 0.3^{a}$ | $12.9 \pm 0.2^{a}$ | $34.5 \pm 5.4^{ab}$ | $0.73 \pm 0.05^{b}$ |
| Significance | | *** | *** | ** | *** |

ns, *, **, *** indicate no significance or significant at p-values of ≤0.05, 0.01, or 0.000; 1[†]fruit flesh firmness data from destructive quality analysis; n=15

## Figures

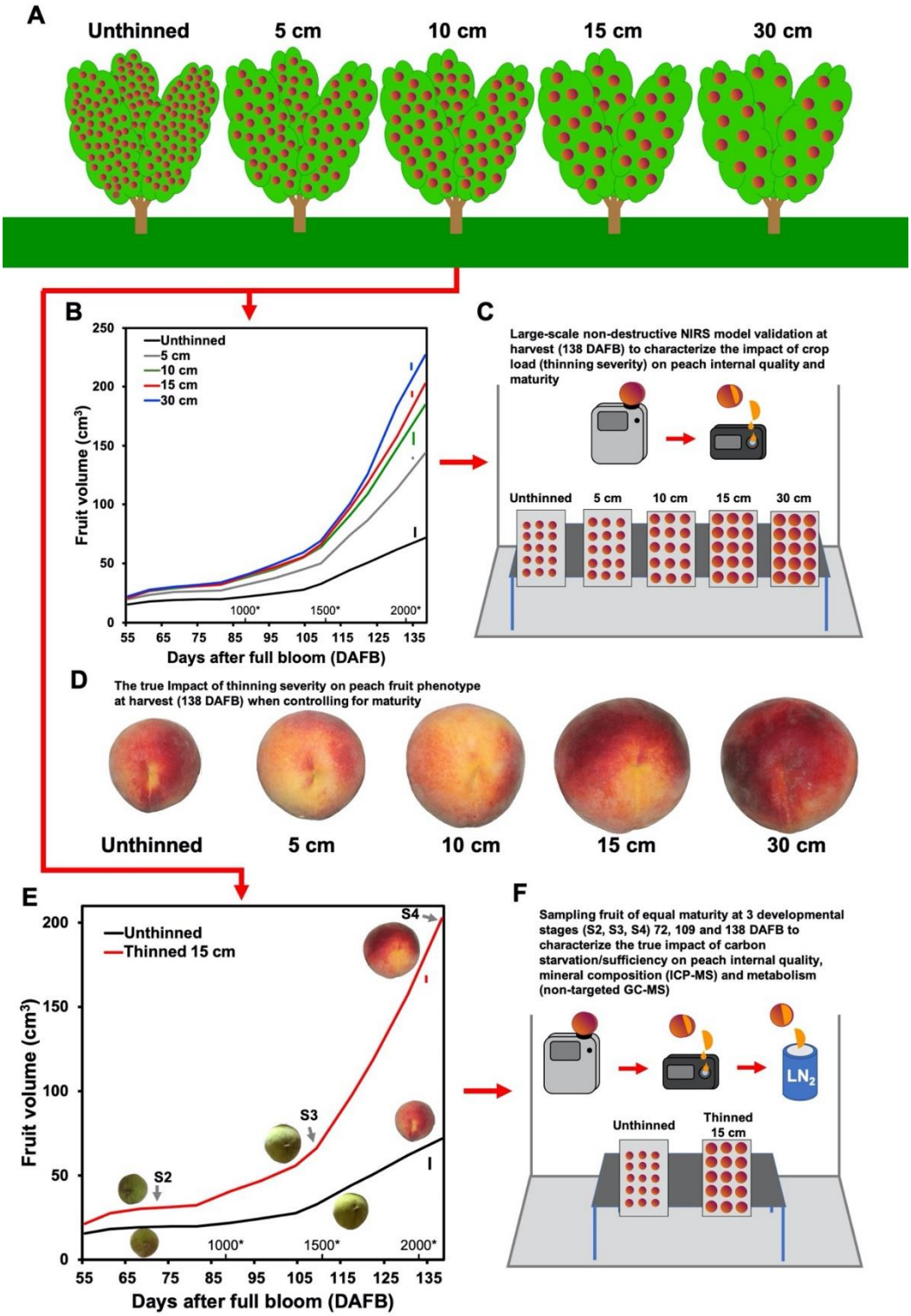


**Figure 3.1. Carbon supply manipulation to assess peach fruit quality, elemental composition and metabolism.** A carbon supply manipulation study was conducted to understand the true

impact of thinning severity on fruit quality, along with how fruit quality development is linked with ionome and metabolome. Trees were thinned to different fruit spacing distances (5, 10, 15 and 30 cm) and compared with unthinned control trees (A). Thinning was conducted 52 days after full bloom (DAFB). Fruit from these trees were evaluated non-destructively throughout the growing season for volumetric growth. Fruit growth assessment time is indicated in DAFB and growing degree days (GDD) with asterisks above. Vertical colored bars represent the least significant difference (LSD) per treatment based on Tukey's HSD test ($P \leq 0.05$) (B). Fruit coming from distinct thinning severities were assessed for quality and physiological maturity at harvest (S4; 138 DAFB) with a non-destructive near infrared spectrometer (NIRS), along with destructive analyses for model validation (C). Fruit were evaluated for their phenotype while controlling for equal maturity to understand the true impact of thinning severity on quality across all treatments (D). Two thinning treatments were selected for further biological analyses: the carbon starved, unthinned treatment, and the optimal thinned at 15 cm fruit spacing treatment, carbon sufficient. Volumetric growth is displayed between the unthinned and thinned treatments across growth and development (S2-S4), while controlling for equal maturity. Vertical bars represent LSD according to Tukey's HSD test ($P \leq 0.05$) (E). At each developmental stage (S2, S3, S4) 72, 109 and 138 DAFB, respectively, fruit were harvested to characterize the true impact of carbon supply on peach internal quality, which allowed for further investigation into the fruit ionome and metabolome. Samples were immediately frozen with liquid nitrogen ($LN_2$) and lyophilized at each developmental stage to quench the metabolism, until molecular analyses (ICP-MS and non-targeted GC-MS) could be conducted (F).

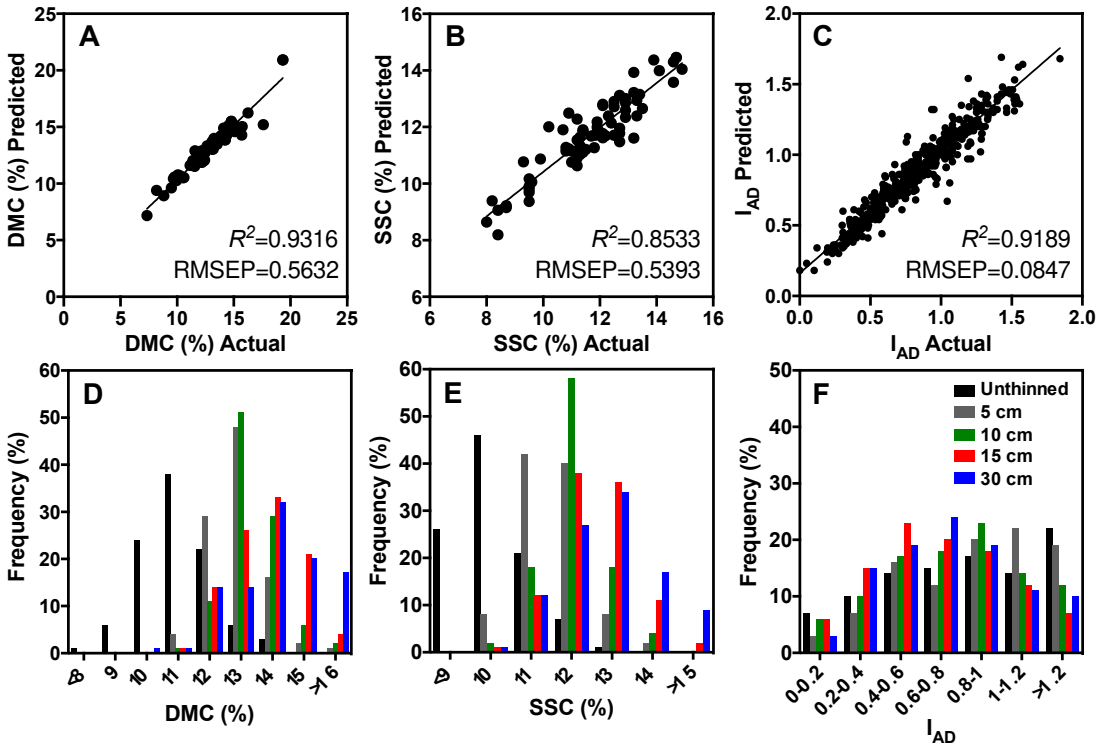


**Figure 3.2. Non-destructive quality model validation and distribution of quality parameters at harvest across variable carbon supply conditions.** Multivariate regression models for non-destructive estimations of peach internal fruit quality and maturity were validated with fruit

samples at harvest (138 DAFB) from the various carbon supply treatments. Non-destructive predicted estimations were plotted against actual destructive values for validation of quality and maturity parameters: dry matter content (DMC) (A), soluble solids concentration (SSC) (B) and index of absorbance difference ($I_{AD}$) (C). The created models for these parameters were evaluated for linearity ($R^2$) and root mean square error of prediction (RMSEP) to demonstrate the accuracy of the model in estimating maturity and various quality parameters with near-infrared (NIR) spectroscopy (A-C). The impact of thinning severity on 'Cresthaven' fruit distributions across classes of DMC, SSC and $I_{AD}$ are shown (D-F) and were assesses using the non-destructive NIRS multivariate models. Various thinning treatments' frequencies for each parameter are visualized as: unthinned (black), 5 (grey), 10 (green), 15 (red) and 30 cm (blue) (D-F).

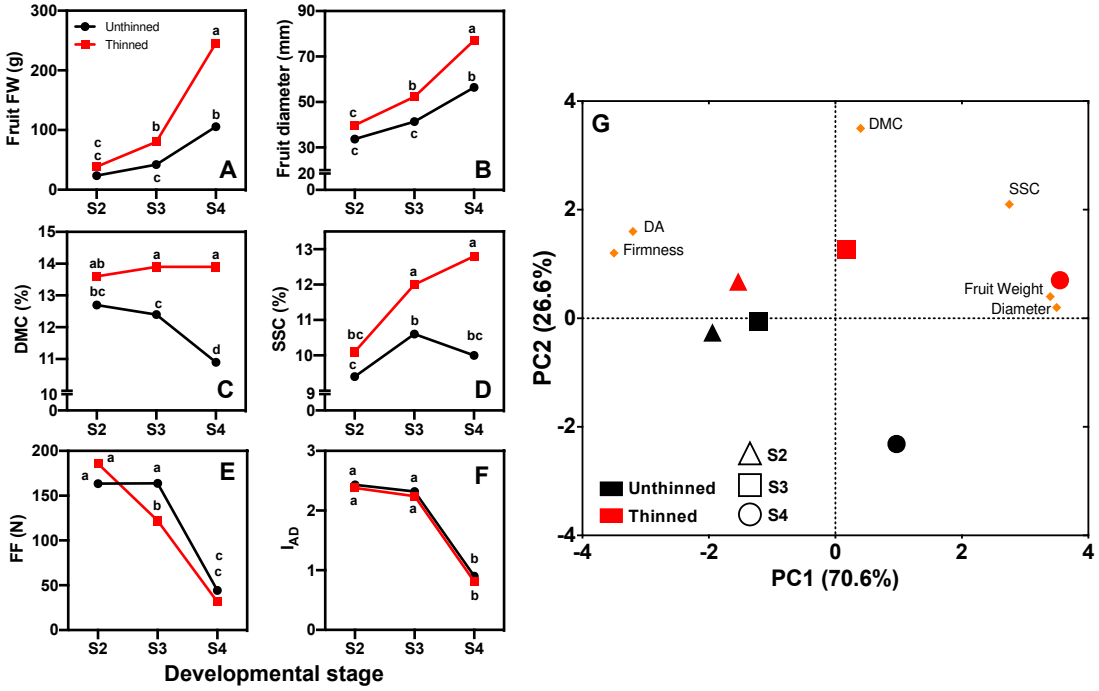


**Figure 3.3. The true impact of carbon supply on fruit internal quality development.** Impact of two carbon supply treatments: unthinned (starved) and thinned (15 cm, sufficient) on fruit fresh weight (FW, g; A), diameter (mm; B), dry matter content (DMC, %; C), soluble solids concentration (SSC, %; D), flesh firmness (FF, N; E), and physiological maturity ($I_{AD}$; F) from destructive quality analysis on 10 fruit per treatment at each developmental stage. Means of fruit plotted at three developmental stages: stage 2 (S2, pit hardening, 72 DAFB), S3 (cell elongation, 109 DAFB), and S4 (harvest, 138 DAFB). Error bars are not visualized as they were too small to be seen in the figure. Fruit quality was assessed on fruit of equal maturity, assessed with $I_{AD}$, at each developmental stage (F). One-way ANOVAs (by thinning treatment and developmental stages) were used to detect differences across means with lettering or asterisks assigned by Tukey's HSD test. Means with the same letter indicate non-significance at $P \leq 0.05$ within thinning treatments across developmental stages. Upper-case letters correspond to the thinned treatment,

whereas the lower-case letters correspond to the unthinned treatment. Significant differences between thinning treatments at each developmental stage are denoted with an asterisk (*) at $P \leq 0.05$. Principal component analysis (PCA) was also conducted to assess the impact of variable carbon supply on peach fruit phenotype. Thinning treatment (red) vs. unthinned fruit (black) and developmental stage (S2 (triangle), S3 (square), S4 (circle)) scores are scaled on the PCA with peach fruit quality parameters (loadings, orange diamonds). Large symbols on the PCA are the averaged ten fruit per each thinning x developmental stage treatment.

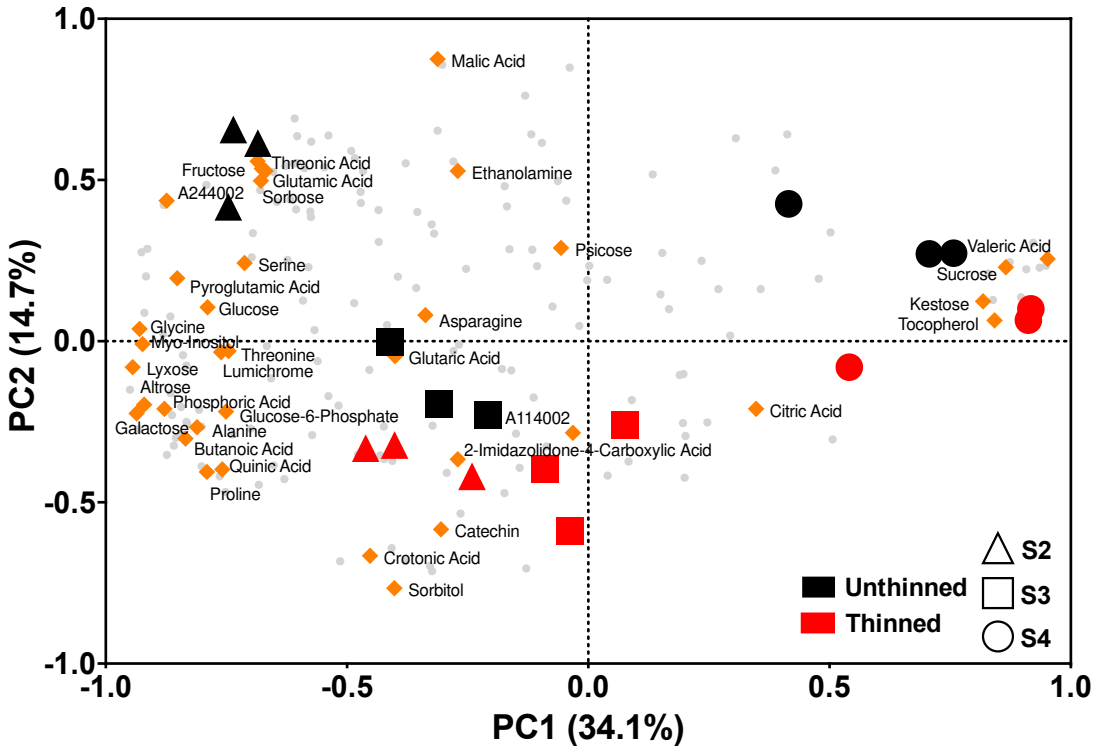


**Figure 3.4. Principal component analysis biplot of variable carbon supply on peach fruit metabolism.** Large symbols indicate the scores for the thinning treatments (unthinned (black) vs. thinned (red)) and developmental stages (S2 (triangle), S3 (square), S4 (circle)) and are scaled with the metabolites detected in the peach mesocarp (loadings). Principal component analysis (PCA) of the three reps per each thinning x developmental stage treatment demonstrate that developmental stage was a major contributor for metabolome variation as indicated by separation on PC 1 (~ 34%). Additional metabolome variation due to the carbon availability and other factors is visualized on PC 2 (~ 15%). The 36 annotated metabolites are shown on the PC loadings as orange diamonds. The remaining 152 peaks that were detected, but not annotated, are shown as grey circle loadings in the background. PCA shows wide variation in metabolome between carbon supply treatments at S2, while at S4, fruit metabolome scores are minimally different.

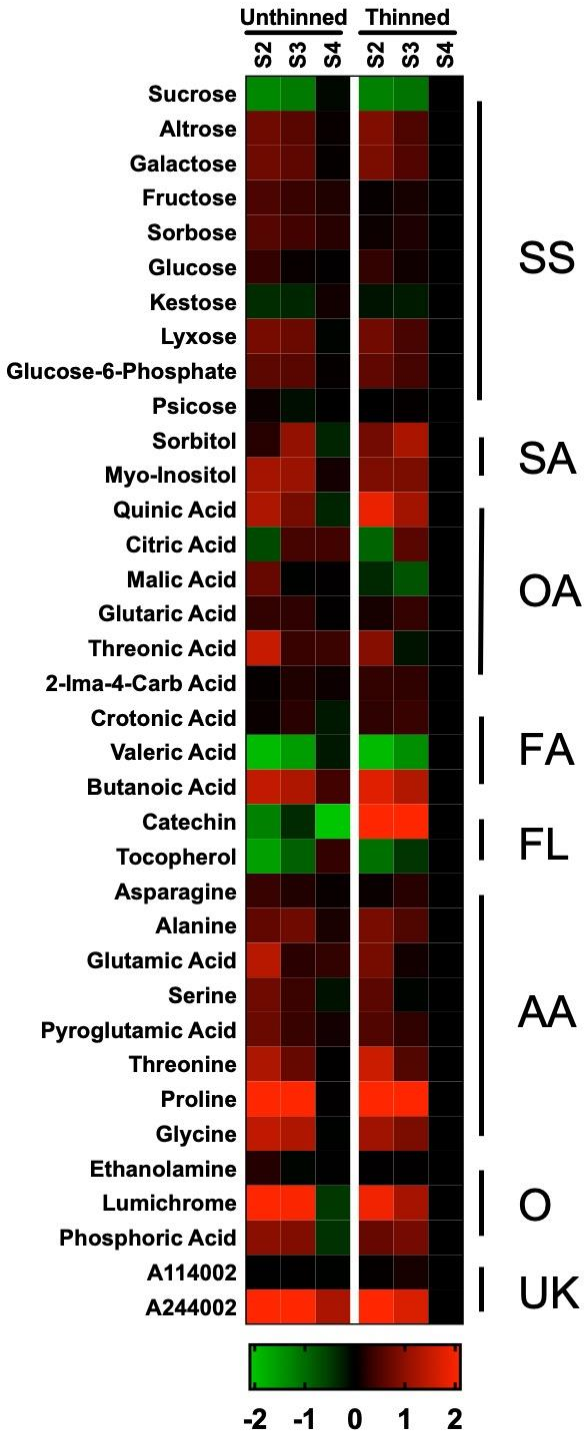


**Figure 3.5. Heat map of metabolite profiles across development under two carbon supply conditions.** Profiles of metabolism changes across two carbon supply conditions during growth and development in peach fruit mesocarp. Figure shows comparisons of the metabolite abundance across S2, S3 and S4 in the carbon starved (unthinned) and sufficiency (thinned) fruits. The ratio between thinning x developmental stage treatments were compared to the

"optimal" conditions of thinned-S4 (far right column). Each of the 36 annotated metabolites were transformed into $\log_2$ and shown with the following color scale (green to red) according to Lombardo *et al.*, (2011). Fruits at each developmental stage were of equal maturity according to the $I_{AD}$ measured by the DA meter (Figure 3F). Annotated metabolites are organized by chemical class: soluble sugars (SS), sugar alcohols (SA), organic acids (OA), fatty acids (FA), flavonoids (FL), amino acids (AA), other (O) and classified unknowns (UK).

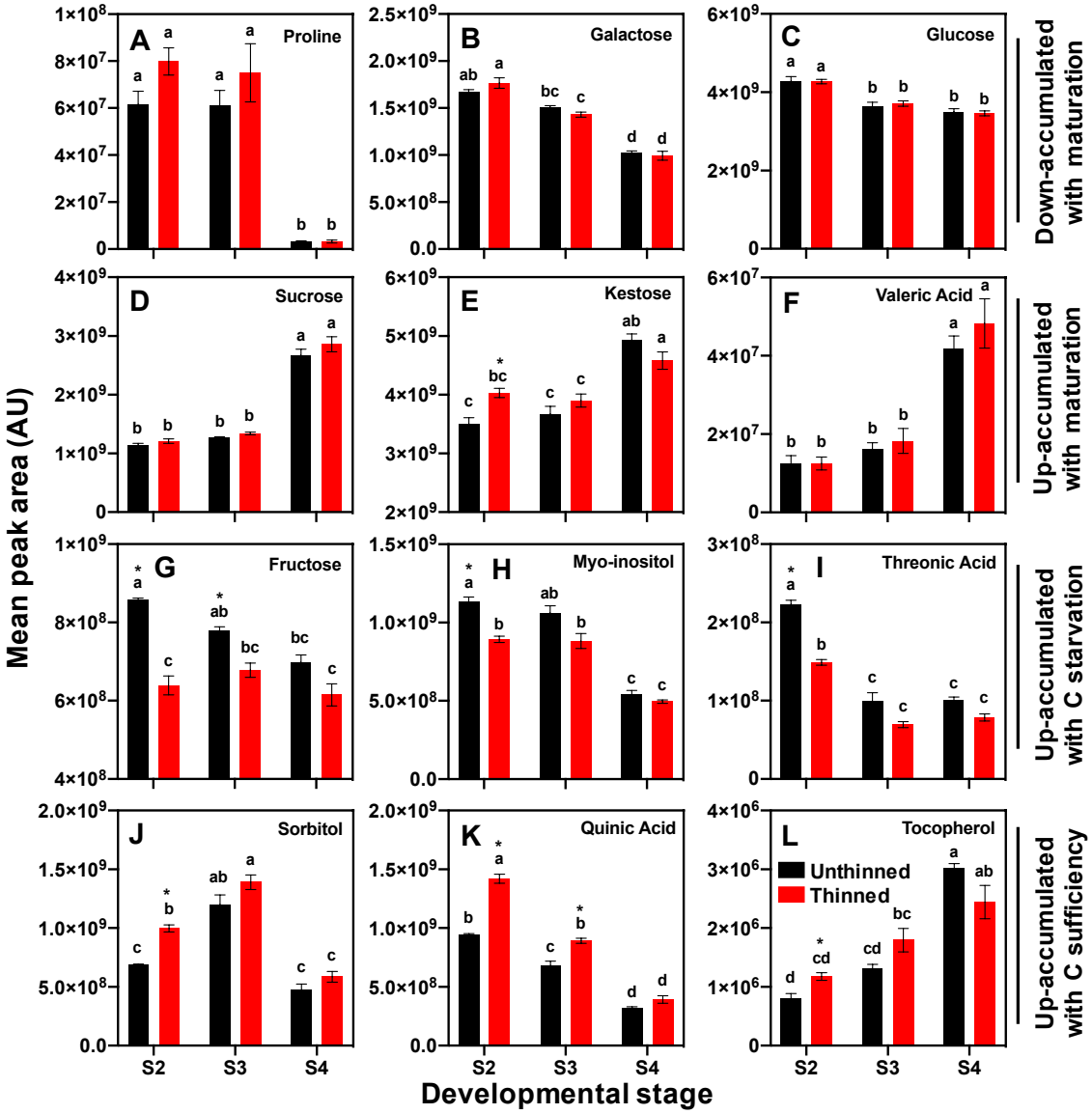


**Figure 3.6. Accumulation trends of metabolite abundances throughout fruit maturation and across variable carbon supply treatments.** Figure showcases the mean peak area (AU) of selected metabolites that are down-accumulating (A-C) or up-accumulating (D-F) throughout peach fruit development and maturation (S2 – S4) in two carbon supply conditions. The mean peak area of selected metabolites that are up-accumulating with carbon starvation, e.g. decrease in

thinned vs. unthinned (G-I) or with carbon sufficiency, e.g. increased in thinned vs. unthinned (J-L) are also shown across development. The bars indicate carbon supply treatments: unthinned (black) and thinned (red, 15 cm fruit spacing). Samples were controlled for equal maturity ($I_{AD}$) across development and between carbon supply treatments. Mean values ± S.E. are displayed. Means followed by the same letter are not statistically different according to Tukey's HSD test ($P \leq 0.05$) within each thinning treatment across developmental stages (thinned=upper-case, unthinned=lower-case). * indicates a significant difference between carbon supply treatments at a singular developmental stage according to Tukey's HSD test ($P \leq 0.05$).

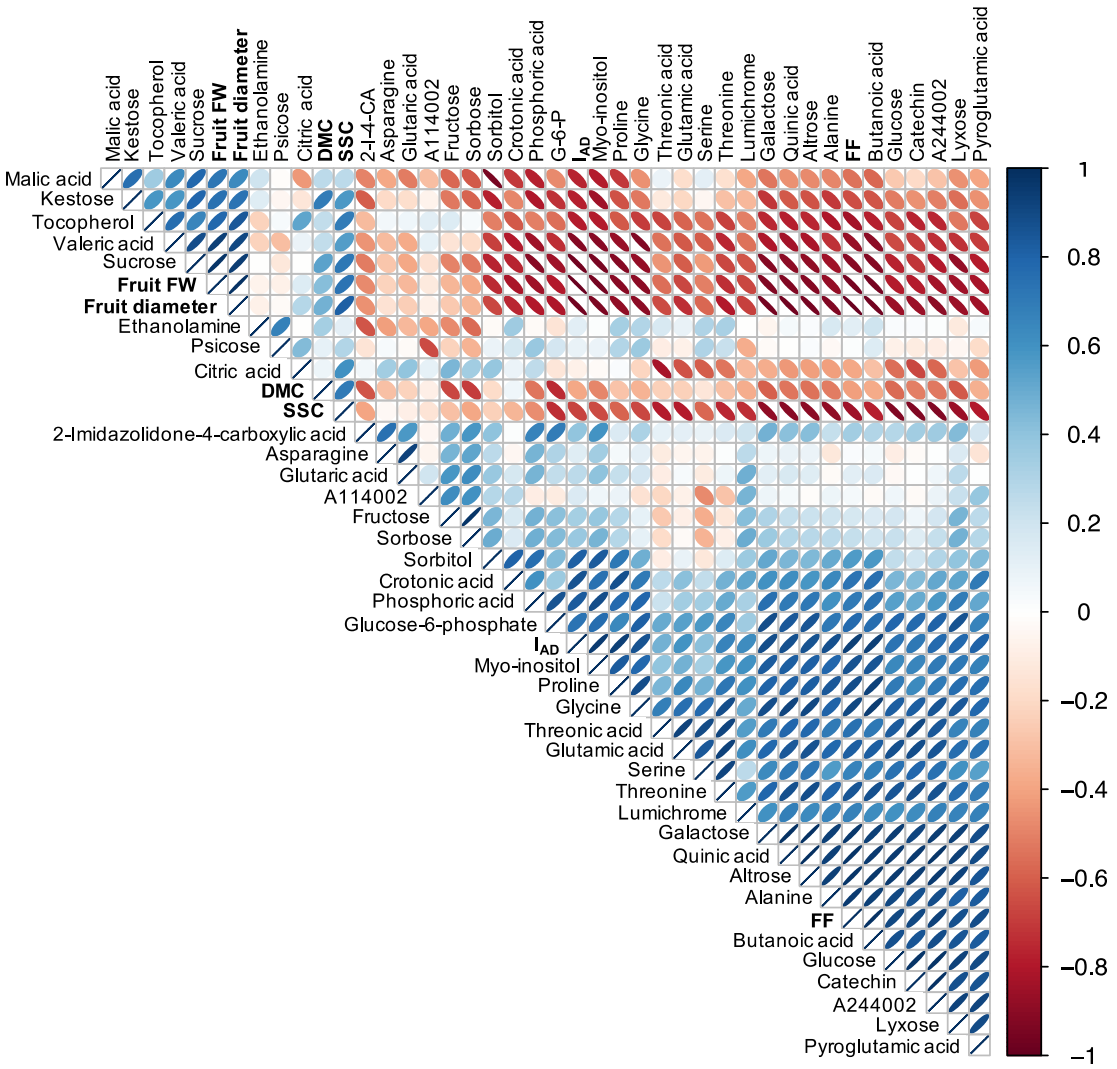


**Figure 3.7. Heat map of correlation matrix between fruit physiological characteristics and metabolite abundances.** Heat map for 42 physiological (6) and metabolic (36) characteristics was created based on hierarchical clustering on the Spearman's rank correlation ($r_s$) values. Colors and ellipse eccentricity visualize the direction and strength of the relationship between characteristics. Data used for relationships are from the carbon sufficient (thinned, 15 cm) treatment only, across all developmental stages (S2, S3 and S4). Correlations show the relationship between the

accumulation or degradation of fruit physiological parameters and metabolite abundances throughout maturation.

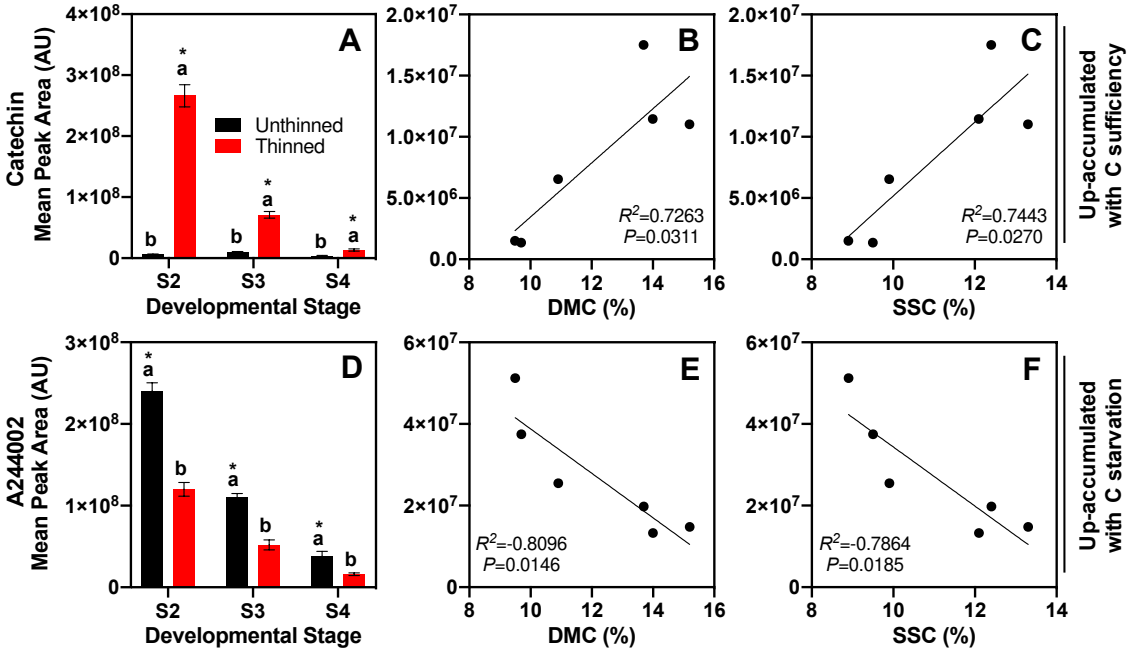


**Figure 3.8.** Abundance of proposed metabolite primers and their relationship with peach internal quality parameters at harvest. Mean peak area (AU) of catechin (A) and of a classified unknown (A244002, D), respectively, at each developmental stage (S2, S3 and S4) between carbon sufficiency treatments (unthinned vs. thinned (15 cm fruit spacing)). Mean values ± S.E. are displayed. Means followed by the same letter are not statistically different according to Tukey's HSD test ($P \leq 0.05$). * indicates a significant difference between carbon supply treatments at a singular developmental stage according to a one-way ANOVA ($P \leq 0.05$). The relationships between the mean peak area of catechin and A244002 with dry matter content (DMC, %; B and E, respectively) and soluble solids concentration (SSC, %; C and F, respectively) at harvest (S4) with three replicated samples from both the carbon availability treatments are plotted. $R^2$ values are displayed to demonstrate the linearity of the relationships, along with *P*-values to indicate the significance of the relationship.

## Supplementary data

**Table S3.1.** Effect of thinning severity on peach leaf mineral composition.
**Table S3.2.** Temperature program from Titan MPS peach mesocarp microwave digestion.
**Table S3.3.** Element concentrations in peach mesocarp under carbon sufficient/starved conditions throughout development.
**Table S3.4.** Relative abundances of the 36 annotated metabolites in peach mesocarp under carbon sufficient/starved conditions throughout development.

**Figure S3.1.** Fruit size distribution by thinning treatment.
**Figure S3.2.** Principal component analysis biplot of variable carbon supply on peach fruit ionome.
**Figure S3.3.** Heat map profiles of peach ionome across development between thinning treatments.
**Figure S3.4.** Butanoic acid metabolite abundance across treatments throughout fruit development.
**Figure S3.5.** Sorbose metabolite abundance across treatments throughout fruit development.

# CHAPTER FOUR

# PHENYLPROPANOIDS SERVE AS METABOLIC SIGNATURES OF SUPERIOR QUALITY PEACH FRUIT UNDER SUFFICIENT CARBON SUPPLY

## 1. Introduction

Peach (*Prunus persica* L. Batsch) consumption and production have been in decline globally throughout recent years due to poor fruit quality and reduced consumer demand (Minas et al., 2018). Peach fruit quality is of utmost importance to ensure consumer retention, repeat sales and a profitable industry. Optimization of preharvest factors alone is responsible for improving fruit quality at harvest, while postharvest management can only maintain fruit quality (Crisosto et al., 1997). Crop load management is one of the most influential preharvest factors in regulating/achieving fruit quality standards (Minas et al., 2018). The crop load, or carbohydrate source:sink ratio, dictates the abundance of photosynthates available for developing fruits to achieve high levels of dry matter content (DMC) and soluble solids concentration (SSC), two parameters that relate well to consumer satisfaction and high fruit quality (DeJong and Grossman, 1995; Crisosto and Costa, 2008; Minas et al., 2018, 2021; Anthony et al., 2020). By removing flowers or fruitlets, the leaf-to-fruit, or source:sink ratio is increased, improving the supply and abundance of carbohydrates, as well as other metabolites, to the remaining fruit (DeJong and Grossman, 1995). By doing so, developing fruit are better able to reach both their: 1) maximum fruit growth potential (Grossman and DeJong, 1995), and 2) maximum quality potential (Anthony et al., 2020). High crop loads may increase yields, but will sacrifice fruit quality, in respect to size, weight, color, DMC and SSC, as carbohydrates become scarce (Anthony et al., 2020). While, reducing the crop load will enhance these quality characteristics, as the total pool of carbohydrates receives less demand and competition for resources (Grossman and DeJong, 1995). However, an

increased supply of carbon, or reduced crop load, can also advance fruit maturity (Anthony et al., 2020). Therefore, to better understand the direct impact carbon supply has on internal fruit quality, selecting fruit of equal maturity to reduce this confounding variable's impact is required.

Historically, selecting fruit of equal maturity could not be conducted accurately or on large sample sizes, as it was evaluated through destructive flesh firmness (FF) measurements or subjective background color assessments that are ineffective for cultivars that develop a fully red overcolor early in the season (Minas et al., 2021). More recently, the development of a new maturity index, the index of absorbance difference ($I_{AD}$), which non-destructively assesses the chlorophyll degradation beneath the skin, better describes physiological maturity (Ziosi et al., 2008; Costa et al., 2009; Minas et al., 2021). However, the $I_{AD}$ values will vary based on cultivar and flesh textural typology (Anthony et al., 2021). This index has effectively been integrated into open-source handheld visible-near infrared spectroscopy (Vis-NIRS) sensors to enable simultaneous predictive assessments of both physiological maturity ($I_{AD}$) and internal fruit quality (DMC and SSC) in a single scan (Minas et al., 2021). This technology and experimental approach were used in a recent study, which underscored the true impact of crop load on peach fruit quality, revealing significant quality improvements in fruit that received a sufficient supply of carbon throughout development (Anthony et al., 2020).

Selecting fruit of uniform maturity status is critical in pomological experiments to best understand the impact of preharvest factors on quality without confounding variables (Anthony et al., 2020; 2021; Minas et al., 2018; 2021), but it is also critical for evaluating how these factors influence biological characteristics, as maturity is a heavily regulated process at the molecular level (Giovannoni et al., 2017). As a climacteric fruit, peach, undergoes several sensorial, textural and metabolic changes throughout maturation and ripening that relate to consumer satisfaction,

organoleptic characteristics and nutraceutical properties (Anthony et al, 2020; Minas et al., 2018; Crisosto and Costa, 2008). Therefore, the use of large-scale molecular tools ("-omics" fields), paired with maturity control, can help unravel the complexities inherent in the biological processes involved in quality development (Anthony et al, 2020; Minas et al., 2018). This comprehensive physiological and biological approach can help determine connections between metabolic shifts and internal quality development, as metabolites serve as translators of physiological stimuli for the fruit's biology. In particular, the use of non-targeted metabolomics can be applied in horticultural research to determine the regulatory networks that are influenced by preharvest factors, which provide the underpinnings for priming fruit quality at harvest (Guy et al., 2008; Anthony et al., 2020).

Investigating the central carbon metabolism, or primary metabolism, is critical for understanding the dynamics involved in fruit growth and development, along with the fruit's nutrition, taste and quality (Cirili et al., 2016). But, as documented recently, the primary metabolism appears to be heavily regulated by development and maturation (Anthony et al., 2020). Therefore, minimal metabolic differences are present at harvest when preharvest factor investigations into the primary metabolome are conducted on fruit of equal maturity levels (Anthony et al., 2020). A previous experiment validated this trend with only two metabolites demonstrating significant differences at harvest, between two distinct carbon supply treatments (C-sufficient vs. C-starved) that were controlled for maturity (Anthony et al., 2020). These metabolites were catechin, a small secondary metabolite related to antioxidant activity, and a classified unknown (A244002) related to stress in previous studies (Anthony et al., 2020). Therefore, it was hypothesized that secondary metabolites may be more influenced by preharvest factors like carbon manipulation and less coupled with maturation. This is perhaps due to these

chemical classes being less integral for fruit growth and survival, but more critical for "luxury" investments in plant defense/protection, color, hormones and anti-microbial properties (Anthony et al., 2020; Tiwari and Rana, 2015). Furthermore, catechin, a flavonoid, demonstrated levels 47-fold higher early in development (i.e., Stage II) in the C-sufficient treatment, when compared to the C-starved treatment, perhaps priming the superior phenotype at harvest (Anthony et al., 2020). Additional preharvest factor research also demonstrated superior quality peach fruit in association with elevated catechin levels at harvest (Anthony et al., 2021; Karagiannis et al., 2016). It was suggested that this metabolite and its' chemical class (e.g., flavonoids) might connect fruit quality and the metabolome.

Flavonoids are a product of the phenylpropanoid pathway, which synthesizes a multitude of secondary metabolites in plants and contribute to plant defense, color, flavor, aroma and overall quality. These chemical compounds are synthesized through intermediates, such as aromatic amino acids (AAAs), like L-phenylalanine (Phe) and L-tyrosine (Tyr), which regulate the development of several specialized secondary metabolites (Maeda and Dudareva, 2012). AAA deaminases (AAADs) are critical enzymes, which mediate the flux of carbon from the primary metabolism to the secondary metabolism in plants (Barros and Dixon, 2020). Greater than 30% of all synthesized carbon is directed to the development of AAAs, which are important in both protein and secondary metabolite biosynthesis (Maeda and Dudareva, 2012). Two key AAAD enzymes that are especially responsible for facilitating the development of phenylpropanoids, include Phe ammonia-lyase (PAL) and Phe/Tyr ammonia-lyase (PTAL), which form cinnamate and coumarate (i.e., hydroxycinnamates), the building blocks for flavonoids, coumarins and lignans (Barros and Dixon, 2020). Therefore, the phenylpropanoid pathway may provide a link between the primary metabolism, secondary metabolism and fruit quality development, as increased carbohydrates help

facilitate the up-accumulation of phenylpropanoids, which contribute to the fruit's aroma, flavor and overall quality.

Overall, in the initial carbon supply experiment, minimal metabolic differences were noted at harvest (i.e., Stage 4, S4) due to maturity control, while several metabolites showcased vast distinctions between treatments early in development (i.e., Stage 2, S2), indicating that early metabolic shifts (e.g., elevated/suppressed catechin) are priming the vast phenotypic variation (e.g., enhanced/reduced DMC and SSC) exhibited at harvest (Anthony et al., 2020). In short, when phenotypes were similar, metabolite profiles were distinct between carbon supply treatments, while the distinct phenotypes at harvest were not associated with differences in the primary metabolism. Further, since the only metabolite (e.g., catechin) demonstrating positive trends with quality parameters, and exhibited significant differences between treatments at each developmental stage was a secondary metabolite, it was concluded that more in depth investigations into the fruit's secondary metabolome was required.

The present study seeks to determine if the secondary metabolism is coupled with maturation's regulation, like the primary metabolism, or if it is more heavily influenced by preharvest factors, like carbon supply manipulation (i.e., crop load). The objective of this study was to characterize the true physiological and biological impacts of distinct carbon supply conditions on fruit internal quality and the secondary metabolism.

## 2. Materials and methods

### *2.1. Plant material and experimental approach*

Six 'Cresthaven' (late-ripening cultivar) peach (*Prunus persica* L. Batsch.) trees of uniform size and health were selected from Colorado State University's experimental orchard at the Western Colorado Research Center-Orchard Mesa, Grand Junction, CO (39°02'31.3"N,

108°27'56.8"W). The trees were nine years old, grafted on 'Lovell' rootstock, and trained to an open vase system at a planting density of 509 trees ha-1 (4 × 5 m spacing). Two distinct thinning treatments were administered fifty-two days after full bloom (DAFB: April 9, 2016) to shift the photosynthate source:sink ratio and create distinct carbon supply levels. The two thinning treatments to achieve varying levels of photosynthate sufficiency for the growing fruits were: 15 cm spacing between fruits on a growing shoot and an unthinned control. Each thinning treatment was conducted on three replicate trees. Thinning was conducted during early Stage 2 (S2) to ensure no further abscission would occur. In this way, the spacings, crop loads and predetermined levels of inter-fruit competition for carbon were maintained throughout development. The trees were managed according to industry standards and practices for irrigation, fertilization and pest management.

Fruit from each crop load treatment (thinned (15 cm) vs. unthinned) were sampled at three developmental stages (S2, Stage 3 (S3), S4) and were selected for each for maturity status ($I_{AD}$). Fruit of equal maturity were selected to characterize the impact of carbon supply more directly on peach quality development and secondary metabolome without the confounding impact of maturation status. The two treatments represent distinct carbon supply conditions: sufficiency and starvation. In particular, the unthinned treatment represents a carbon "starved" condition (C-starved) due to the high demand and competition between fruits, while the 15 cm fruit-to-fruit spacing (thinned) treatment, represents an adequate and sufficient carbon supply (C-sufficient) throughout development due to reduced photosynthate competition.

Five fruit of equal maturity (determined non-destructively using NIRS) per experimental tree (n=15 per treatment) were evaluated for quality (destructively as described below) at three development stages: S2 (72 DAFB), stage 3 (S3, 109 DAFB) and S4 (138 DAFB).

Five homogenized fruit mesocarp samples of equal maturity from each of the two treatments (thinned vs. unthinned) were taken from each of the biological replicates (n=15) at the three developmental stages (S2, S3, S4). In total, there were 18 homogenized samples (three trees x two treatments x three developmental stages = 18). After mesocarp samples were homogenized, they were flash frozen (quenched) with liquid nitrogen, freeze dried with the lyophilizer (Freezone 4.5, Labconco, Kansas City, MO, USA) at -40 °C for 12 h, and stored at -80 °C until analysis by liquid chromatography mass spectrometry (LC-MS). Immediately prior to analysis, samples were pulverized into a powder with a bead beater (Bullet Blender Storm, Next Advance, Troy, NY, USA) for five minutes. These samples were then kept in -20 °C until extractions could take place. With these data, two primary hypotheses were tested to determine which metabolites accumulate in accordance to: 1) fruit with a sufficient carbon supply or 2) fruit starved of carbon throughout development.

*2.2. Fruit quality analysis*

After harvest, fruit physiological maturity ($I_{AD}$) was assessed non-destructively using near-infrared spectroscopy (NIRS) on an "open-type" near-infrared spectrometer (F-750 Produce Quality Meter, Felix Instruments Inc., Camas, WA, USA) (F-750) (Minas et al., 2021). The Vis-NIR wavelengths used for measuring IAD were 600-750 nm (in 3 nm intervals) (Anthony et al., 2021). $I_{AD}$ ($R^2$=0.96; RMSEP=0.08) had been predicted with high accuracy using cultivar specific models that were created in Minas Lab following a Vis-NIRS calibration protocol (Minas et al. 2021; Minas et al., unpublished). Fruit was scanned on the sun-exposed cheek of the fruit. Fruit of equal maturity ($I_{AD}$) were selected to evaluate quality destructively. Fruit was selected based on previously determined cultivar specific calibrations for commercial harvest maturity (Anthony et al., 2020).

Destructive quality analysis was conducted according to the protocol laid out in Minas et al. (2021). The following parameters were evaluated during quality analyses: fruit diameter (cm), SSC (%), and DMC (%), from the sun- and shade-exposed cheeks from each fruit (Minas et al., 2021). Fruit volume (FV, $cm^3$) was calculated from fruit's radius as: $FV = (4/3)\pi r3$.

*2.3. Non-targeted metabolite profiling using liquid chromatography mass spectrometry (LC-MS)*

Metabolite extraction was conducted by first homogenizing freeze-dried mesocarp samples with a bead beater (Bullet Blender Storm, Next Advance, Troy, NY, USA) for five minutes. Extraction was conducted according to Anthony et al. (2020), by suspending 25 mg of each sample tissue in a two mL autosampler glass vial (VWR, Radnor, PA, USA) with one mL of 80% methanol (MeOH) in water solution. After centrifuging samples, supernatant was transferred into a new vial. A pooled quality control (QC) was created by transferring 50 µL of each sample into a separate glass vial (0.9 mL). One hundred µL of each sample's supernatant were also transferred into new vials.

LC-MS analysis was conducted at Colorado State University's Analytical Resources Core-Bioanalysis and Omics (ARC-BIO) facility. One µL of each extracted sample was injected onto a Waters Acquity UPLC system in a randomized order, with a pooled quality control (QC) injection run after every 6 samples. Separation was achieved using a Waters Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm), using a gradient from solvent A (Water, 0.1% ammonium formate) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 99% A, held for 1 min, ramped to 98% B over 12 minutes, held there for 3 minutes, and then returned to starting conditions over 0.05 minutes. Samples were then allowed to re-equilibrate for 3.95 minutes, with a 200 µL/min constant flow rate. The column and samples were held at 65 °C and 6 °C, respectively. The column eluent was infused into a Waters Xevo G2-XS Q-TOF-MS with an

electrospray source in positive mode, scanning 50-1200 m/z at 0.1 seconds per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 700 V, with a source temperature at 150 °C, and a nitrogen desolvation temperature at 600 °C with a flow rate of 1000 L/hr.

MS files were processed by XCMS (Smith, 2006; Tautenhahn, 2008, v3.9.3) in R version 3.6.2. RAMClustR (Broeckling, 2014) was used to normalize, filter, and group features intro spectra. Samples were normalized to total extracted ion signal. To account for instrument drift samples were further normalized by linear regressing run order versus QC feature intensities. Molecular weight was inferred from in-source spectra (Broeckling, 2016) using interpretMSSpectrum (Jaeger, 2016). MSFinder (Tsugawa, 2016) was used for spectral matching, formula inference and tentative structure assignment. Metabolite annotation confidence was based on guidelines of the Metabolomics Standards Initiative (Sumner et al., 2007). Compounds were assigned to chemical ontogenies using the ClassyFire API (Djoumbou, 2016).

*2.4. Statistical analysis*

All parameters were assessed for statistical differences between thinning treatment at each developmental stage using proc-GLM in SAS (SAS Inc., Cary, NC, USA). The effect of carbon supply and developmental stage on fruit quality and mean peak area of metabolites were each assessed for significance by two-way ANOVA ($P \leq 0.05$). Tukey mean comparisons were used to assign different lettering groups where the model was significant at $P \leq 0.05$ across thinning treatments within each developmental stage. An ANOVA was used to denote significance between carbon supply on quality and annotated metabolites by Type III sum of squares at $P \leq 0.05$ and Student's t-test ($P \leq 0.05$). Biological significance was indicated for metabolites that greatly shifted

in abundance at each stage of development (i.e., fold change), as classified as log2(C-sufficient/C-starved) < -2 or >2. Principal component analyses (PCA) were performed on metabolites in the mesocarp using SIMCA (Umetrics, Umea, Sweden) and on fruit quality spectra characteristics using JMP (SAS Inc., Cary, NC, USA). PCAs, and graphs were visualized using Prism v8.2.1 for Windows OS (Graph Pad Inc., San Diego, CA, USA).

## 3. Results

### *3.1. Carbon supply true impact on fruit quality*

Fruit from each carbon supply (C-supply) treatment were sampled at each developmental stage (S2, S3, S4) for quality, while controlling for physiological maturity ($I_{AD}$) through the application of Vis-NIRS technology (Figure 4.1). Equal maturity across treatments in each developmental stage is demonstrated by uniform levels of $I_{AD}$ (Figure 4.1a). However, significant differences were noted in fruit volume and DMC at each developmental stage, with increasing differences between treatments as development progressed (Figure 4.1). C-sufficient fruit volume grew exponentially with 64, 97 and 153% larger fruit at S2, S3 and S4, respectively, when compared to the C-starved fruit (Figure 4.1). Similarly, DMC values were 0.9 (7%), 1.5 (12%), and 3.0% (28%) higher in the C-sufficient fruit at S2, S3 and S4, respectively, when compared to the C-starved fruit as well (Figure 4.1). Soluble solids concentration revealed differences at S3 and S4, but significant differences ($P \leq 0.05$) were not detected between carbon treatments at S2 (Figure 4.1). At S4, C-sufficient SSC values were 2.8% (28%) higher than C-starved fruit, on average (Figure 4.1). Fruit quality differences intensify throughout development, with smaller phenotypic differences at S2, and extreme quality (DMC, SSC and fruit volume) differences at S4 (i.e., harvest).

After scanning each fruit with Vis-NIRS for maturity control, the second derivative absorption spectra (285-1200 nm) of the fruit utilized in destructive quality analyses were plotted in a PCA. The plotted absorption spectra appeared to visualize the incremental separation of fruit quality and phenotypic differences between the carbon supply treatments (red scores = C-sufficient; black scores = C-starved) throughout development (triangle = S2; square = S3; circle = S4) (Figure 4.1e). The distinct treatments demonstrated proximate clustering at S2, while there is vast separation at S4 (Figure 4.1e). In total, the PCA explained 37.2% of the total variation (Figure 4.1e). PC1 (21.3%) explained the differences due to development, while PC2 (15.9%) appeared to explain the differences due to carbon supply, especially later in development (Figure 4.1e).

Overall, the data clearly demonstrates that, even with controlling the confounding variable of maturity, carbon supply impacts quality development. Although this is evidenced early on (at S2), the vast distinction between treatments in fruit quality and phenotype is most apparent at harvest (at S4). Given this experimental approach, further investigations into the biological impacts of carbon supply manipulation were achievable. Therefore, an investigation into how these carbon conditions influenced the fruit's (mesocarp) secondary metabolome throughout development was conducted.

*3.2. Secondary metabolite profile composition across variable carbon supply treatments throughout peach fruit development*

In total, 1,971 metabolites were detected across the peach mesocarp samples (Table 4.1). Of those, 1,141 were confidently annotated and classified by chemical "superclass" (Djoumboi, 2016; Table 4.1). Although 42.3% of the compounds were unable to be annotated, the majority of the annotations were comprised of lipids and lipid-like molecules (32.7%) (Table 4.1). The next dominant chemical superclasses detected in these samples included: phenylpropanoids and

polyketides (8.2%), organic oxygen compounds (4.5%) and organoheterocyclic compounds (4.3%) (Table 4.1).

When evaluating the secondary metabolome composition across C-supply treatments throughout development, area graphs reveal four unique trends across the 12 chemical superclass categories: elevated in C-starved fruit, elevated in C-sufficient fruit, shift with development and uniform at developmental stage(s) (Figure 4.2).

Lipid and lipid-like molecules, along with organic nitrogen and unknown compounds appeared to be up-accumulated in C-starved fruit throughout development (Figure 4.2). Lipids appeared to increase throughout development, regardless of C-supply treatment, although again they comprised a much larger percentage of the secondary metabolome in the C-starved fruit (Figure 4.2). Organic nitrogen compounds nearly doubled their percentage from S3 to S4 in C-starved fruit, while percentages remained stable across development in C-sufficient fruit (Figure 4.2). Phenylpropanoids and polyketides, benzenoids and organic acids and derivatives appeared to retain higher percentages of the metabolome in C-sufficient fruit, when compared with C-starved fruit (Figure 4.2). In addition to this trend, these chemical superclasses also appeared to exhibit a dip in composition at S3, while incurring a spike in accumulation in S4, regardless of C-supply (Figure 4.2). Nucleosides and nucleotides, along with hydrocarbons demonstrated an increasing trend associated with development, while organoheterocyclic compounds decreased in composition percentage throughout development (Figure 4.2). Lastly, three chemical superclasses showcased uniformity in composition across C-supply treatments at one, two or all developmental stages. Lignan and neolignane compounds indicated downward accumulation throughout development, with uniform levels across C-supply treatments at S4 (Figure 4.2). Organic oxygen compounds demonstrated uniform levels at S2 and S3, but then diverged at S4, with an elevated

composition in C-sufficient fruit (Figure 4.2). Finally, alkaloids and derivatives uniformly decreased across developmental stages across both C-sufficient and C-starved fruit (Figure 4.2).

*3.3. Secondary metabolite profiles shift in response to variable carbon supply conditions begins early in development*

Principal component analysis of the metabolomic data revealed separation between carbon supply treatments at each developmental stage (Figure 4.3). The primary variation in the PCA is attributed to PC1 at 44.4% and visualized the metabolic variation across fruit development and maturation. PC2 explains a lesser amount of variation at 13.2% and appeared to showcase the variation attributed to carbon supply manipulation (Figure 4.3). Overall, the PCA explains 57.6% of the total variation across these samples (Figure 4.3). In general, the bulk of the loadings (grey circles, representing the 1,971 metabolites detected) appeared to be associated with S2 and S4, with less clustering at S3 (Figure 4.3).

Of the detected metabolites, 684 (34.7%), 616 (31.3%) and 276 (14.0%) metabolites demonstrated statistical differences ($P \leq 0.05$) between carbon supply treatments at S2, S3 and S4, respectively (data not shown). However, not all statistical differences equate to biological significance, so an additional threshold was administered to highlight specific metabolites/chemical classes demonstrating variability, both statistically and biologically. A fold change (FC) threshold for biological significance was set at: log2(C-sufficient/C-starved) < -2 or >2 (-2>FC>2) was then paired with a statistical threshold ($P \leq 0.05$) to evaluate how metabolites changed in respect to carbon supply conditions in each developmental stage (Figure 4.4). When investigating the variability within each stage by chemical superclass, the vast majority of significant metabolic changes occurred early, at S2, with a decreasing amount of variation throughout development (Figure 4.4). Seventy-four (4.0%), 59 (3.0%) and 36 (1.8% of all

metabolites detected) metabolites met the statistical and biological change thresholds at S2, S3 and S4, respectively (Figure 4.4). Although the number of metabolites varied at each stage, the predominant annotated chemical superclass that exhibited statistical and biological changes between carbon supply treatments across all stages were the phenylpropanoids and polyketides (Figure 4.4). The next largest chemical superclasses that demonstrated changes between treatments were either the lipids and lipid-like molecules and/or the benzenoids (Figure 4.4).

Volcano plots are displayed for each developmental stage to visualize the global changes between carbon supply treatments, with each data point representing a metabolite. Metabolites above the line are significant at $P \leq 0.05$ (Figure 4.5). These volcano plots demonstrate that the large majority of variation occurring between carbon supply treatments is occurring early on in fruit development at S2 (Figure 4.5). Further, the metabolites that are demonstrating large fold changes early on and throughout development appeared to be up-accumulating with the C-sufficient treatment (marked in red), instead of the C-starved (marked in black) (Figure 4.5).

*3.4. The majority of metabolic shifts are metabolites up-accumulating with increased carbon supply and demonstrate superior phenotypes*

When evaluating metabolites that were statistically different and exhibited large fold changes (-2>FC>2) across development between carbon conditions, there appears to be a larger number of metabolites up-accumulating with the C-sufficient treatment (Figures 4.5-4.6). At S2, 60 metabolites experienced a FC>2, while only 14 metabolites demonstrated a FC<-2 (Figure 4.6). The metabolites that up-accumulate with C-sufficiency at S2 represent a mix of chemical superclasses including phenylpropanoids and polyketides (35.0%), unknown compounds (26.7%), lipids and lipid-like molecules (13.3%), benzenoids (13.3%), organic oxygen compounds (10.0%) and organoheterocyclic compounds (1.7%) (Figure 4.6). Of the 60 metabolites with FC>2 at S2,

44 were annotated, with 21 of them being phenylpropanoids and polyketides. At S2, only 5 of the 14 metabolites that demonstrated a FC<-2 were classified and annotated. These were also comprised of a mix of chemical superclasses: one lipid, one organic acid, one organoheterocyclic compound and two phenylpropanoids and polyketides (Figure 4.6).

At S3 and S4, a similar trend was noted, with a much larger number of metabolites up-accumulating with C-sufficient fruit than up-accumulating with C-starved fruit (Figure 4.6). Fifty two and 29 metabolites were up-accumulated with the C-sufficient treatment (FC>2) at S3 and S4, while only 7 metabolites up-accumulated with the C-starved treatment at these stages (FC<-2), respectively (Figure 4.6). At S3, 38 of the 52 metabolites that were up-accumulating in the C-sufficient treatment were annotated and were largely comprised of phenylpropanoids and polyketides (32.7%) (Figure 4.6). Of the seven metabolites that were up-accumulated in the C-starved treatment (i.e., FC<-2) at S3, only two were annotated: one lipid and one phenylpropanoid, hexandraside E (Figure 4.6). At S4, 23 of the 28 metabolites that up-accumulated with the C-sufficient treatment were annotated (Figure 4.6). Similarly, these were made up of phenylpropanoids and polyketides, primarily (41.4%) (Figure 4.6). Four metabolites were annotated and up-accumulated (FC<-2) with the C-starved treatment at harvest. These two lipid and lipid-like molecules and two phenylpropanoids and polyketides, one of which was hexandraside E again (Figure 4.6).

Overall, a larger number of metabolites were up-accumulated in the C-sufficient treatment throughout fruit development, with a large amount of these metabolites being classified as phenylpropanoids and polyketides. Inversely, the majority of the annotated metabolites that were up-accumulated in the C-starved treatment throughout development were lipids and lipid-like

molecules. Although, it is worth noting that several lipids, along with benzenoids, also demonstrated up-accumulation in the C-sufficient treatment throughout development as well.

*3.5. Metabolic signatures of peach fruit supplied with abundant carbon supply throughout development*

When evaluating secondary metabolites that were demonstrating significant differences (both statistically and biologically) at every developmental stage between C-supply treatments, 27 metabolites were identified as commonly changed (Figure 4.6). Of the 27, 25 demonstrated up-accumulation with C-sufficient fruit, while only two metabolites up-accumulated consistently with C-starved fruit (Figure 4.6). Twenty of the 25 metabolites up-accumulating with C-sufficient fruit throughout development were annotated, with 11 (55%) of which being classified as phenylpropanoids and polyketides (Figure 4.6). These compounds were highlighted as signatures of carbon sufficiency throughout peach fruit development and a subset of these compounds, namely the flavonoids, are presented in detail in Figure 4.7.

The eight highlighted compounds, specified as flavonoids in the phenylpropanoid superclass, demonstrated statistical ($P \leq 0.05$) significance between carbon supply treatments at every developmental stage (except for steppogenin), with FCs that ranged from 1.7 to 6.3 (Figure 4.7). These include: steppogenin, trilobatin, catechin, epicatechin, dihydromorelloflavone, robinetinidol, cinnamtannin A2 and eriodictyol (Figure 4.7).

Catechin demonstrated the largest FC at S2 (6.3) with levels in the C-sufficient treatment 8,011% higher than those in the C-starved, on average (Figure 4.7). In fact, when averaging all of the flavonoids, abundances were 3,672% higher in the C-sufficient fruit when compared to the C-starved fruit at S2 (Figure 4.7). At S3 and S4, eriodictyol had the highest percent increase in the C-sufficient fruit, with levels 1,428 and 1,037% higher than the C-starved fruit, respectively

(Figure 4.7). At S4, although collective levels decreased throughout development, the flavonoids in C-sufficient fruit sustained abundances 534% higher than the starved, control fruit, on average (Figure 4.7).

## 4. Discussion

### *4.1. The true impact of carbon supply on peach fruit quality development*

Several previous studies have demonstrated crop load's effect on fruit quality in various tree fruit species (Marini and Reighard, 2008; Anthony et al., 2019; Anthony et al., 2020; Minas et al., 2021). Shifting source-sink relationships by removing fruitlets enables a more abundant pool of photosynthates to be available to the remaining developing fruit. Increased crop load diminishes fruit quality, increases photosynthetic activity due to high sink demand and delays fruit maturation and ripening. Whereas, reduced crop loads may sacrifice yield for the sake of enhanced fruit quality, in respect to larger fruit size and elevated levels of overcolor, DMC and SSC (Anthony et al., 2020; Minas et al., 2018; Alcobendas et al., 2012). However, reduced crop load also advances fruit maturity, which translates to several sensorial and quality changes, such as increased internal quality characteristics, aroma volatilization, pigment accumulation and mesocarp softening (Minas et al., 2018; Crisosto and Costa, 2008). As a result, thinning or crop load treatment effects on quality characteristics are difficult to ascertain if the confounding variable of maturity status is not controlled for.

This study was conducted to better understand the role preharvest factors have on quality and promote more accurate investigations into the biological characteristics of peach fruit, by selecting fruit of equal maturity (i.e., reduce confounding variables). Through the use of Vis-NIRS, fruit maturity was controlled for by maintaining uniform levels of $I_{AD}$ across C- sufficiency treatments at each development stage (Figure 4.1a). These results presented herein support the

hypothesis that increased C-sufficiency, through the elimination of inter-fruit competition (via thinning), result in enhanced fruit quality and phenotype throughout development and at harvest (Figure 4.1; DeJong and Grossman, 1995). This is evidenced with elevated levels of DMC and fruit volume in the thinned, C-sufficient fruit at each developmental stage, when compared to the unthinned, C-starved fruit (Figure 4.1). Additionally, levels of SSC were elevated at S3 and S4 (i.e., harvest) in C-sufficient fruit as well (Figure 4.1). In respect to all quality parameters, the severity of differences is exacerbated later in development, with the largest phenotypic differences occurring at S4.

When plotting the NIRS spectra into a PCA, both a developmental and phenotypic trend was elucidated. PC1 demonstrated the developmental shift, while PC2 showcased phenotypic differences between C-supply treatments (Figure 4.1e). Overall, the PCA reflects the shifting quality differences between carbon condition treatments throughout development, with close proximity at S2 and vast separation at S4. These developmental and qualitative changes are to be expected, as the spectra modeled in this PCA are utilized in the Vis-NIRS prediction of quality and physiological maturity (Minas et al., 2021; Anthony et al., 2021). The spectral range of 600 – 750 nm is utilized for physiological maturity ($I_{AD}$), while DMC and SSC are predicted using the spectral range of 729 – 975 nm (Minas et al., 2021). Both of these ranges lie within the spectra plotted in the PCA and are visualizing the developmental and quality separations presented in the model.

Enhanced fruit quality in the C-sufficient fruit is evidence of an abundant pool of carbohydrates available throughout development, while inferior fruit quality in the C-starved fruit is the result of diluted photosynthate availability and increased inter-fruit competition for resources

(Anthony et al., 2020; DeJong and Grossman, 1995). These results underscore the true impact of carbon supply on peach fruit quality, without the confounding variable of maturation status.

*4.2. Carbon starvation elicits elevated levels of lipids, while enhanced carbon supply prioritizes composition of phenylpropanoid pathway products throughout peach fruit development*

Peach maturation and ripening are regulated at the molecular level (Giovannoni et al., 2017) and should therefore be controlled for when investigating biological characteristics of the fruit, such as the metabolome. This novel maturity control approach has been validated in recent preharvest factor studies investigating their true impact on fruit quality and the primary metabolome in peach (Anthony et al., 2020; 2021). However, this study sought to expand metabolome coverage, while maintaining the integrity of maturity control, to further elicit a more comprehensive understanding of how the metabolome shifts under variable carbon supply conditions. Twelve "superclasses" of chemical compounds were identified, including unknown compounds, amongst the 1,971 metabolites detected (Table 4.1). Excluding the unknown compounds, the lipid and lipid-like compounds were the most abundant in peach mesocarp tissue (Table 4.1). Lipid composition (out of 100%) increased from 40 and 36% at S2, to 49 and 45% at S4, in the C-starved and C-sufficient treatments, respectively (Figure 4.2). Lipids are a major component of biological membranes, with their physical state and composition contributing to structural and functional attributes (Izzo et al., 1995). Interestingly, a previous study in peach, demonstrated a reduction of lipids throughout development (Izzo et al., 1995), while in the present study it appears mean abundance of this superclass increases with advanced development (Figure 4.2). This appears to be largely due to the increasing composition of glycerophospholipids, which was the predominant class in the lipid superclass category, accounting for 45% of the 644 lipids detected in the mesocarp samples. Glycerophospholipid metabolism was documented to be closely

related to postharvest softening in pears (Xu et al., 2021). In peach, it has been suggested that postharvest chilling tolerance can be enhanced through inhibiting lipid degradation (Wang et a., 2019). Preharvest carbon supply conditions may have implications for postharvest management of peach fruit via lipid composition and glycerophospholipid metabolism, with higher levels in C-starved fruit. Regardless, minimal literature is available connecting preharvest treatments with lipid composition in tree fruit. But, with an understanding of the final phenotype at harvest in C-starved fruit, perhaps an elevated level of lipids may have a connection with inferior quality.

The next most abundant superclass of compounds were the phenylpropanoids and polyketides. The abundance of these compounds appears to be elevated in the C-sufficient fruit, with a slight dip at S3, and an overall increase at S4, in both C-supply treatments (Figure 4.2). These treatment and developmental trends were also documented in the benzenoid superclass, as well (Figure 4.2). The phenylpropanoid metabolism pathway is responsible for synthesizing a large number of secondary metabolites and has been attributed with several disease resistance traits (Li et al., 2016). Products of the phenylpropanoid pathway (PP) include lignans, flavonoids, anthocyanin, proanthocyanidins and hydroxycinnamic acids (Ma and Constabel, 2019). In particular, the PP is regulated through MYB transcription factors, such as R2R3-MYBs (Ma and Constabel, 2019). In plants, R2R3-MYBs are the most common type of MYB factor (Yadav et al., 2020), responsible for secondary plant metabolite synthesis, such as terpenoid, glucosinolate, phenylpropanoid and benzenoid pathways (Ma and Constabel, 2019). The similar compositional trends documented in the mesocarp accumulation of phenylpropanoids and benzenoids may be due to similar transcriptome regulation via R2R3-MYBs. Phenylpropanoid compounds provide an array of plant defense mechanisms, such as chemical and physical barriers to infection, along with signal molecules that trigger gene induction for additional defense functions that are antimicrobial

or hormonal in nature (Dixon et al., 2002; Yadav et al., 2020). Further, these defense mechanisms are not exclusive to any class of chemicals, as these protective properties have been documented in simple compounds such as hydroxycinnamic acids, ranging to more complex molecules, like flavonoids and isoflavonoids (Dixon et al., 2002). The prioritization of these compounds certainly provides a benefit to the developing fruit and appear to be a "luxury" investment for peaches supplied with an abundant pool of carbohydrates (i.e., C-sufficient fruit) (Anthony et al., 2020). As a result, the triggering of phenylpropanoid pathway products in peach, through exogenous applications of various gases, has been the objective of recent research studies to promote natural biological resistance to postharvest diseases (Li et al., 2016; Ji et al., 2021).

Further, these PP products may indicate a relationship with fruit quality in peach, as anthocyanins and flavanols have demonstrated relationships to berry quality in grape (VanderWeide et al., 2020). Saccharide hexoses are fundamental for the biosynthesis of these chemical classes, as their biosynthesis follows the accumulation of hexoses in fruit (Zheng et al., 2009; Castellarin et al., 2011). This may explain why the C-sufficient fruit demonstrate elevated abundances of these chemical compounds, as they maintain elevated levels of sugars throughout development (e.g., DMC and SSC) (Figure 4.1). It has been documented that phenylpropanoid biosynthesis is largely affected by environmental factors, such as radiation and temperature (VanderWeide et al., 2020), but it now appears that it is also largely affected by carbon supply and leaf-to-fruit ratios. In short, reduced inter-fruit competition and elevated source:sink ratios plays a fundamental role in assimilating higher levels of hexoses in developing fruit, which triggers an up-accumulation of phenylpropanoid pathway products, like anthocyanins and flavanols. Ultimately, there appears to be a connection between the central carbon metabolism, PP products and fruit quality development in peach.

*4.3. Early secondary metabolic variation primes fruit quality through up-accumulation of several compounds in C-sufficient peaches*

Metabolic shifts occur dramatically early in fruit development, with a diminishing amount occurring towards harvest (Anthony et al., 2020). In the present study, 74 metabolites characterized statistical and biological shifts at S2 between C-supply treatments, while only 36 met this criterion at S4 (i.e., harvest). Further, these metabolic shifts are demonstrating up-accumulations in the C-sufficient fruit, with minimal chemical compounds increasing their abundance in C-starved fruit (Figures 4.5-4.6). Of these selected metabolites undergoing significant changes, 81, 88 and 81% of the metabolites were up-accumulating in C-sufficient fruit at S2, S3 and S4, respectively (Figures 4.5-4.6). This indicates that when fruit are under stress conditions, such as a limited C-supply, they cannot accumulate and invest in chemical compounds that are integral for plant defense, stress mitigation, pigmentation and overall fruit quality. Therefore, there appears to be a connection between the metabolome composition, especially early in development, and the at-harvest phenotype (Anthony et al., 2020). Early metabolic shifts that up-accumulate these secondary metabolites, especially phenylpropanoids and polyketides, appear to prime the fruit quality characteristics present at harvest. This is reflected with the elevated levels of the PP products at S2 (and throughout development) and the elevated levels of DMC and SSC at S4. As a result, these chemical compounds can serve as a signature of C-sufficiency and an indicator of a balanced source:sink ratio within the tree.

In a previous study investigating the primary metabolism, only two metabolites were statistically ($P \leq 0.05$) different between C-supply treatments at S4 (Anthony et al., 2020). However, one of these compounds was catechin, a secondary metabolite, and the other was an identified unknown compound. As a result, it appeared that there were no primary metabolites that

were significantly different at harvest. It was hypothesized that this was due to the experimental approach of controlling for maturity, as the primary metabolism appears to be heavily regulated by development, contributing to the fundamental growth and survival of developing fruit (Anthony et al., 2020). Therefore, one of the objectives of this study was to determine if the secondary metabolism would be more affected by preharvest factors than the primary metabolism at harvest, when controlling for maturity. In the present study, 1,138 metabolites were annotated, with 184 metabolites demonstrating statistical differences ($P \leq 0.05$) between C-supply treatments at S4 (data not shown). This equated to 16.2% of the annotated metabolites exhibiting statistical differences between treatments at S4. As a result, it appears that although the secondary metabolism is more dramatically affected early on by carbon manipulation (like the primary metabolism), it still exhibits substantial differences at harvest (unlike the primary metabolism). This is perhaps due to secondary metabolites being less integral for fruit growth and survival, and so the shifts in metabolite accumulation and investments are more pronounced under variable conditions and stresses throughout the totality of development. This supports the hypothesis that secondary metabolites are key translators of physiological stimuli for the fruit's biology, demonstrating a more dynamic relationship with abiotic and biotic stress signals.

Although less fundamental for survival, the increased levels of secondary metabolites certainly help improve the growth and enhance the quality of developing fruit. Secondary metabolite content is heavily associated with color, flavor, aroma and tissue dynamics (Tohge et al., 2014) and translate to influencing quality characteristics that consumers value. The up-accumulation of particular secondary metabolites, such as phenylpropanoids and benzenoids, appear to play a role in contributing to superior fruit quality in peach. In particular, the flavonoid

chemical class appears to remain highly elevated in the C-sufficient fruit throughout each stage of development (Figure 4.6-4.7).

*4.4. Flavonoids serve as metabolic signatures of carbon sufficiency in peach and highlight the connection between primary and secondary metabolism*

Flavonoids promote an array of defense, stress-mitigation and antimicrobial strategies in plants, and have demonstrated numerous health-promoting benefits and pharmacological activities in humans (Andersen and Markham, 2005). Flavonoids in fruit can exist in a free state or in a glycosidic form (Liu et al., 2020), and contribute to plant coloration, catalyze the light phase of photosynthesis, regulate iron channels in phosphorylation, scavenge radical oxygen species (ROS) and provide UV radiation protection (Pietta, 2000). Flavan-3-ols, such as catechin and epicatechin, are widely distributed in plants, especially in tea leaves (Pietta, 2000). Their innate astringency serves as a defensive mechanism repelling herbivorous insects (Pietta, 2000). In sweet cherry, the presence of flavonoids has been hypothesized that promotes superior fruit quality maintenance and disease resistance during storage (Liu et al., 2020).

In the present study, the abundance of flavonoids was markedly superior, both statistically and biologically, in the C-sufficient fruit, when compared to the C-starved fruit (Figure 4.7). Of the 25 metabolites that demonstrate significant up-accumulations in C-sufficient fruit throughout all developmental stages (Figure 4.6), 11 of them were flavonoids. These flavonoids were therefore noted as metabolic signatures of a sufficient C-supply throughout peach fruit development, as these compounds consistently up-accumulate in C-sufficient fruit (Figure 4.7). As this treatment exhibited a superior phenotype throughout all developmental stages as well, it was further supported that this chemical class (flavonoids) has a relationship to superior fruit quality in peach (Anthony et al., 2020). This relationship between flavonoids and fruit quality is further supported

with similar results in grape, sweet cherry and blueberry as well (Ge et al., 2019; VanderWeide et al., 2020; Liu et al., 2020). In a recent peach study, positive relationships between catechin and DMC and SSC were demonstrated at harvest (Anthony et al., 2020). In fact, catechin was the only annotated metabolite that demonstrated differences at harvest between two distinct C-supply treatments, demonstrating significantly higher levels in the C-sufficient fruit (Anthony et al., 2020). It is hypothesized that given the exceptional ecological and physiological importance of flavonoids in plant biology, that fruit are benefited by elevated levels of these compounds, and so prioritize the investment of these metabolites under beneficial environmental conditions (e.g., abundant C-supply or light availability) (Anthony et al., 2020; 2021). This contributes to not only an enhanced competitive advantage for C-sufficient fruit, but also translates to superior quality, as flavonoids contribute substantially to the sensorial and nutritional properties of fruits (Labadie et al., 2020).

Of the flavonoids in the present study, catechin demonstrated the largest difference between C-supply treatments, with abundances 8,011% higher than those in the C-starved at S2 (Figure 4.7). Previous investigations have hypothesized that the vast early metabolic shifts in catechin may play a role in priming the fruit quality phenotype at harvest, which has now been supported again in the present study as well, along with various other flavonoids demonstrating this similar trend (Figure 4.7; Anthony et al., 2020). Eriodictyol was the flavonoid with the greatest increases in the C-sufficient fruit during the later stages in development (S3 and S4) (Figure 4.7). It has been documented as an antioxidant, anti-inflammation, anti-cancer, anti-obesity and anti-diabetic agent (Islam et al., 2020). Eriodictyol is a flavonoid commonly found in several medicinal plants, citrus fruits and vegetables, and it has been associated with modulating a number of cell-signaling cascades and masking bitter flavors (Islam et al., 2020; Mehmood et al., 2021).

Phenylpropanoids comprise a multitude of secondary metabolites in plants and contribute to plant defense, and fruit color, flavor, aroma and overall quality. These chemical compounds are synthesized through the phenylpropanoid pathway, which may provide a bridge between the primary and secondary metabolism.

Beginning upstream, the central carbon metabolism synthesizes carbohydrates via photosynthesis, which are first used in the pentose phosphate pathway (PPP) and later in the shikimate pathway to synthesize several precursors of several secondary metabolites (Walker and Faminai, 2018). The shikimate pathway undergoes seven metabolic steps (Herrmann and Weaver, 1999), which results in chorismate, an intermediate that can be utilized to form plant hormones like auxin, or produce L-arogenate (AGN) (Barros and Dixon, 2020). Arogenate dehydratase then converts AGN to Phe or Tyr, where several secondary metabolites can be derived from, such as tocopherol, glucosinolate, phenols and alkaloids (Barros and Dixon, 2020). Phe and Tyr can also be deaminated via PAL and PTAL to form cinnamate and coumarate (i.e., hydroxycinnamates), from which flavonoids, coumarins and lignans are then synthesized (Barros and Dixon, 2020).

The phenylpropanoid pathway showcases the interconnection between the primary and secondary metabolism, with the abundance of carbohydrates fueling the PPP and shikimate pathways to form aromatic amino acids, which provide the building blocks for phenylpropanoid and flavonoid biosynthesis. Enhancing the accumulation and biosynthesis of these chemical compounds in fruits in fundamental for improving fruit quality (Tzin et al., 2013). Therefore, the authors hypothesize that by optimizing preharvest factors, where quality and the assimilation of carbohydrates occurs, this can help facilitate the up-accumulation of phenylpropanoids, which will prime and enhance the taste, flavor, aroma and quality of the fruit. Additionally, several postharvest studies have demonstrated that these phenylpropanoids will also help maintain fruit

quality postharvest, by reducing the incidence of chilling injury and storage diseases (Gao et al., 2016). In sum, the optimization of the source:sink ratio preharvest enhances the carbon availability in developing fruit for the synthesis of metabolic signatures, such as phenylpropanoids (e.g., flavonoids), to prime and contribute to the improvement of fruit quality in peach.

## 5. Conclusion

Improving peach fruit quality is imperative for ensuring a sustainable and profitable industry. Enhancing fruit quality can be done through the optimization of preharvest factors, such as balancing the crop load, or source:sink ratio. Few studies have controlled for confounding variables, such as the fruit maturity status, in their preharvest factor assessments, which limits their findings. Further, it inhibits investigations into how these preharvest factor treatments influence the biological characteristics of fruit, as maturity is heavily regulated at the molecular level. In the present study, the true impact of crop load is demonstrated, as fruit of equal maturity were assessed for quality at harvest, revealing superior fruit quality in fruit that had been sufficiently supplied with carbon throughout development (i.e., thinned fruit). While investigating how the secondary metabolome is affected by carbon manipulation, lipids and organic nitrogen compounds maintained higher compositions in the C-starved fruit, while phenlypropanoids and polyketides, bezenoids and organic acids were elevated in C-sufficient fruit. The majority of metabolic shifts occurred early in development (i.e., S2), and were characterized primarily by up-accumulations of phenylpropanoids and polyketides in C-sufficient fruit. In particular, flavonoids were a specific chemical class that demonstrated consistent differences throughout each stage of development between C-supply treatments and were marked as signatures of carbon sufficiency. Similar to the primary metabolism, the early metabolic shifts in the secondary metabolome appear to prime the fruit quality phenotype at harvest. Unlike the primary metabolism, notable secondary metabolites

remain distinct at harvest, responding more directly to preharvest factors like crop load. Enhanced carbon availability in developing fruit facilitates increased synthesis of metabolic signatures, such as flavonoids via the phenylpropanoid pathway, to prime and improve peach fruit quality at harvest.

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## Tables

**Table 4.1. Total number of secondary metabolites detected and annotated in peach mesocarp samples throughout fruit development.** The impact of thinning severity on fruit secondary metabolite profiles was assessed through non-targeted liquid chromatography – mass spectrometry in 'Cresthaven' peach. Chemical compounds were classified by chemical superclass (ClassyFire API, Djoumbou, 2016).

| Chemical Superclass | No. of Metabolites | Percent of Total |
|---|---|---|
| Unknown compounds | 833 | 42.3 |
| Lipids and lipid-like molecules | 644 | 32.7 |
| Phenylpropanoids and polyketides | 161 | 8.2 |
| Organic oxygen compounds | 89 | 4.5 |
| Organoheterocyclic compounds | 84 | 4.3 |
| Organic acids and derivatives | 67 | 3.4 |
| Benzenoids | 38 | 1.9 |
| Lignans, neolignans and related compounds | 18 | 0.9 |
| Alkaloids and derivatives | 13 | 0.7 |
| Organic nitrogen compounds | 11 | 0.6 |
| Nucleosides, nucleotides, and analogues | 7 | 0.4 |
| Hydrocarbons | 6 | 0.3 |
| Total | 1,971 | 100 |

## Figures

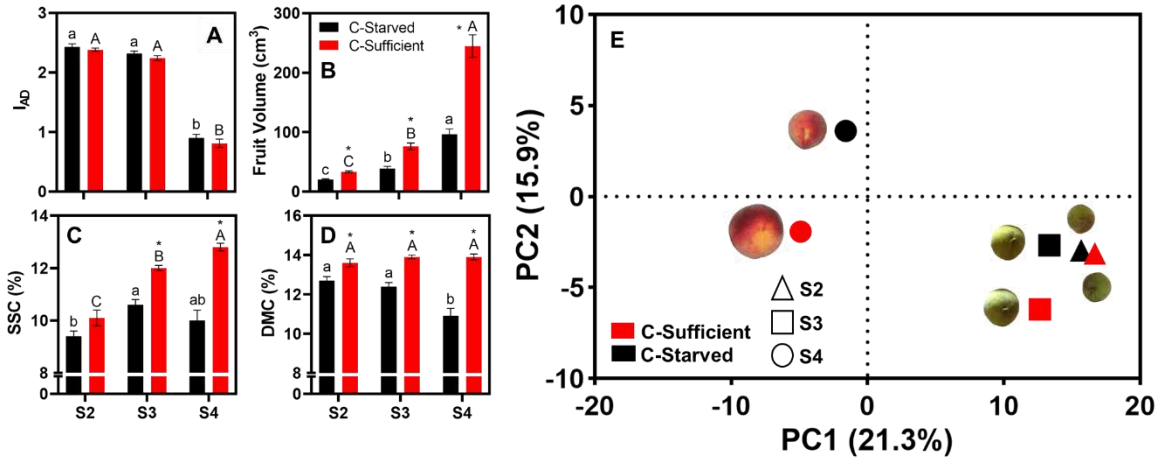


**Figure 4.1. The true impact of carbon supply on fruit internal quality development.** Impact of two carbon supply treatments: unthinned (C-starved) and thinned (15 cm, C-sufficient) on fruit physiological maturity ($I_{AD}$, A), fruit volume ($cm^3$; B), soluble solids concentration (SSC, %; C) and dry matter content (DMC, %; D) from destructive quality analysis on 10 fruit per treatment at each developmental stage. Means of fruit plotted at three developmental stages: stage 2 (S2, pit hardening, 72 DAFB), S3 (cell elongation, 109 DAFB), and S4 (harvest, 138 DAFB). Error bars are ± standard error. Fruit quality was assessed on fruit of equal maturity, assessed with $I_{AD}$, at each developmental stage (A). Two-way ANOVAs (by carbon supply treatment and developmental stages) were used to detect differences across means with lettering or asterisks assigned by Tukey's HSD test. Means with the same letter indicate non-significance at $P \leq 0.05$ within thinning treatments across developmental stages. Upper-case letters correspond to the C-sufficient treatment, whereas the lower-case letters correspond to the C-starved treatment. Significant differences between thinning treatments at each developmental stage are denoted with an asterisk (*) at $P \leq 0.05$. Principal component analysis (PCA) was also conducted to assess the impact of variable carbon supply on peach fruit phenotype and spectral absorbance profiles (E). C-sufficient fruit (red) vs. C-starved fruit (black) and developmental stage (S2 (triangle), S3 (square), S4 (circle)) scores are scaled on the PCA (E). Large symbols on the PCA are the averaged profiles of 10 fruits' $2^{nd}$ derivative absorption across a UV – NIRS spectra (285 – 1200 nm, in 3 nm intervals) per each carbon supply x developmental stage treatment (E). Images in PCA are from a representative fruit from each carbon supply x development stage treatment to highlight phenotypic differences (E).

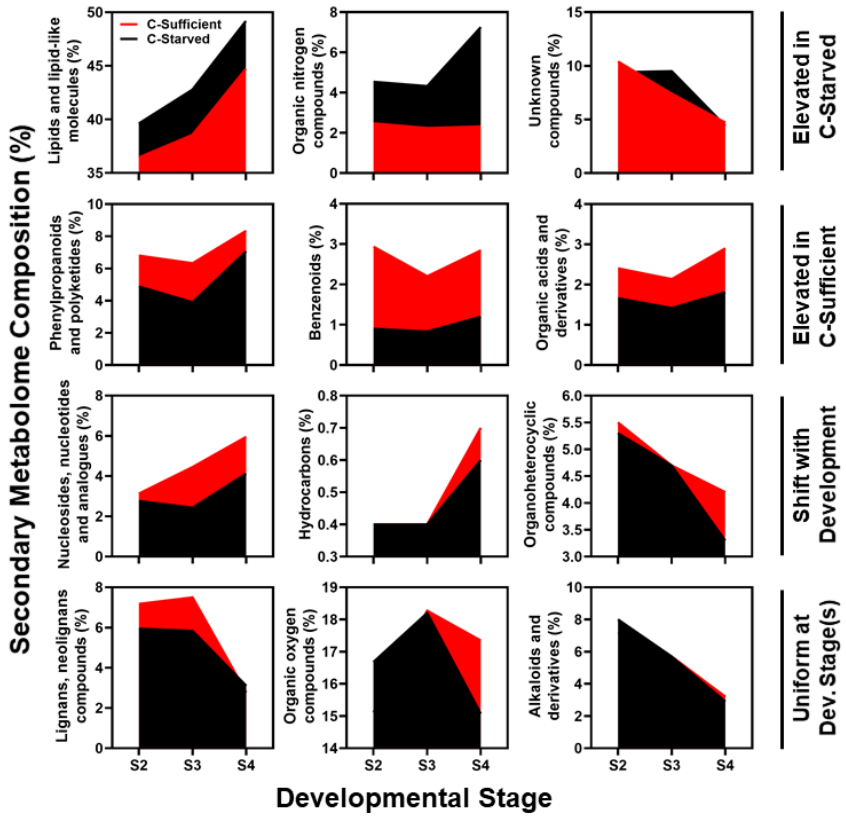


**Figure 4.2. Area plots of metabolite superclass composition in peach mesocarp samples throughout development across carbon supply treatments.** The impact of variable carbon supply on peach fruit metabolite profile composition by chemical superclass are demonstrated at Stage 2, 3 and 4 (S2, S3, S4, respectively) across C-sufficient fruit (red) vs. C-starved fruit (black). Four trends were noted across the metabolite compositional shifts throughout development and carbon supply: elevated composition in C-sufficient fruit (top row), elevated in C-starved fruit (second row), compositional shifts throughout development (third row) and compositions that were uniform at particular developmental stage(s) (fourth row). Composition is presented as a percentage, with each carbon supply x developmental stage treatment contributing to a part of a whole (i.e., 100%).

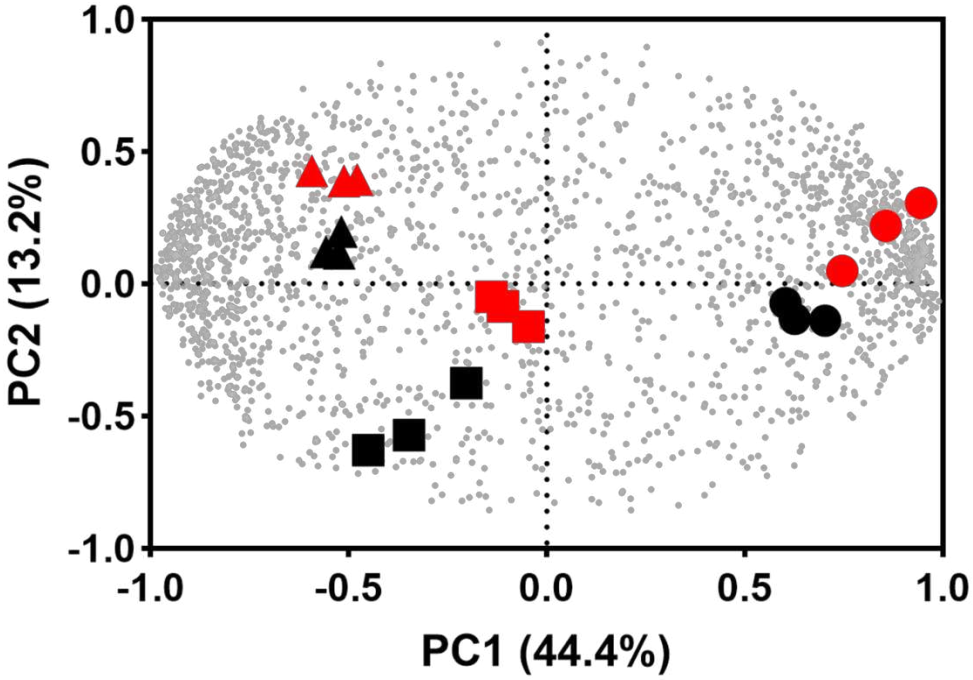


**Figure 4.3. Principal component analysis biplot of variable carbon supply on peach fruit secondary metabolism.** Large symbols indicate the scores for the carbon supply treatments (C-starved (black) vs. C-sufficient (red)) and developmental stages (S2 (triangle), S3 (square), S4 (circle)) and are scaled with the metabolites detected in the peach mesocarp (loadings). Principal component analysis (PCA) of the three reps per each carbon supply x developmental stage treatment demonstrate that developmental stage was a major contributor for metabolome variation as indicated by separation on PC 1 (~ 44%). Additional metabolome variation due to the carbon availability and other factors is visualized on PC 2 (~ 13%). The 1,971 annotated metabolites are shown on the PC loadings as grey circles. PCA shows similar variation in the secondary metabolome between carbon supply treatments throughout development.

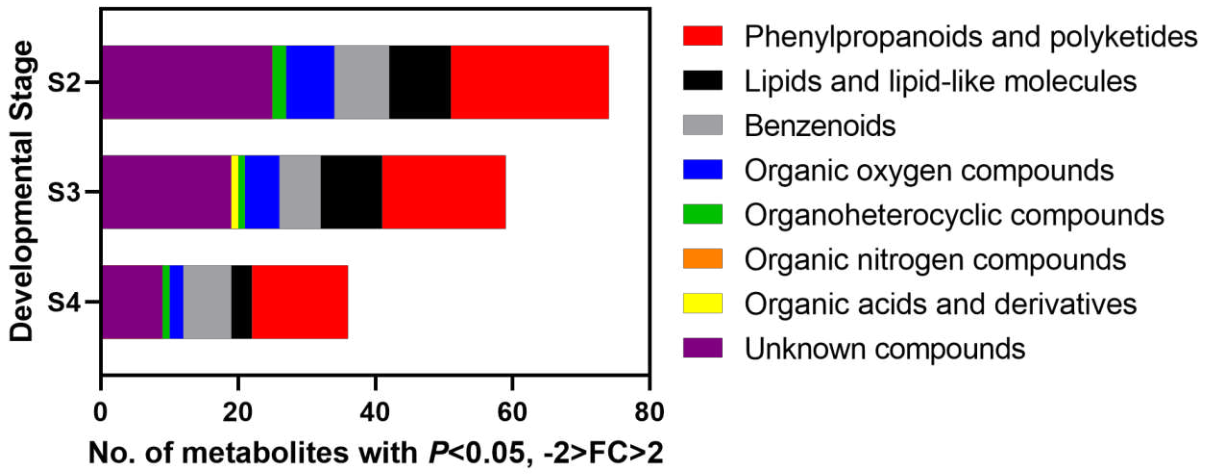


**Figure 4.4. Metabolites that shift significantly across carbon supply treatments throughout development.** Metabolites plotted in each bar demonstrated both statistical ($P \leq 0.05$) and biological (log2(C-sufficient/C-starved) < -2 or >2) significance between carbon supply treatments at each developmental stage. Metabolites were annotated and classified by chemical superclass (ClassyFire API, Djoumbou, 2016). The vast majority of metabolites meeting these criteria are shifting early in development at S2 and are characterized primarily by phenylpropanoids and polyketides. Although, a substantial number of metabolites are still significantly different at S4 (i.e., harvest).

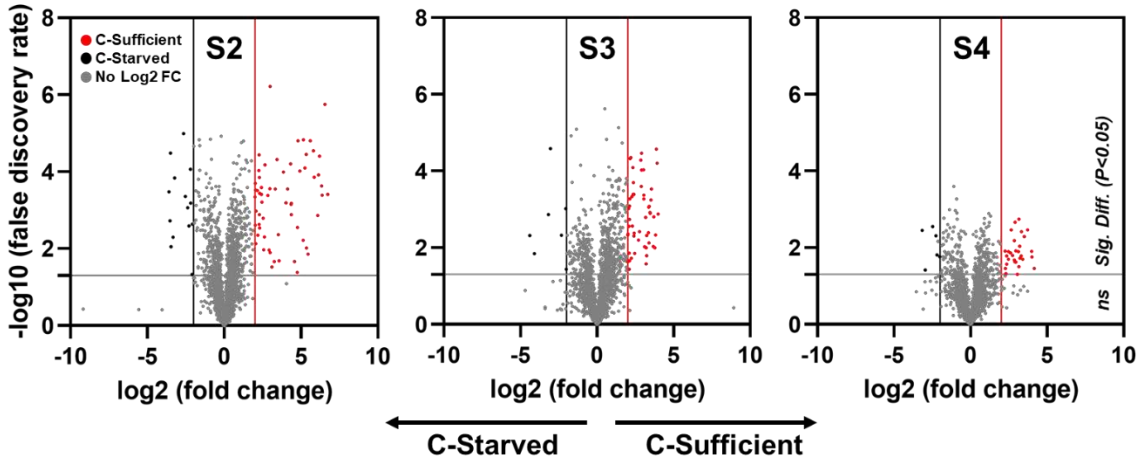


**Figure 4.5. Volcano plots demonstrating statistically and biologically significant metabolites shifting in abundance between carbon supply treatments throughout development.** All 1,971 metabolites are represented with a dot. Grey dots below the grey line (Y-axis<1.3) indicate non-significantly different ($P \leq 0.05$) metabolites between carbon supply treatments. Grey dots above the grey line, indicate statistical difference between carbon supply treatments, but do not shift in

abundance at fold changes < -2 or >2 (i.e., biological significance). Red and black dots represent metabolites that satisfy both statistical ($P \leq 0.05$) and biological (log2(C-sufficient/C-starved) <-2 or >2) significance between carbon supply treatments at each developmental stage. Red dots to the right of the right line (X>2) indicate metabolites up-accumulating in C-sufficient fruit, while black dots to the left of the black line (X<-2) represent metabolites up-accumulating in C-starved fruit. The vast majority of metabolites meeting these criteria are shifting early in development at S2 and are characterized primarily by metabolites up-accumulating in C-sufficient fruit. Few metabolites up-accumulated in C-starved fruit throughout development.

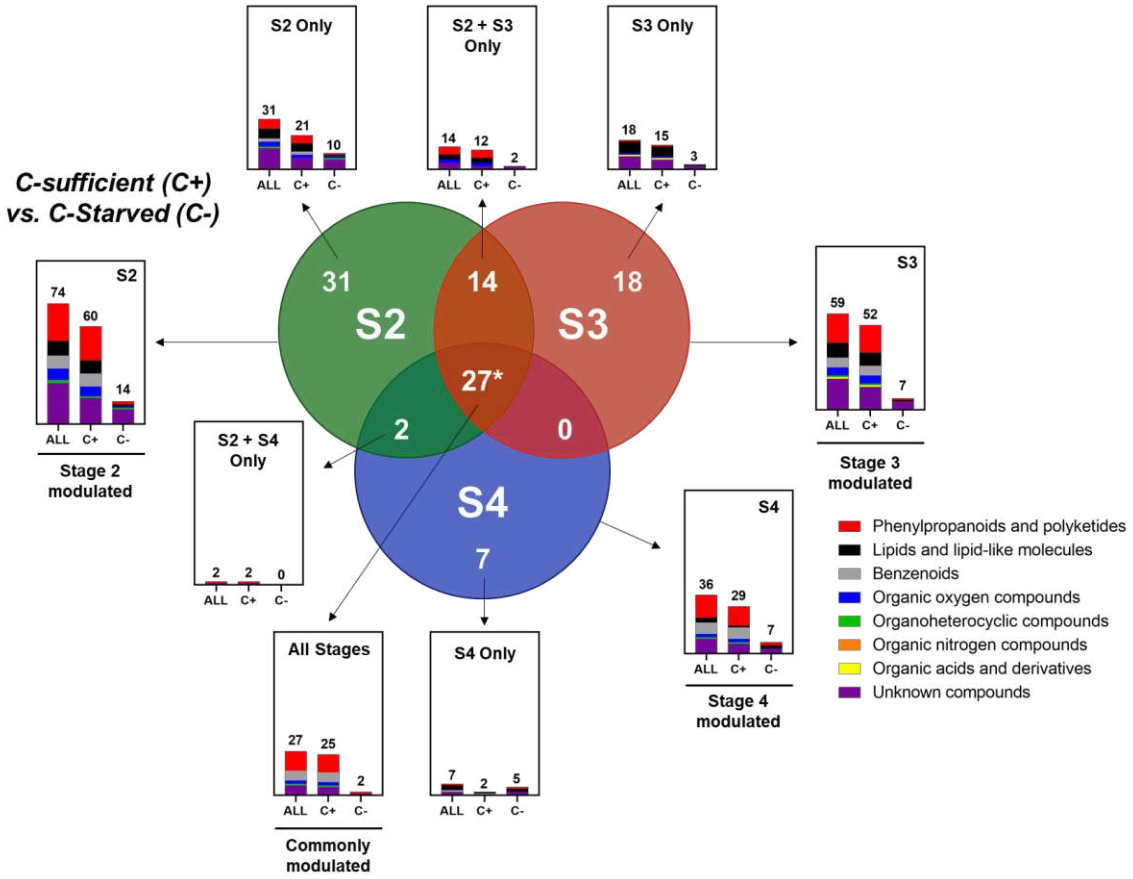


**Figure 4.6. Analysis of differentially accumulated secondary metabolites in response to carbon supply throughout development in peach.** Venn diagrams displaying the number of differentially accumulated metabolites in peach subjected to carbon supply treatments (C-sufficient (C+) and C-starved (C-)). The count of unique or overlapping metabolites are presented. Superclass chemical classification of the identified peach metabolites that changed in abundance due to carbon supply treatments are shown at each stage or overlapping stages of development. The symbols (+) and (−) indicate identified metabolites of peach that shown up-accumulating with the C-sufficient or C-starved treatment, respectively.

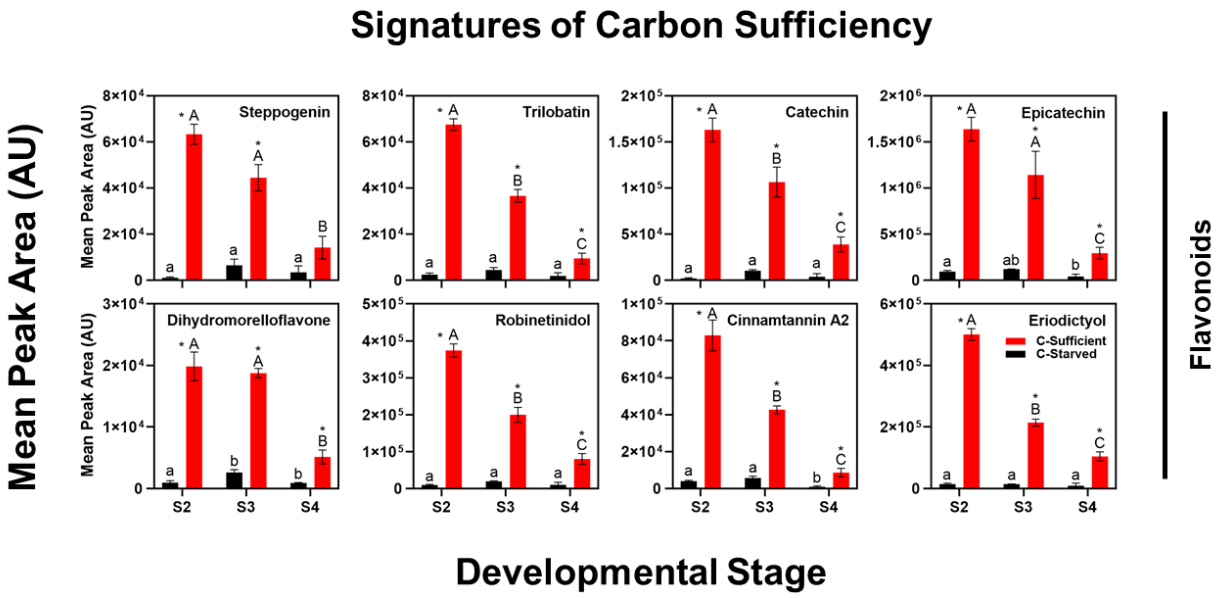


**Figure 4.7. Signature metabolites with consistently elevated abundances in carbon sufficient fruit throughout development in the peach mesocarp.** Figure showcases the mean peak area (AU) of selected metabolites that are consistently up-accumulated in the C-sufficient peach fruit throughout development, when compared to the C-starved fruit. The bars indicate the carbon supply treatments: C-starved (black) and C-sufficient (red). Samples were controlled for equal maturity ($I_{AD}$) at harvest and between carbon supply treatments. Mean values ± S.E. are displayed. Means with the same letter displayed above the bar are not statistically significant according to Tukey's HSD test ($P \leq 0.05$) throughout development (C-starved=lower-case, C-sufficient=upper-case). * indicates a significant difference between carbon supply treatments at each developmental stage (S2, S3, S4) at a $P \leq 0.05$, according to Student's T-test.