Environmental and Genetic Influences on Growth, Flowering, and Nectar Production in Mānuka (*Leptospermum scoparium* J.R. Forst. & G. Forst.)

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Abstract

Dihydroxyacetone (DHA) in floral nectar of the Mānuka shrub, *Leptospermum scoparium* (J.R. & G. Forst.) is a direct precursor to methylglyoxal (MGO) the bioactive compound of manuka honey. Accumulation of DHA in Mānuka nectars varies between trees, localities, and years. However, the reasons for this variability are largely unknown and its origins in nectar are unclear. Since high DHA to total sugar ratios (DHA/TSugar) in fresh honey result in high MGO in mature honey, it follows that nectars with high DHA/TSugar will produce high-value honey indicated by a high Unique Mānuka Factor (UMF®) attracting premium returns for the NZ honey industry and NZ economy. It is key to further optimise both nectar and DHA production by selecting for high producers. Selecting/developing varieties for maximum nectar potential (NP) needs an understanding of the relative influences of genotype (G), environment (E), and their interactions (GEI) on relevant trait expression. The responses of genetic clones from three high yielding Mānuka lines expressing varying levels of nectar DHA to temperature, light, and soil moisture were studied in controlled environments. The relative performances of the clones were evaluated and contributions of G, E, and GEI to aspects of their growth, flowering and nectar production quantified. The effects of long-term (one complete annual growth cycle) Cool and Warm temperature treatments were observed as changes in magnitude and shifts in phenology, such that the growth and flowering curves were unique (r^2 = 0.63; P < 0.001). The observed responses appeared to be independent of nectar DHA concentration. Nectar production in the clones was a strongly genetically determined trait. While nectar quantities were little affected by short (48 hour) or long-term ambient temperature changes, both G and E (temperature, light, soil moisture) influenced the DHA to total sugar ratio (DHA/TS) of the nectars. That is, temperature and light quality appeared to be major determinants of the quality (DHA/TS) of the Mānuka nectars ($P \leq$ 0.05) and potentially the honey derived from that nectar.

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Abbreviations

Ab	Antibacterial
ANOVA	Analysis of variance
BC	Black Cool
BSD	Basal stem diameter
Bud	Floral bud number
BW	Black Warm
CDL	Critical day length
CI	Confidence Interval
Corr.	Correlation coefficient
DHA	Dihydroxyacetone
DL	Day length
DHA/TS	Ratio of DHA to total nectar sugars
FF1	Floral flush one
FF2	Floral flush 2
GC	Green Cool
GLM	General linear mixed model
GMM	Gaussian mixed model
GR	Growth rate
GW	Green Warm
h	Hour
HP	Hydrogen peroxide
Int	Internode length
L	Main axis stem length
LD	Long day
LMM	Linear mixed model

MAE	Main-axis extension
MGO	Methylglyoxal
MIL	Mean internode length
Nod	Node (on primary axis)
NPA	Non-peroxide activity
OC	Orange Cool
OW	Orange Warm
P	P-value (statistical)
PGU	Plant Growth Unit
RGR	Relative growth rate
SD	Short day
SER	Shoot elongation rate
ShDi	(Primary) shoot diameter
ShL	(Primary) shoot length
$\mathbf{T_b}$	Base temperature
TDR	Time domain reflectometry
$\mathbf{T_m}$	Mean daily average temperature
$\mathbf{T_{MA}}$	Mean annual temperature
TSugar	Total nectar sugars
Tmax.	Maximum temperature
Tmin.	Minimum temperature
TTM	Thermal Time Model
χ	Chi (statistical)
VBA	Visual Bud Appearance

Chapter 1 Introduction & Literature Review

1.1 Industry Background

Bioactive manuka honey sourced from the New Zealand (NZ) Mānuka shrub (*Leptospermum scoparium* J.R. Forst. & G. Forst.*)* is a major driver of the international success of the NZ honey industry as an highly innovative, science-based and high value export focused sector. In the year ending June 2017, the revenue to NZ from pure honey exports (bulk and retail packs, comb honey and honeydew of all honey types) was $NZ329 million. This is a 5% increase on the previous year with the average export price per kilogram of honey up by 9%. This is a realised value for the unique manuka honey product produced in NZ of around $10.80 to $127.00 per kg[1].

This PhD project was part of the '*High Performance Manuka Plantations'* Primary Growth Partnership (PGP) funded research programme, a collaboration between the NZ government and industry (established 2011–2018). The aim of the programme as a whole was to create a $1.2b honey industry (a 16-fold increase of the 2010 value) by the year 2028 (10 years post-programme), through productivity gains in nectar standing crop capability. In addition to its high value honey crop, the NZ Mānuka shrub has significant sustainability benefits, for example, in erosion control for use in hill country remediation and vegetation restoration, improved water quality as a component of riparian plantings, and in carbon sequestration[2].

[1] https://www.mpi.govt.nz/

[2] www.mpi.govt.nz/funding-and-programmes/primary-growth-partnership/primary-growth-partnership-programmes/high-performance-manuka-plantations/

1.2 Plant Growth & Development

Growth and *development* changes across a plant's lifespan in response to internal and external cues are due to heritable factors. The *quality* and *quantity* of this *growth* and *development* is controlled by a combination of hereditary potential (genetics) and the environment, and it is the interaction between these that control the expression of all characters (Turner & Kramer, 1980; Figure 1.1).

The extent to which an individual genotype can be 'modified' by its environment is termed *phenotypic plasticity* (Bradshaw, 1965). That is, some characters (morphological or physiological) are more responsive to their immediate environment than others. An unresponsive character will remain relatively unchanged over a wide range of environments, and is described as lacking in plasticity or as *phenotypically constant*. Examples are water storage tissue and thick cuticles of succulents, and low osmotic potential and dehydration tolerance of many xerophytes (Turner & Kramer, 1980). In contrast, a character that is responsive to the environment is regarded as *phenotypically flexible,* or *phenotypically plastic*. For example, the reversible decrease in osmotic potential observed in mesophytes when water stressed, and the differences between sun and shade leaves (Turner & Kramer, 1980).

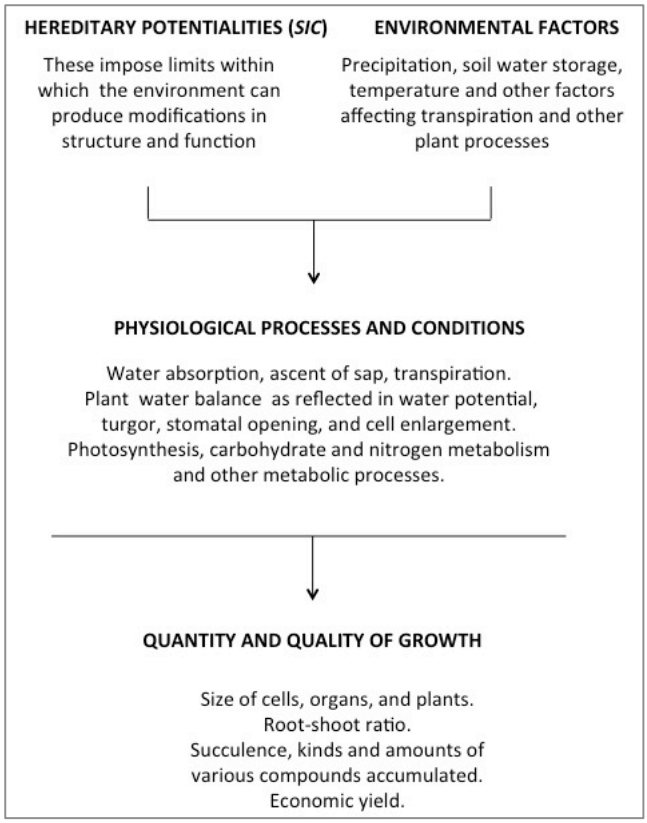

Figure 1.1 The quantity and quality of plant growth is controlled by an organism's hereditary potential and its environment (reproduced from Turner and Kramer, 1980; Copyright © 1980 by John Wiley & Sons, Inc.)

1.3 Plants Under Stress – A General Overview

Plants rarely attain their full genetic potential because of imposed environmental limitations especially unfavourable temperatures and insufficient water. There exist optimum conditions for plant growth and development as well as for individual metabolic pathways (e.g. photosynthesis) in plants. These optima differ between individuals depending on their genetic determination and habitat. Seldom do plants experience full optimal conditions, that is, it is rare for all environmental factors (e.g. temperature, mineral content, water supply, light intensity) to be in the optimum range for growth. Hence, day-to-day deviations from '*physiological optimality'* in any particular environment are quite normal. Plants are able to partially compensate for low stress events by the processes of *acclimation*, *adaptation,* and *repair mechanisms.* Strong or chronic stress events cause considerable damage that may lead to cell and even plant death.

Stress is limiting to plant growth and prolonged durations can have marked effects on vegetative and reproductive characteristics. The resulting productivity losses from the effects of stress are measured as reductions in *yield*. Stress may be environmental in origin (termed *'abiotic'* stress, e.g. cold, heat, wet soils, dry soils, alkaline soils, wind, freezing and salt exposure) or *biotic'* originating from the action of other living organisms (e.g. bacteria, viruses, fungi, parasites, beneficial and harmful insects, herbivores, weeds, and species competition from other cultivated or native plants). Often stresses occur together, for example, high temperature and drought or waterlogging and mineral toxicities. Combinations of stress are the most harmful to plants (Mittler, 2006).

Stress exists as a continuum in plants and was considered, by Lichtenthaler (1988) and others, to be dose-dependent. Plants respond quickly to changing environmental conditions by metabolic readjustments to alter photosynthetic rates, or respiration and transpiration rates. For example, changes in photon flux density (by sunlight and clouds), a decrease in temperature, or an increase in air humidity can act as re-occuring switches of cell metabolism and physiological activities. Lichtenthaler (1996) termed such responses *'fast acclimations'* and considered them not to be stress since they are a normal part of functioning in plants. Similarly, diurnal changes in cell metabolism and growth activities do not represent stress. In addition, plants respond to environmental changes not only by fast acclimation but by particular longer-term adaptations, for example leaf size and thickness, stomata density, and structure and function of chloroplasts. These adaptations typically take place within one to two days or one week at the most. With such adaptation responses plants can avoid the constraints of stress and adapt in an optimal way to new and changing growing conditions.

1.3.1 Stress Physiology (Resistance Mechanisms)

1.3.1.1 Adaptations

Adaptations are defined as '*heritable modifications in structures or functions that increase the probability of an organism surviving and reproducing in a particular environment'* (Turner & Kramer, 1980). While the term 'adaptation' implies a *heritable* modification, in the literature it is often used to describe both unstable and stable adaptations that result from short- or long-term stress effects respectively. For example, it may be used to describe the contribution of a character to the fitness of an organism in its current environment or to describe the evolutionary origins of a character. The dual understanding illustrates the different approaches to stress biology by ecologists and physiologists. In this study, 'adaptation' is used to describe heritable and 'acclimation' to describe non-heritable modifications (see below).

Adaptations by higher plants to resist periods of stress during their life cycles include three main 'strategies'[3] *escape* (also called *evasion*), *avoidance* or *tolerance.* The different 'strategies' are explained as: plants that *'escape'* stress do so by avoiding being subjected to the stress; plants that '*avoid'* stress avoid their tissues from being subjected to the stress even though the stress is present in the environment; and true '*tolerance'* exists at a biochemical or physiological level (Jones, 2014). These categories are not mutually exclusive and a particular plant may possess more than one category of adaptation. Quantifying adaptation is difficult because it is unlikely that any plant is in a perfect state of perfect adaptation to its environment since it is made up of a collection of ancestral characteristics and the process of adaptation is occurring continually (Jones, 1989).

[3] Definition of 'strategies' is '*genetically programmed series of responses that can favour survival of that genotype in a given environment*' (Jones, 1989).

Adaptations to *escape* or *evade* a stress are not considered a kind of resistance, but simply the ability to ensure reproduction when the stress is replaced by suitable growing conditions, i.e. able to complete the vegetative stage before the stress appears (Levitt, 1980). Because a plant cannot alter the environment external to itself, its only course of action is to either prevent or decrease penetration of the stress into its tissues (‘*Avoidance’*), or, to be resistant in spite of the stress entering its tissues provided that it can decrease or eliminate the strain (*‘Tolerance’*). Levitt further explains that ‘*tolerance mechanisms permit plants to maintain high metabolic activity under mild stress, and reduced activity under severe stress. Avoidance mechanisms permit plants to reduce autotrophic activity and become dormant in the face of extreme ‘stress’* (Levitt, 1980*).*

To explain the differences between the terms ‘*avoidance*’ and ‘*tolerance’,* Levitt (1980) used the concept of thermodynamic equilibriums. *Stress tolerance* is the ability to come to thermodynamic equilibrium with the stress without suffering injury (Levitt, 1980). A plant with *stress tolerance* has the ability to prevent, decrease or repair the injurious strain induced by the stress through tolerating the stress; it may either avoid or tolerate the strain. Thus even plastic (injurious) strain may be tolerated, provided that the injury is not irreversible and the plant possesses a repair mechanism capable of reversing it (Levitt, 1980). *Stress avoidance* is stress resistance by avoiding thermodynamic equilibrium with the stress. The plant with stress avoidance is able to exclude the stresses either partially or completely, either by means of a physical barrier which its insulates its living cells from the stress, or by a steady-state exclusion of the stress (a chemical or metabolic barrier). By avoiding the stress, it also avoids the strain (Levitt, 1980). Avoidance mechanisms include stomatal closure, leaf movement, stomatal closure, leaf movement and leaf shedding.

1.3.1.2 Acclimation

'Acclimation' also plays a role in plant performance under conditions of stress and differs from adaptation in that it is '*non-heritable' i.e.* not genetically determined. Acclimation or 'hardening' occurs during the life of a plant and confers some resistance by previous exposure to stress, for example, in drought tolerance, flood tolerance, cold hardiness, heat tolerance, stimulation of reproductive growth, breaking of dormancy, seed storage and seed dispersal. Emphasis In the literature has predominantly been on the harmful effects of environmental stresses on the growth of (woody) plants, and this has obscured some very beneficial effects of such (Kozlowski & Pallardy, 2002). It is well known, that by slowly increasing stresses in plants, often the induced physiological adjustments protect them from subsequent adverse responses that would occur if such stresses were imposed abruptly and continued for a long time. Indeed, Kozlowski & Pallardy (2002) claim that 'short judicious' exposures of plants to extremes of water supply, temperature and humidity, as well as some combinations of these, are essential for optimal plant development and/or protection of plants from subsequent environmental injury. However they emphasize that the proper timing of such exposures is crucial. Short exposures of woody plants to extreme environmental conditions at critical times in their development can often improve their growth.

1.3.2 Stress Responses in Plants

1.3.2.1 A common pathway

Analyses of stress responses in plants are complex, for example, most of the observed effects of water deficit are secondary resulting from specific plant regulatory responses (Jones, 2014). The causal responses by plants to stress vary depending on the plant species and it's evolutionary adaptations to selective (local) environmental pressures. Responses may be at the eco-physiological level (e.g. reduced leaf area, greater stomatal resistance to gas exchange), the biochemical level (e.g. solute and pH changes in cells, increased or decreased enzyme activity in cells) or the molecular

level (e.g. changes in gene expression in organelles). It is difficult to quantify the 'genetic potential' of plants and therefore to measure the impact of stress. Jones (1989) believed it unlikely that a common unit to quantify different stresses would be possible. He points out the difficulty in measuring stress responses in terms of yield (a 'genetically' complex trait) since this will depend on the severity of the stress, the time over which it is imposed and whether the plant can fully recover from the effects. Other considerations are the temporal and spatial variations of the stress, the plants' potential to acclimate to the stress and the genetic variation in stress responses (Osmond et al., 1987).

Plant hormones act as long-distance signalling molecules and are critically involved in the integration of plant responses to stress. For example, cytokinins and gibberellins are involved in response to low temperatures. Abscisic acid (ABA) is involved in dehydration responses during seed maturation. ABA is central to drought responses, but is also involved in integrating whole-plant responses to a wide range of other environmental stresses such as salinity and high temperatures (Wilkinson and Davies, 2002 cited in Jones 2014). It is a central regulator of plant adaptations to both biotic (e.g. wound or pathogen response) and abiotic stressors, (in particular dehydration, salinity and high/cold temperature). It has a protective role in the production of osmoprotective proteins and metabolites, and the regulation of stomatal conductance (Zhu, 2002). *De novo* synthesis of ABA during stressful conditions is responsible for stomatal closure, and acts as a protective mechanism against the potentially damaging effects of water loss. The regulation of stomatal aperture by ABA is rapid and reversible. Leaf epidermis peeled off in a single layer when floated on ABA solutions shows a stomatal closure response within minutes. The magnitude of opening or closing is dependent on the ABA concentration across a wide range. There appears to be no gene expression changes involved in this mechanism, it is purely a physiological

response i.e. change in aperture as a function of cell turgor and volume, involving the rapid movement of water and solutes out of the guard cells (Atwell et al., 1999). ABA synthesis in leaves is not the only process occurring during water stress. Studies are consistent with (ABA) being transported from droughted roots providing the root-sourced signal causing stomata to close. Root-derived ABA causing reduced stomatal conductance during periods of water-deficit is true in many cases, but not all. For example in apricot, ABA levels in leaves are positively correlated with stomatal conductance. This is contrary to normal expectations since apricot is considered a drought tolerant species and is often grown alongside grapevine, which adheres to the normal model as a drought tolerant species. In apricot then, osmotic adjustment provides a protection mechanism during drought and ABA appears to play little part. Apricot is a species which can osmoregulate and thereby maintain stomatal opening (Loveys et al., 1987).

Zhu (2002) discussed stress-signalling pathways involved in cellular homeostasis for ionic and osmotic stress, and growth regulation and detoxification pathways as a result of injury *(a 'derived'* stress), and the interactions between all three. He considered that while some of the observed changes may clearly be adaptive, some might simply just be the '*pathological consequence of stress injury'*. He considered that knowledge of the active signalling pathways to activate these pathological responses is also important in drought response studies. Major advances in recent years have improved our understanding of ABA metabolism and signal transduction (Zhu, 2002) and the ABA receptor identified (Klinger et al., 2010).

1.3.2.2 Plant Physiological Responses to Drought

Of all the environmental factors that limit plant growth, drought or water deficit[4] is the *most* limiting on plant performance (Boyer, 1982). For example, even in relatively moist climates such as that of Southern England, barley crop yield losses due to drought (water stress) are lowered by an average of 10 to 15% each year, while the yields of more sensitive crops such as salad greens and potatoes may be reduced even further if not irrigated (Jones, 2014). The commonly used terminology for 'drought' is '*an environmental stress of sufficient duration to produce a plant water deficit or stress which in turn causes disturbance of physiological processes'* (Turner & Kramer, 1980). Plant water stress may also occur in situations where transpiration is excessive or water absorption is inhibited by for example, cold soil, an excess of salt in the soil solution, deficient aeration, or injury to roots.

At the whole plant level, water deficiency may have a strong effect on shoot growth, biomass production, and patterns of dry matter distribution. Water stressed plants achieve maximum water absorption by partitioning more carbon assimilates into the root system (e.g. deeper rooting tap roots) and minimising the loss of water from above ground plant parts (e.g.stomatal closure and reduced transpiration). Plant growth is often more inhibited than root growth when the soil water supply is limited because of exposure to the dehydrating effects of the aerial environment (Sharp and Davies, 1989). This can lead to large increases in root to shoot ratios.

[4] It is important to note that 'drought' is a field or environmental term. Under controlled studies such as in a glasshouse or in a growth cabinet, its equivalent is 'water deficit'.

1.3.2.3 Temperature Influences on Plant growth

Temperature is one of the most important components of the plant environment. It has a major impact on the functioning and distribution of plant species, and their ability to adjust to temperature fluctuations within and beyond (stress) the ambient range often limits their geographic distribution (Walther, 2003). Plants experience considerable (temporal) variations in temperature within a growing-environment, both on a daily basis, i.e. short-term or 'momentary' changes (intermittent cloud cover), and annually i.e. 'seasonal' changes (e.g. spring warming). There may also be diurnal differences in day and night time temperatures (temperature cycles).

Temperature varies with latitude, altitude, size of and position in a landmass, atmospheric conditions and local topography. Regional variations in mean air temperature may seem slight, yet be biologically significant. For example, a 1 °C increase in a northern temperate climate may be expected to increase plant productivity of the natural vegetation by around 10%, provided all other factors (water and nutrients) are non-limiting (Grace, 1988). Sometimes for annual crop species, a negative temperature effect on dry matter content has been observed due to faster maturation i.e. reduced crop duration (Monteith, 1981).

Optimal growth temperatures vary among species, with rates of dry matter production decreasing above and below the optimum. Extremes of temperature can cause production to cease altogether (Grace, 1988). According to Porter and Gawith (1999), plants seem to respond to absolute rather than relative changes in temperature, that is, there are discontinuous threshold responses to temperature unlike those to water. Temperature influences plant growth at many points in a growth model. Rates of many plant developmental processes, and hence the timing of phenological stages, are strongly temperature dependent. Even small changes in temperature can have marked effects. For example, a 1°C temperature shift elicited responses in cytosolic calcium

levels in plant cells (Knight & Knight, 2000). 1°C to 2°C shifts were sufficient to completely inhibit seed germination in lettuce (Argyris et al., 2011), and the circadian clock is sufficiently entrained by a 4°C diurnal temperature cycle in Arabidopsis (McClung et al., 2002). Each vital developmental process is adjusted to a certain temperature range. For optimal growth to be achieved the diverse processes involved in metabolism and development must be 'harmoniously attuned' to each other. Thus temperature has both an indirect influence on growth and development due to its quantitative effect on energy supply (from basal metabolism and biosynthesis), and a direct effect via regulatory processes.

1.4 Genotype-By-Environment Interactions (GEI)

Genotype-by-environment interaction (GEI) results from different genotypes responding to environmental variation in different ways. For example, some genotypes may perform well across a wide range of environmental conditions while others may perform well in only in a subset of environments. That is, the relative performance of different cultivars depends on the environment in which they are grown. Dickerson (1962) defined GEI as the "*additional variation caused by the joint effects of genotypes and environments not predictable from their separate average effects".* Much scientific inference is conditional because of GEI. G x E interactions are extremely important in the development and evaluation of new plant varieties because they reduce genotypic stability under diverse environments. However, a significant G x E interaction for a quantitative trait, such as grain yield, can seriously limit the efforts of selecting superior genotypes for improved cultivar development. Understanding the GEI relationship is important if plant breeders and agronomists are to target germplasm better suited (adapted) to different production environments.

1.5 Mānuka, the Plant

1.5.1 Ecological Significance of *L. scoparium*

Leptospermum scoparium (J.R. Forst. et G. Forst.), common name Mānuka, is considered the most widely spread and economically relevant indigenous shrub species in New Zealand (Stephens et al., 2005; Figure 1.1). In the wild, Mānuka can be found growing mainly on infertile and poorly drained soils. Early research placed *L.scoparium* ecologically as a permanent dominant species of habitats of extreme environmental stress (swamplands, dry rocks, river beds, cold subalpine moorlands and coastal sand hills); or as a seral species of disturbed habitats (recently cleared forest) (Cockayne, 1910; Stephens et al., 2005).

Figure 1.2 Mānuka growing in the wild. Photo kindly supplied by Alastair Robertson of Massey University and reproduced with permission.

1.5.2 Habitat Range

In NZ, wild populations of Mānuka extend throughout both the North and the South islands, and also, to the Three Kings Islands in the north (latitude 34° 10'S) and to Stewart Island in the south (47°S), and eastwards to the Chatham Islands. *L.scoparium* occurs from sea level in coastal situations to near the regional tree line at a maximum altitude of around 1100 metres. Permanent dominance occurs on sites that are unfavourable for climax forest development as they are too wet, dry, cold, exposed, infertile, or unstable, and across a range of soils. For example, leached infertile clays of the Northland gumlands with perched water tables and sand podzols; edaphically dry-pumice on the central North Island Volcanic Plateau; permanently wet Waikato oligotrophic lowland mires (bogs); Westland pakihi soils; the lowland swamps of Westland and Fiordland; and Southern lake shorelines (Stephens et al., 2005).

1.5.3 Taxonomy

L. scoparium is the only indigenous member of the *Leptospermum* genus in New Zealand (Stephens et al., 2005). There are two recognised variants[5] one non-endemic [*L. s* var. *scoparium;* also found in mainland Australia from the south coast of New South Wales to western Victoria, and widespread in Tasmania (Thompson, 1989)], and one endemic (*L. s* var. *incanum)*[6] to the more northern regions of NZ (Northland; zone 1 of Williams et al, 2014). *L. scoparium* belongs to the Myrtle family (Myrtaceae); other well-known family members include, *Eucalyptus, Melaleuca,* the NZ Pohutukawa *(Metrosideros excelsa),* the common bay tree, and Feijoa (*Feijoa sellowiana).* Of the 140 genera represented worldwide, the family is most developed in Australia where it is suggested that members evolved in response to seasonal droughts and the poor nutrient status of many Australian soils (Beardsell *et al.,* 1993).

[5] Botanical varieties
[6](http://www.nzflora.info/factsheet/Taxon/Leptospermum-scoparium.html).

Classification of *L. scoparium* has been difficult because of a 'diversity of forms'. Cockayne was the first to note this in his 1919 publication; other authors have described *L.scoparium* as a 'variable' species (Stephens et al., 2005). Much of the intraspecific variation is in leaf characteristics and growth habit. Several taxonomic reviews at the family, genus and species levels have been undertaken, however the consensus among biologists currently is that a taxonomic review is urgently needed. Thompson (1989) placed *L.scoparium* in the subgroup *L. myrtifolium*, along with 12 other species. A common feature of this subgroup is an ability to survive periods of low water-availability. Thompson noted that most possessed "rather thick leaves". In a study of the leaf anatomy of 40 species of the *Leptospermum* genus, Johnson (1980) showed *L. scoparium* to have the xeromorphic structure typical of the genus. Variation occurred in stomatal type, sculpturing of the cuticle, persistence of the indumentum and structure of the oil cavities.

1.5.4 Phenotypic Plasticity

There is much intra-specific variation in the morphological and physiological characteristics of Mānuka. Variability in leaf shape and size, flowering phenology and plant form result from habitat-modification and genetic variation (Allan, 1961; Ronghua, et al., 1984; Stephens et al., 2005; Thompson, 1989). In populations from a wide range of geographical regions within New Zealand, significant correlations were made between leaf morphology and the environmental factors of latitude, distance from coast, and annual and winter temperatures (Ronghua et al., 1984). A more detailed account is given in the Introduction of Chapter 3 (Section 3.1).

1.5.5 Floral Characteristics

Flowers may be perfect or imperfect on the same plant i.e. andromonoecious. The perfect flowers (having functional male and female parts) tend to open in the first flush of flowering, followed by the staminate (male) flowers. Andromonoecy in the Myrtaceae is believed to have arisen in response to low fertility soils and to drought, allowing optimal resource allocation for reproduction (Beardsell et al., 1993). Variation in the proportion of male flowers on plants between bushes, years and over a flowering season, (Andersen, 1990; Beardsell et al., 1993; Primack & Lloyd, 1980) is believed to be mostly environmental (Primack & Lloyd, 1980). Flowers are simple (Thompson, 1989), commonly white, rarely pink or red, and are axillary or occasionally terminal on branchlets, usually solitary and sessile, 8 – 12 mm in diameter, occurring from October to February (Stephens et al., 2005). Individual flowers last from one to three weeks. For a detailed description of the floral structure of Mānuka flowers, readers are referred to (Thompson, 1989). Flowers are produced '*in profusion'* near the tips of branches and shoots for optimum display to attract insects that are presumed attracted to the floral nectar (Beardsell et al., 1993). A brief review of the literature regarding what is known about *flowering time* in wild Mānuka populations is presented in Section 4.1, that is, in the Introduction of Chapter 4.

1.6 Mānuka The Story

Medical grade manuka honey is currently widely used in wound care and as a topical antibacterial, antifungal and anti-inflammatory, providing an alternative to conventional antibiotics (Saikaly & Khachemoune, 2017; Noori, Khelod, & Ahmad, 2011). To date, no resistance by common hospital bacteria to honey has been attainable in the laboratory (Johnston et al., 2018; Liu et al., 2015; Lu et al., 2013). The use of honey as a therapeutic substance has a long history and extends into the area of folklore. The first (apparent) report of the antibacterial activity of honey was by Van Ketel in 1892 [cited in Dustmann, (1937)]. Readers are referred to articles by the late Professor Molan associated with the University of Waikato, NZ, for an extensive review of historical honey research including that of manuka (Molan, 1992a & b; Molan, 1995).

1.6.1 Pronounced Antibacterial Activity of Manuka Honeys

There exist only a few naturally occurring substances with antimicrobial action e.g. hydrogen peroxide, ozone and phenol. The antimicrobial (Ab) activity of most generic honeys (first demonstrated by Dold et al. in 1937) is due to the enzymatic production of hydrogen peroxide (HP) by the enzyme glucose oxidase in honey (White et al., 1963) which originates from the salivary glands of bees (Gauhe, 1940 and cited in White et al., 1963). However, honeys sourced from the floral nectar of the NZ Mānuka shrub (*L. scoparium* and some Australian species most notably *L. polygalifolium)* exhibit significantly higher levels of Ab activity (equivalent to 15 -30% w/v phenol) than other honey types (Russell et al., 1990; Allen et al., 1991; Molan 1992b; Windsor et al., 2012) This additional Ab activity is not attributed to HP since activity remains (in some but not all manuka honeys) after removal of HP from diluted honey by the enzyme catalase, hence referred to as 'non-peroxide activity' (NPA; Adcock, 1962; Weston, 2000). This *in vitro* broad-spectrum antibacterial activity of Mānuka honeys is attributed to the carbohydrate metabolite methylglyoxal (MGO). The level of NPA is directly correlated with the MGO content or the UMF[7] of the honey (Mavric et al., 2008).

[7] The UMF® or Unique Manuka Factor is an industry potency rating of bioactivity based on inhibition tests in *Staphylococcus aureus* and is directly correlated to the MGO content of honey.

1.6.2 Variable Bioactivity of NZ Manuka Honeys

The level of bioactivity (and MGO) of NZ manuka honeys has been shown to vary widely (Allen et al., 1991). This variability has been attributed to varietal and environmental differences in the regional populations of *L.scoparium* (Stephens, 2006). Traditionally, the most northern regions of NZ (Northland, Waikato, and some parts of Coromandel) produce better quality Mānuka honeys, that is, with higher grade UMF®. Monofloral manuka honeys sourced from the geographically distinct regions of Northland and Waikato exhibited high non-peroxide antibacterial activity, for example, mean UMF ® (unadjusted for dilution by other honey types) of 14.0 and 14.9 compared with values of 9.1, 8.8, 10.4, 8.7, 6.9 and 10.9 for the Coromandel, East Coast, Gisborne, Taranaki, Wairarapa and the West Coast regions respectively (Stephens, 2006).

1.6.3 DHA is a Direct Precursor to MGO Formation in Honey

Dihydroxyacetone (DHA), a three-carbon sugar, was shown to be a direct precursor to MGO formation in manuka honeys (Adams et al., 2009). DHA coverts to MGO during the storage of honey in a non-enzymatic maturation process. The DHA content of fresh and stored honeys is variable, with the amount of MGO in mature honey ultimately determined by its DHA content (Adams et al., 2009; Atrott et al., 2012). Conversion of DHA to MGO in fresh manuka honey is an irreversible first order chemical dehydration reaction (Grainger et al., 2016). From unpublished data, storage conditions by the apiarist strongly influence the efficiency of the conversion of dihydroxyacetone to methylglyoxal in maturing manuka honey and hence the final UMF® value (Williams et al., 2014).

1.6.4 *In-Planta* DHA

DHA is present in the nectar of Mānuka flowers in varying amounts, and also at high levels in fresh manuka honey. In contrast, MGO is not detectable in Mānuka floral nectar [although see Stephens et al., (2010)] and is present at only relatively low levels in fresh manuka honeys (Adams et al., 2009; Atrott et al., 2012). The trace amounts of MGO in Mānuka nectar reported by Stephens (2010) are believed to be an artefact of nectar storage (Grainger et al., 2016). Historically, the presence of both plant- and bee-derived components in honey has added to the complexity of the origins of MGO and its precursor DHA. DHA is present in Mānuka nectar in varying amounts () and the reasons for this variability are as yet unknown.

Inter-plant variation in the amount of DHA in Mānuka nectar was first reported by Adams et al., (2009) in a small study of trees in Northland & central Waikato. They proposed that high-producing DHA trees should yield honeys with high NPA since typically, high ratios of DHA to nectar sugars in young honey result in high non-peroxide antibacterial activity or UMF® in mature honey. Preliminary studies by Williams et al. (2014) reported both *inter-* and *intra*-regional and annual variation in the DHA to total sugar ratio (DHA/TS) of nectars from *Leptospermum spp.* (*L. s*coparium var. *scoparium, L. scoparium* var. *incanum*, some horticultural cultivars of *L. scoparium,* and some Australian species). Regional variability in DHA/TS in that study aligned with that reported for UMF® by Stephens (2006; see Section 1.8.2) for two of the regions (Coromandel and East Cape) but not for another three (Northland, Wairarapa, and northern South Island), and was lower than expected for one other region (Waikato). Soil composition (order and quantifiable components) was not a

significant factor in determining the DHA content in the study of Williams et al., (2014), in agreement with the more recently published work of Nickless et al., (2017). There were also no apparent trends in DHA/TS relating to botanical variety (*L. scoparium* var. *scoparium* and *L. s.* var. *incanum*). A more detailed account is given in the Introduction to Chapter 5 (Section 5.1). Williams et al., (2014) also reported flower gender and age differences in the DHA/TSugar ratios, for example, male flowers had significantly higher DHA/TSugar due to elevated levels of dihydroxyacetone. Both dihydroxyacetone and total sugar values were higher in flowers with red hypanthia, an indicator of floral age. Factors such as the composition of leaf oil, soil composition, and sooty mould coverage that may relate to DHA/TSugar were also discussed. No correlations were found for the last two, however some degree of correlation occurred between leaf oil and nectar DHA/TSugar in some regions (East Cape and Wairarapa regions).

1.6.5 Origins of DHA in Floral Nectar of Manuka

DHA is a monosaccharide (ketotriose or a three carbon sugar) and an intermediate of carbohydrate metabolism in plants as part of the glycolysis and gluconeogenesis pathway via the reversible isomerisation of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. The reaction is catalysed by the enzyme triose phosphate isomerase. A similar pathway was proposed for sugar metabolism in floral nectaries (Wenzler et al., 2008; Figure 1.3). To date however, the origins of DHA in floral nectar of Mānuka are unknown.

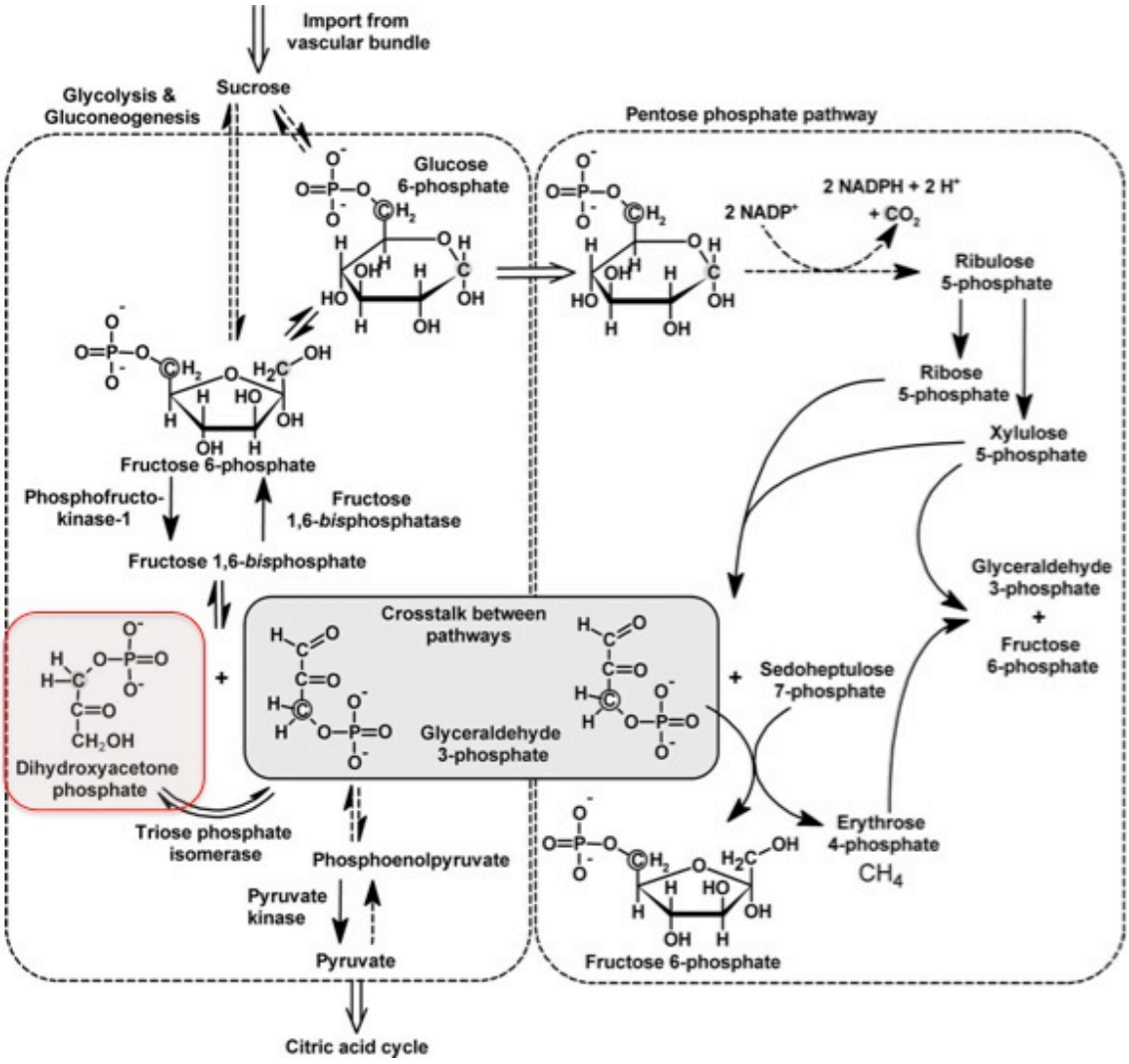

Figure 1.3 Proposed pathway for sugar metabolism in floral nectaries (reproduced from Wenzler et al., 2008). The red region indicates the intermediate dihydroxyacetone-phosphate.

Two possible hypotheses for the origin of DHA have been proposed in the literature, endogenous plant factors (Adams et al., 2009) and exogenous microbial activity (Williams et al., 2014). Adams et al., (2009) were the first to propose that soil and climatic conditions, often experienced by Mānuka growing in marginal land areas, may be contributing to high stress levels in plants and that plant effects were being transferred to honey. The biological significance of the 'shunt' or MGO detoxification pathway[8] in plants under stress was only just beginning to be explored at that time.

[8] Detoxification of MGO by the catalyzing enzymes glyoxalase I and glyoxalase II to D-lactate using reduced glutathione (GSH) as the cofactor and in the process GSH is recycled back.

This was also before DHA was known to be the precursor to MGO formation in honey and nectar-derived. Adams et al., (2009) based their hypothesis on studies by Yadav et al. (2005) that showed two- to six-fold increases (form base levels of 40 – 75 to 75 -200 μM) in MGO levels in roots and leaves in two monocotyledonous species, *Oryza sativa* and *Pennisetum glaucum* and two dicotyledonous species *Nicotiana tabacum* and *Brassica juncea*, in response to salinity, drought and cold stress. More recent studies have confirmed that MGO accumulation is a common response to abiotic stress in plants. It is believed to act as a signal molecule (Hossain et al., 2009), a function shown in yeast (*Saccharomyces cerevisiae*; Maeta et al., 2005). MGO has cytotoxic effects hence it needs to be tightly regulated in cells. Further support for the hypothesis is that microclimatic factors are generally known to influence nectar composition (Nicolson et al., 2007). The effect of soils however, as a contributor to high levels of DHA in *Leptospermum spp.,* was ruled out by two later studies (Williams et al., 2014 and Nickless et al., 2017). In the alternative hypothesis, Williams et al. (2014) proposed that microorganisms, specifically yeasts, introduced by visiting pollinators, for example ants, may be responsible for high DHA (> 0.002 mg/mg). The basis for their proposal is the predominance of fructose in nectars of *Leptospermum spp.* that may indicate the effect of a microorganism, specifically yeast, transported by visiting insects such as ants.

In a very recent study, Smallfield et al. (2018) reported that an absence of DHA from phloem exudate suggests that DHA biosynthesis is closely linked to nectar production in floral tissues. It was also recently reported by another group (Clearwater et al., 2018) that while temperature (in the previous 24 hour period) influenced nectar yield, water stress did not. Soil chemistry had no influence on nectar production or composition in individuals of wild or cultivated varieties (Nickless et al., 2017; Williams et al., 2014).

1.7 Overview of Nectar

1.7.1 Nectary Structure and Function

Nectar is the aqueous, sugar-rich reward offered by plants to attract potential pollinators (birds, bats, bees, moths, flies, ants etc.). That is, there exists a complex plant-pollinator interaction that is mediated by the floral nectar (Heil, 2011). Thus, the primary function of nectaries (specialised tissues that secrete nectar) is considered by some to be an ecological one, and not physiological as first thought (see Lorch, 1978; De la Barrera and Nobel, 2004). Nectary localisation, morphology and secretory mechanisms are extremely diverse among plant species and differences can even occur within the same plant (Nicolson et al., 2007; Heil, 2011). Early work by Shuel (1956) showed nectaries to be (real) glands with an active metabolism evidenced by the presence of metabolic enzymes in nectar. For example, invertase is involved in the post-secretory modification of nectar sugars (Nepi et al. 2012). Additionally, nectar sugar volume and concentration can be separately regulated by the nectary (Nepi & Stpiczynska, 2008).

1.7.2 Nectar production

Nectar production may use up to 37% of a plant's available energy (Pleasant & Chaplin, 1983; Southwick, 1984) and may entail a cost to the plant in terms of growth and/or reproduction. Nectar production is a dynamic process involving many tissues simultaneously. The processes of nectar secretion, cessation and reabsorption follow temporal patterns that are usually linked to the foraging behaviour of pollinators (Pacini & Nepi, 2007). Nectar production is strongly affected by microclimate (air temperature, air humidity, evaporation, wind, water balance), physical and chemical soil properties (structure, composition, fertility, moisture and acidity), and irradiance (altitude, latitude; Percival, 1965; Petanidou, 1999; Jaric et al., 2010). The volume and concentration of nectars depend on ambient humidity and temperature, selective reabsorption of solutes

or water, and changes in the concentration at which nectar is secreted (Nicolson, Nepi, & Pacini, 2007). Post-secretory changes to sugar concentrations are dependent largely on physico-chemical and microclimatic effects and to a smaller extent on nectar composition (Corbet et al., 1979).

Nectar secretion describes the release of nectar from the protoplasm of the nectary parenchyma cells i.e. at a cellular level. In contrast, *nectar production* relates to the nectary as a whole, comprising sugar unloading from the vascular bundle, transport of molecules into the nectary tissue, transformation of molecules, and nectar release from the nectary leading to nectar release (or exudation) from the nectary (Nepi, 2007). Endogenous rhythms for secretion are species-specific and depend on the characteristics of flowers and nectar e.g., size, shape, and position of flowers, timing and length of flower lifespan, flower development phase, beginning and duration of anthesis. Variability in the volume, concentration and composition of nectar, within or among plants i.e. at the flower, plant, species and population levels, may result from the interaction between the plant's pattern of nectar secretion and the pollinator's foraging strategies (Nicolson et al., 2007). Nectar secretion varies with flower age. Secretion volumes are greatest at the beginning of a flower's life and decrease with flower age (Fahn, 1949; Percival, 1965). The effects of relative humidity on nectar are primarily connected with flower shape (Percival, 1965). Tubular flowers in which the nectar is largely protected from the external environment, are unaffected by changes in ambient relative humidity. Conversely in shallow flowers, changes in relative humidity, rain and air movement may affect the concentration and this may markedly alter the pollination potential of the flowers.

1.7.3 Nectar Composition

Nectar is composed primarily of sugars, predominantly sucrose (a disaccharide) and its breakdown products glucose and fructose (hexose monosaccharides), in varying amounts. Nectar may also contain much smaller quantities of sugars such as raffinose, maltose, melibiose, trehalose (in *Carex*), and melezitose, together with small amounts of organic acids, ethereal oils, polysaccharides (i.e. dextrin), protein and enzymes, boron (in *Nymphaea)* and alkaloids. Nectar type tends to be family specific with closely related families often having the same kind of nectar. Nectar sugars may occasionally vary within a species but sugar proportions have been found to be comparatively stable (or "remarkably constant" Percival, 1965) for most species (Galetto & Bernardello, 2003; Wykes 1952 cited in Percival, 1965). The proportions of sucrose to glucose and fructose vary from an almost pure solution to a differently balanced combination. The presence and activity of enzymes in nectar may explain inter-intra specific variation in composition (Zimmerman, 1953, 1954 cited in Nicolson & Thornburg, 2007). All of the components of nectar may be important for maintaining pollinators. Nectar constituents (quality) have been shown to be strongly correlated with pollinator preference (Sturmann, 2004).

Secondary metabolites in nectars have an important function in repelling less specialised or illegitimate visitors (such as robbers or pathogens; Brandenburg et al., 2009). However, they may also regulate the length of time a pollinator visits and as a consequence the number of plants visited (Kessler & Baldwin 2007; Irwin & Adler, 2008; Kessler et al., 2008). Nectar sugars (carbohydrates) may be derived directly from photosynthesis (by active nectary chloroplasts in some species) or more usually from storage material such as starch grains in nectary parenchyma tissue (Pacini et al., 2003; De la Barrera and Nobel, 2004).

1.7.4 Nectar Research

As a whole, nectar is an under-studied area of plant research because of its complex physiology, complex polygenetic structure and strong environmental variability. For example, there are few described genes relating to nectar production; the role of hormones in floral nectar production has been investigated in only a few studies; little is known about the non-carbohydrate components of nectar; and even less is known about nectary development, synthesis of nectar components, and the regulation of secretion. What is known about nectar, is that both endogenous and exogenous factors affect the qualitative (composition) and quantitative (concentration, volume, secretion) characteristics of nectar (Jaric *et al*. 2010; Farkas *et al.* 2012).

The potentiality for nectar production is hereditary i.e. genetically determined. Whether it is fulfilled depends on the plant's environment which is composed of climatic and soil factors (Percival, 1965). For example, it is often observed by beekeepers that a species may heavily secrete nectar in one part of a geographic range but lightly in another. The reasons for this are unknown.

GEI for nectar production has been studied in relatively few species only. For example, extensively in the crop species, alfalfa (*Medicago sativa),* and in several herbaceous perennials (*Epilobium canum, Echium vulgare, Ipomopsis aggregata,* and *Campanula rapunculoides).* Of these studies, only two examined the influence of temperature on nectar traits (Vogler et al., 1999; Walker et al.,1974); most were concerned with the effects of changes in soil moisture.

1.8 Gaps in existing knowledge

- The reasons for the observed regional and seasonal variation in the amount of DHA in nectars of *L. scoparium* are as yet unexplained.

- At the time of starting this study, the relative (quantifiable) influences of G, E and GEI on nectar characteristics in *L. scoparium* were unknown since there were, to the author's knowledge, no published G x E nectar yield studies for *L. scoparium*.

In an attempt to explore the G x E interactions on nectar characteristics of selected inter/intra-specific cultivars *of Leptospermum scoparium,* it was necessary to separate the external environmental from the internal plant effects. Therefore, the use of clones to eliminate genetic variation within the genotypes was key to this study. By comparing the variability in the responses of the genotypes (expressing low, medium, and high amounts of nectar DHA) to the imposed temperature, light and soil water treatments, inferences about the source of the phenotypic variation were made, aided by statistical modelling. The use of controlled environments (glasshouses and growth rooms) allowed the manipulation of a single environmental factor to the exclusion (ideally) of others. This study will contribute toward a greater understanding of the relative influences of genotype and environment on nectar quantity and quality in *L. scoparium*.

1.9 Research Objectives

A main objective of this study was to assess the effects of the environmental factors of ambient temperature, light, and soil moisture on growth, flowering, and nectar quantity and quality in selected lines of *L.scoparium* expressing varying levels of nectar DHA.

1.10 Thesis Layout

Following this chapter, General Materials and Methods are presented in Chapter Two. Chapter Three examines long-tem temperature effects on aspects of growth, Chapter Four reports on long-term temperature effects on the flowering process, and Chapter 5 examines both short and long-term temperature effects on nectar production. Finally, a General Discussion is presented in Chapter Six of combined findings and their scientific and commercial implications. Each chapter is presented with Abstract, Introduction, Materials & Methods, Results, Discussion, Conclusions, and Reference sections, together with Appendices, and in that order.

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Chapter 2 General Materials & Methods

2.1 Experimental Design Overview (Experiments #1 - #4)

Four controlled environment experiments were attempted in this study. Experiments #1 & #2 were temperature experiments of short- and long-term durations [48 hours (h) and 12 months respectively]. Experiments #3 & #4 were water-deficit experiments carried out in the spring and autumn respectively. All experiments were pot-based using clonal plant material and conducted in glasshouses or growth rooms. Results from Experiments #1, 2 & 4 only are reported in this thesis. Experiment #3 was discontinued (see Section 2.4. below). Growth, flowering and nectar aspects of Experiment #2 are covered in separate chapters (Chapters 3, 4 & 5 respectively). The nectar aspects only of Experiments #1 & #4 are also covered in Chapter 5. Further details of experimental design and treatment structure are documented below for each experiment.

2.2 Short-term Temperature Experiment (Experiment #1)

Experiment #1 was carried out in October (spring) of 2015 using one-year-old plants in flower, and was conducted in temperature-controlled rooms (CTRs). In a crossover experiment (two-period/two treatment) eight replicated clones in full bloom (at *peak flowering*) from three clonal lines (Green, Orange, and Black; see Section 2.5.1 for the basis of line selection) were placed in one of two CTRs in a randomised block design (Figure 2.1).

Figure 2.1 The short-term (48 h) Cool (15/10°C D/N) and Warm (25/15°C D/N) temperature treatments were administered to clones in peak flower in controlled temperature rooms under artificial lighting (187 µmol m^{-2} s^{-1} output at flower height) and with a 12 h photoperiod. In Run 1 treatments are as labelled; in Run 2 the temperature treatments were switched i.e. a room-crossover experimental design.

The treatment structures were regimes of Cool followed by Warm (Sequence 1; Table 2.1), and Warm followed by Cool (Sequence 2). The individual treatments (Runs) were administered for a period of 48 h. The temperature regimes were 25/15°C day/night (D/N) in Warm and 15/10°C D/N in Cool. The photoperiod was 12 h under fluorescent lights (an average light intensity of 187 µmol m^{-2} s^{-1} at the top of plants near to where flowers were sampled from) and 102 µmol m^{-2} s^{-1} at the base of plants. An eight-day window in time separated Run 1 from Run 2. For this 8-day period, the clones were returned to the ventilated glasshouse from which they originated in mild temperatures close to ambient. The same clones were then returned to their same positions in the CTRs and temperature treatments in the rooms were switched (Table 2.1). A blocking structure was used to account for any local position effects in the CTRs. The nectar sampling method is explained in Section 2.6.

Table 2.1 Treatment structure (2 period/2 treatment) for the short-term crossover temperature experiment (Experiment #1). The set of sequences for applying the two treatments 'Cool' (D/N = 15/10°C) and 'Warm' (D/N = 25/15°C) to the experimental units are as shown. CTR 3,5 are Controlled Temperature Rooms 3 and 5 respectively, at the Massey University Palmerston North Plant Growth Unit.

	Time/Run	
Sequence	1	2
1 (CTR 3)	Cool	Warm
2 (CTR 5)	Warm	Cool

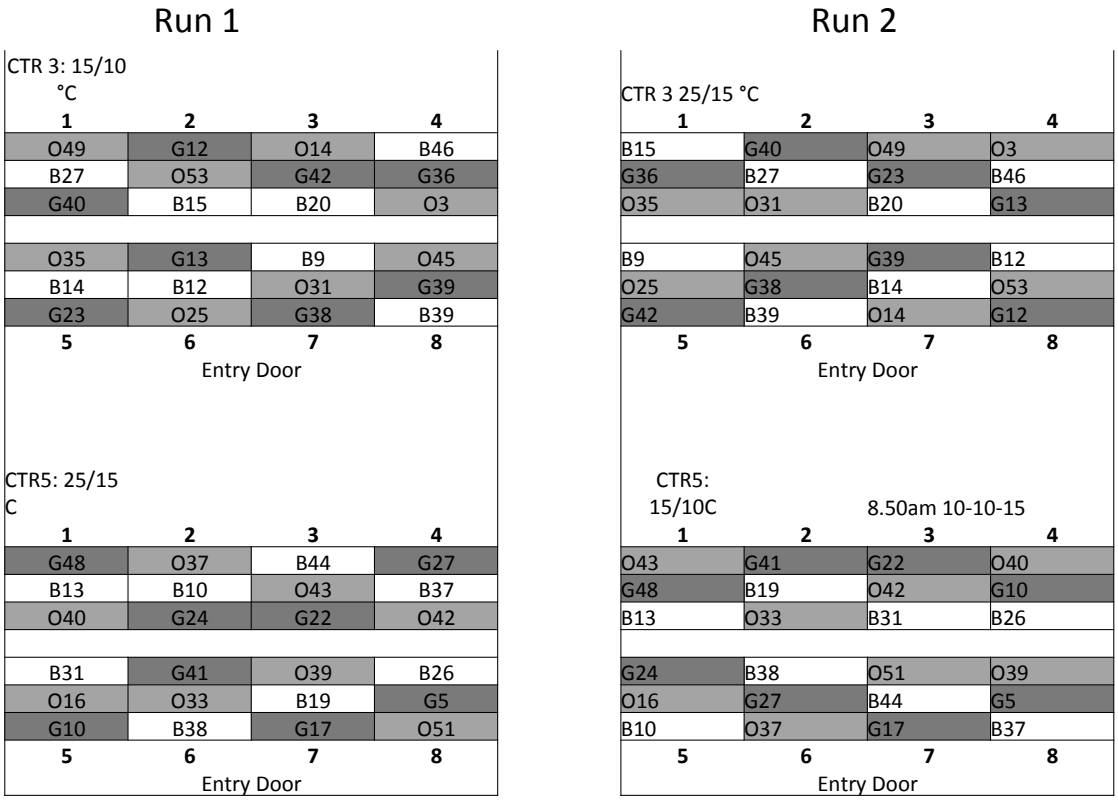

Figure 2.2 Short-term Temperature Experiment #1 layout showing experimental and treatment structures. Clones were blocked (#1 –8 blocks) in CTR '3' and '5'. Each clone was individually coded e.g. G48 was clone #48 from line Green.

2.3 Long-term Temperature Experiment (Experiment #2)

Experiment #2 was a long-term temperature trial conducted in the 2015/16 growing season (from the 3rd of November 2015 to the 3rd of November 2016) on plants in their second year of growth and flowering. This trial was conducted in glasshouses. Healthy clones not previously used in other experiments were selected from the same three clonal lines used in Experiment #1 i.e. Green, Orange, and Black, representing low, medium and high nectar DHA producing lines respectively. The lines were selected on a similar basis to that experiment (refer to Section 2.5.1). Individuals of similar 'size' were selected from the initial plant measurements (just prior to Day zero) of main-axis stem length (L) and basal stem diameter (BSD). Within a line, plants were paired for size and randomly assigned to one of two glasshouse-growing environments, that is, 'Cool' and 'Warm' temperature regimes under conditions of natural light and day length.

The two glasshouses, situated approximately 10 metres apart, were identical in construction, floor area, aspect, heating (hot water circulating from a central boiler), and cooling systems (staged fans; Figure 2.3). To achieve a significant temperature difference between the Cool and Warm glasshouses, the night time target temperatures were above and below a threshold of around 10°C (in Warm and Cool respectively), that is, a known physiological threshold for Mānuka growth (Zeislin & Gottesman, 1986). To achieve this, the minimum set temperatures (single thresholds so that heating would generally trigger at night) were 5 –7.5°C (heating ON and OFF resp.) in Cool, and 17.5 –19.5°C in Warm, year-round. As an attempt to control the upper temperature limits in the glasshouses and to maintain a year-round temperature differential, the cooling fans were set to turn on at 18°C and 18.5°C for Fans 1 & 2 in Cool, and 29°C and 30°C for Fans 1 & 2 in Warm. In addition to the standard fans and vents in the cooler glasshouse, an evaporative cooling system (water cascading over paper foils) was used to maintain a temperature differential between the glasshouses during the warmest months of the year (December through to March). A small mixing fan was running continuously at ceiling height in each of the glasshouses to achieve a

uniform air temperature. Treatments were administered for one complete cycle of growth and flowering i.e. a full 12-month period. The experimental design was a two-way factorial (two treatments x three genotypes) in a randomised block design. The experimental units were blocks (rows and columns) based on initial plant size (the largest assigned to Block 1 and the smallest to Block 8; Figure 2.4). Experimental units consisted of one specimen from each of the three clonal lines.

Figure 2.3 The two experimental glasshouses with 'Warm' and 'Cool' temperature environments (left & right images resp.) were located at the Massey Plant Growth Unit, situated approx. 10m apart, and identical in size and aspect.

Temperature and % relative humidity (RH, defined as the amount of water vapour present in air expressed as a percentage of the amount needed for saturation at the same temperature) data were collected using two electronic data loggers (HortPlus High Capacity Minilogger) in each glasshouse, one located on the North-side (air vent side) and one on the South-side (fan side), at plant height. Data loggers were suspended in custom-built (Lindsay Sylva, Massey University) wooden boxes to avoid direct sunlight with a small electric fan at one end to pull air through the box past the data logger. Recordings were taken every minute and averaged over a 10-minute time interval. Records were downloaded every two weeks at which time the dataloggers were reset. Temperature data and its analysis are kept within the respective chapters in which it is first presented. That is, Chapter 3 (Growth) for the Long-term Experiment #2 and Chapter 5 (Nectar) for Short-term Experiment #1. Details of the plant data for aspects of growth, flowering, and nectar are provided in the respective chapters.

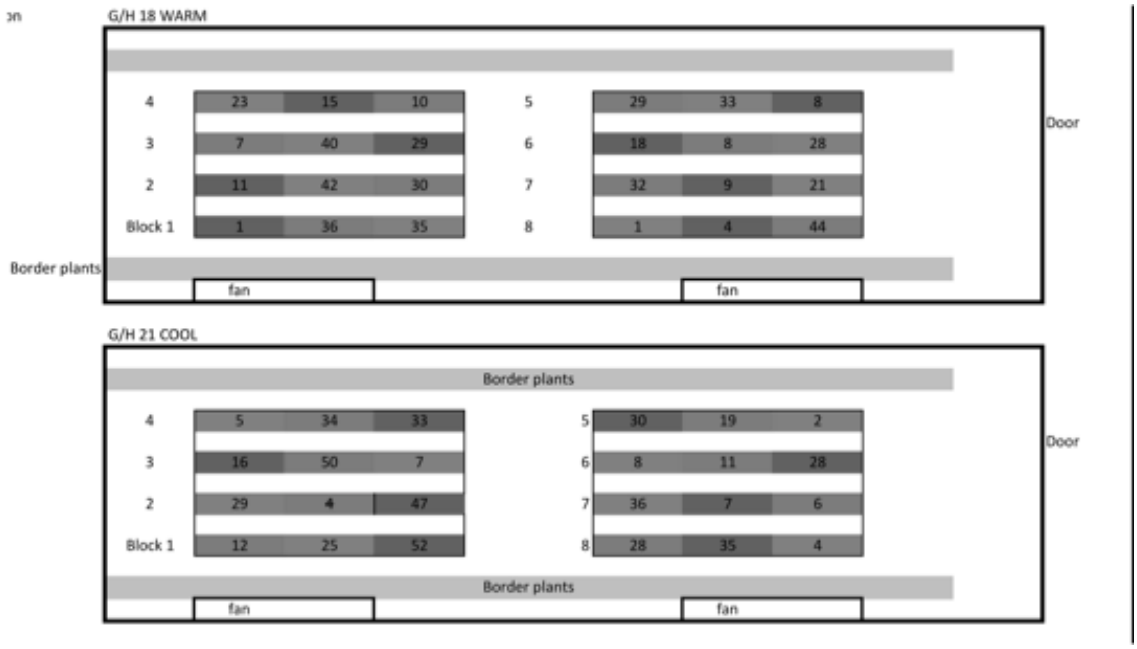

Figure 2.4 Layout of the Long-term Temperature Experiment showing experimental and treatment structures. Replicates were blocks based on plant size. Positions of entry door, fans and floor vents are as indicated.

2.4 Water-deficit Experiments (Experiments #3 & #4)

Experiment #3 was a spring dry-down trial carried out in November of 2014 using plants in their first year of flowering. Potted clones from three clonal lines representing low, medium and high nectar-DHA producing lines (Yellow, Orange and White respectively; refer to Section 2.5.1) were subject to two different watering regimes. The treatments were soil water regimes of 'Water Sufficient' and mildly 'Water-Deficient', and these were administered gravimetrically. That is, after bringing all trial pots up to field capacity (FC; see below for calculations), each pot (of 8 litre capacity) was weighed every two days and the appropriate volume of water added back or withheld, as required, to bring pots to a pre-determined gravimetric soil water content (SWC). SWC was measured, by weight, as a percentage relative to an empirically determined container capacity (CC), equivalent to the field term field capacity (FC), for the soil. The 'Water-Sufficient' pots were held at or near 100% FC while the 'Water-Deficient' pots were held at or near 70% FC. Percentage FC for the Water-Deficient treatment was arrived on from a preliminary dry-down study to ascertain, by visual observation, the

wilting points for each of the clonal lines i.e. the SWC at which free water is no longer available for plant uptake. Day zero of the experiment was deemed to be when all of the Water-Deficient pots had reached their 70% FC target weights.

CC defined as the 'maximum water holding capacity' of the soil in use (standard PGU soil mix; Section 2.5) was determined by oven drying (80°C for 7 days) of six representative soil samples, that is, one central core sample (150 –200g soil) from each of six individual pots containing soil of similar compaction and volume. Prior to sampling, the pots were thoroughly watered to saturation, the soil surface covered with polythene to prevent evaporation, and then left to drain naturally in air (raised 10mm above floor level on wooden dowels) for a period of 24 hours. The average maximum water holding capacity i.e. at 100% CC, of the soil was determined to be 0.52 or 52% saturation (52% of soil air spaces filled with water) by weight from the equation:

$$CC = \frac{\text{(wet weight of soil sample – dry weight of soil sample)}}{\text{(dry weight of soil sample)}}$$

Accordingly then, 70% FC is calculated as 0.70 * 52% = 36.4% saturation by weight.

The experiment was terminated early (after 9 weeks) due to an unexpected heat wave that resulted in wet-dry cycling of root zones as indicated by tip dieback (*pers. comm.,* Trevor Jones, Plant & Food) in all experimental plants of both treatments. Non-uniformity of flowering during the experimental period also meant that not enough nectar was available for robust statistical analysis. It was decided to rewater plants, and to undertake an autumn dry-down trial (May of 2015; Experiment #4) on the same plants i.e. in year two.

In the Autumn Dry-down Trial (Experiment # 4), treatments of 'Water-Sufficient' and mildly 'Water-Deficient' were administered to root zones by an automatic watering system using an automatic timer (Hunter NODE-100-VALVE B). Volumes of water delivered to individual pots were calibrated from the gravimetric data collected in Experiment #3 (volumes of around 250 mL and 175 mL administered four times daily resp. for the two treatments at flow rates of 8 litres $hour^{-1}$ regulated by Netafin compensating emitters). Soil moisture content was measured using a standard Time Domain Reflectometry (TDR) Instrument (MiniTrase 6050X3). Nectar was collected as per the standard protocol reported in Section 2.7 below.

2.5 Experimental Plant Material

All experiments were undertaken at the Plant Growth Unit (PGU) located at Massey University, Palmerston North, New Zealand (latitude -40.353687; longitude 175.612771). Experimental plants were pot-grown clones, propagated from cuttings, of *elite* Mānuka cultivars selected from Comvita NZ Ltd.'s breeding programme. Plantlets approximately six-months-old were supplied in 10 x 10 mm plastic pots (Figure 2.5) by Ashhurst PGP Nurseries, Palmerston North. New plant material was supplied in the autumn of each year, that is, the same batches of plantlets were used for Experiments #1, & 2, and for Experiments #3 & 4. Plantlets were potted-on into larger 8 litre 23.7 x 24 cm; diameter x height) plastic pots (Experiments #1 & 2), or 25 litre (Experiments #3) and 45 litre (Experiment #4) planter bags (Super-lift®; Primehort NZ). Plants were allowed to establish in glasshouses under mild conditions close to ambient before being selected for experimental studies.

Figure 2.5 Clonal plantlets around six months old were supplied in 10 x 10 mm pots from the cutting nursery.

All experiments were pot-based. Soil was the PGU standard potting mixture identified in a previous study (Nickless et al., 2017) as giving the best overall growth and flowering in the Mānuka clones It was labelled as the 'Control' medium in that study, and was described as an 'organic' profile texture group with parent materials of peat and pumice. To every 100 litres of soil, 300g of long-term fertilizer (Daltons), 60g of short-term fertilizer (Daltons), and 150g of dolomite (Daltons) were added and thoroughly mixed.

2.5.1 Plant Genetics

Clones for each of the experiments were selected from thirteen available lines (clone-cultivars). While the lines were chosen primarily on their nectar DHA content, sufficient numbers of plants for the factorial experiments was also a practical consideration. The relative nectar DHA production of the clonal lines used in this study, together with parentage, appears in Table 2.2. For each experiment, three clonal lines were selected to represent low, medium, and high nectar DHA accumulators (Green, Orange, and Black for Experiments #1 & #2; Yellow, Orange and White for Experiments #3 & #4).

The low to high category labels for DHA content were arrived on from the best information available (*pers. comm.* with Massey University Manuka Research Team; MUMRT, established 2011), and were relative rather than definitive.

Table 2.2 List of clonal lines used in this study (developed and supplied by Comvita NZ Ltd.). Three lines were chosen for each Experiment (#1–4) to represent low, medium and high DHA accumulators. Superscript 'a' is code relating to parental hybrid crosses (specific details confidential due to Plant Variety Rights). Superscript 'b' is DHA content as previously described by Nickless (2015).

Arbitrary Colour Code	Genotype Code[a]	Parentage	Relative DHA Content[b]
Black	28064-007	*L. scoparium* var. *incanum* cultivar *x L. scoparium* var. *scoparium* selection	High
White	28059-030	*L. scoparium* var. *scoparium* selection x *L. scoparium* var. *incanum* cultivar	High
Orange	26011-820	*L. scoparium 'Nicolsonii' x L. scoparium* var. *scoparium* selection	Medium
Green	28076-029	*L. scoparium* var. *incanum* cultivar open cross (pollen donor unknown)	Medium
Yellow	27059-944	*L. scoparium* var. *scoparium* field selection	Low

2.5.2 Plant Maintenance

Insects were excluded from all experiments by keeping entry doors closed at all times, covering air vents of glasshouses with mesh, using ant bait, and the occasional (when required) spraying of insecticide (Orthene, Ovation, mineral oil) at the recommended rates to control predominantly scale insects (*Acanthococcus spp*.), and other insect species. The use of sprays was avoided for a 14 day window prior to all nectar sampling. To allow direct comparisons of the *growth* measurements across the clonal lines of varying plant forms, plants were trained into an upright position by tying the single most dominant leader on each to a long bamboo stake (Figure 2.6).

Figure 2. 6 The single most dominant leader on each experimental plant was trained into an upright position by tying it at intervals to a tall bamboo stake.

2.6 Nectar collection

Nectar collection followed the standard protocol developed by Massey University (see Nickless, 2015). That is, where possible, flowers at development Stage IV (Figure 2.7) were chosen at random from each plant. Floral discs were gently rinsed with a small amount of distilled water using a pipette, to aid recovery of all nectar. A more detailed methodology appears in Chapter 5.

Figure 2.7 Stage IV of flower development at which nectar was sampled. Petals and anthers open, nectar production at a maximum. Photo courtesy of G. Hamilton, Massey University Manuka Research Team (MUMRT).

2.7 Nectar Analysis (DHS and Sugars)

DHA levels in (diluted) nectar samples were measured using aqueous extraction, derivatisation, and analysis using the method of Nickless et al., (2017). Hydroxyacetone (HA; 3.01 mg ml^{-1}) formed the internal standard solution. The derivatising reagent was O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine [PFBHA; 19.8 mg ml^{-1} in citrate buffer (0.1 M) adjusted to pH 4 with NaOH (4M)]. DHA (stock 3.88 mg ml^{-1}) formed the DHA standard solution (Sigma-Aldrich, 1,3-Dihydroxyacetone dimer; 97%). DHA standard solutions of concentrations 0, 25, 50%, 60%, 75%, and 100% were prepared from the stock solution in sterile water (MilliQ). For sugar analysis, 1% stock solutions were prepared for each of the sugars (sucrose, glucose and fructose). Standard sugar solutions of 0%, 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% (in MilliQ) were prepared from the stock solutions.

2.7.1 Sample preparation

For DHA analysis, 20 µL of a nectar sample (nectar extracted from flowers with sterile water using the 'pipette method' described above (Section 2.7) was added to a 2.0 mL Eppendorf tube. 25 µL of HA standard solution was added to each. Tubes were vortexed and allowed to stand for 1 hour to allow for complete dissolution. 40 µL of PFBHA derivatising solution was added to each tube, mixed, and left stand for 1 hour at room temperature to allow for complete derivatisation. ACN (1.5 ml) was added to each tube and thoroughly mixed. Sterile water (MilliQ; 0.5 ml) was added to each tube and again thoroughly mixed. Samples were then filtered through HPLC grade 4 mm PTFE membrane syringe filters with a 0.2 µm pore size (Phenomenex®). Approximately 1.5 mL aliquots of each sample were placed in an HPLC vial for analysis. For the analysis of sugars, 30 µL of nectar samples were made up to a total volume of 1.5 mL in MilliQ water. Diluted samples as well as sugar standards were filtered using 0.2 µm syringe filters into HPLC vials.

2.7.2 High performance liquid chromatography (HPLC)

Reversed phase high performance liquid chromatography (HPLC) with diode array detection was used for the quantitation of PFBHA derivatives of DHA. Analyses were performed on a Perkin Elmer Series 200 Pump and Autosampler using a Flexar UV diode array detector (λ = 263 nm; UVD340U) and a Synergi Fusion column (75 x 4.6 mm, 4 µm particle size). The column was maintained at 30°C to ensure stable conditions. Mobile phase A was water: acetonitrile (ACN), 70/30 v/v and mobile phase B was 100% ACN. For the sugar analysis, the mobile phase solution was prepared by dissolving ethylene-diamine-tetra-acetic acid (50 mg mL^{-1}) in deionised MilliQ water and run using an isoelectric elution system and a constant flow rate of 0.6 mL $minute^{-1}$. 20 µL of sample or standard was injected onto the column using the following 23 minute elution gradient: A: B = 90:10 (isocratic 2.5 minutes), graded to 50: 50 (8.0 minutes), graded to 0:100 (1.5 min), 0:100 (isocratic 7 minutes), graded to 90:10 (1.0 minutes), 90:10 (isocratic 3.5 minutes), detection at 263 nm.

For sugar analysis, the mobile phase solution was prepared by dissolving ethylenediaminetetraacetic acid (50mg L^{-1}) in deionised MilliQ water and run using an isocratic elution system and a constant flow rate of 0.6 ml $minute^{-1}$. Sucrose, glucose and fructose were analysed using a RI detector (Shodex RI-101) with a Sugar-Pak I column (Waters, size 6.5 × 300 mm) kept at 75°C.

2.7.3 DHA and Sugar Analysis

The relative DHA and sugar levels were calculated from calibration curves, generated for the standard solutions by linear regression using the HPLC peak area ratios of DHA: HA plotted against the known concentrations of the DHA and sugar standards.

2.8 Statistical Approach

All data analyses in this study were completed using a GenStat® statistical software package (VSNI; Version 18). Where possible, the data sets were analysed as a whole i.e. a single data set including all three clonal lines, as long as the assumptions of the model were satisfied. Combining observations into a single data set avoids the difficulty of checking for overlapping confidence intervals and standard error intervals which has a large type 1 error rate ($\alpha = 0.16$) associated with it (Clark & Thompson, 2011; Payton et al., 2003). The statistical approach was informed by the question: Are temperature effects in the Warm glasshouse different from those in the Cool glasshouse? The null hypothesis is then: temperature has no effect on growth and the transition to flowering, and nectar quantity and quality in clones of low, medium and high DHA producing lines.

A functional approach i.e. based on mathematical predictions or estimates, using linear mixed models (LMM) i.e. fixed and random effects, via the method of residual maximum likelihood (REML) was employed for growth and nectar data analyses. To test for significant differences between factor means, *post hoc* pairwise comparisons of Fisher's least significant differences (LSDs) were carried out, unless stated otherwise in the respective chapters, Firstly, a functional (mathematical) vs. an empirical approach was chosen because of experimental design i.e. clones 'nested'[9] in glasshouse i.e. the 'glasshouse' term was not replicated. Secondly, whilst this study was undertaken in a controlled environment over a single season and each plant was considered independent (replicate) i.e. pots were stand-alone and plants were not large or dense enough for shading effects to occur, the data was unbalanced (missing data), non-orthogonal (the order of fitted terms is important), and there were several error terms. Some individuals had to be removed from the analyses because of severe localised insect attack, atypical growth or plant death. REML is useful in the analysis of

[9] A factor is *nested* within another factor when each category of the first factor co-occurs with *only one* category of the other.

unbalanced data sets, because it can account for more than one source of variation in the data i.e. several error terms generated from multiple treatments or factors (clone, genotype, temperature), and it can provide an estimate of the variance components associated with the random terms of the model (Robinson et al., 1987). The floral data were analysed using a mixture of LMM, General Linear Models (GLM), and Gaussian Mixed Models (GMM). GLMs were used to accommodate non-homogeneity of the floral data, and because flower counts were repeated across time and not independent observations [since the lifespan of a Mānuka flower is 2 –3 weeks in the glasshouse (Nickless, 2015)].

To examine the *structure* of the GEI, Multi- Environment Trial (MET) analyses were performed on some of the variables (growth, flowering, and nectar) to further partition the GEI. Interaction effects are described as the '*combined effects of factors on the measured response parameter i.e. when the effect of one factor depends on the level of the other factor'*. Adding interaction terms into statistical analyses (e.g. LMM) makes the coefficients of the lower order terms *conditional* on the main effects. That is, the effect of one predictor is conditional on the value of the other. Unlike ANOVA, however, in regression analyses (e.g. LMM) the coefficient of the lower order term isn't the effect of that term; it's the effect only when the other term in the interaction equals zero[10]).

The MET analysis approach used was Finlay-Wilkinson (FW) joint regression as decided by the inherent properties of the data set i.e. missing values and heterogeneity of data. The strength of the Finlay-Wilkinson regression model is that no data assumptions need to be met. FW regression computes a measure of *genotype adaptability* where the 'yields' are measured on a logarithmic scale (Finlay & Wilkinson, 1963; Yates & Cochran, 1938). The fitted model investigates the interaction between the two factors 'genotypes' and 'environments'. The intention is to characterise the

[10] https://www.theanalysisfactor.com/testing-and-dropping-interaction-terms/

sensitivity of each genotype to environmental effects by fitting a regression of the environment means for each genotype on the average environment means, producing a high degree of linearity. The strength of MET analysis is in comparing performances of the genotypes (G) across the growing environments (E) relative to an average environment, and in quantifying the contributions of G, E, and their interactions (GEI) to the observed trait responses.

Further details of the models used and any necessary data transformations together with the variables on which MET analyses were performed are given in each of the respective chapters. The models differed between the variables being analysed and are described in the Material and Methods sections of the respective chapters.

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Chapter 3 Growth Responses to Changes in Temperature

Abstract

The ability of NZ Mānuka *(Leptospermum scoparium)* to respond to different environments is reflected in its wide ecological amplitude. Intra-specific variation in growth form has been attributed to habitat-modification and genetic variability (Allan, 1961). In a comparative study of three high yielding cultivars with varying nectar dihydroxyacetone (DHA), developed as part of a breeding programme by *Comvita NZ Ltd.*, two-year old potted plants were grown in glasshouses under conditions of natural light and day length in two different temperature regimes (Cool and Warm) for one complete annual growth cycle. The clones showed markedly different responses to growing temperature, such that, the genotype rank orders differed in the two environments ($P \leq 0.05$), that is, a large Genotype (G) x Environment (E) interaction (GEI) was observed. Average annual growth rates for main-axis stem length (L) and basal stem diameter (BSD) were not significantly different across the growing environments for two of the cultivars but were significantly higher at the warmer temperatures in a third. The relative contributions of G, E, and GEI were quantified using Multi-Environment Trial (MET) analyses. Growing environment accounted for *ca.* 34% ($P < 0.001$) and 10% ($P \leq 0.05$) of the observed phenotypic responses in L and BSD respectively, while the genetic contribution across the environments was non-significant for L (0.4%), and *ca.* 44% for BSD ($P < 0.001$). Phenotypic (developmental) plasticity in the L response to growth temperature in one of the cultivars ($P < 0.001$) resulted in an altered phenotype and a significant shift in phenology. In conclusion, the temperature-induced growth responses in the clones appear to be related to differences in plant form and were not correlated with the relative nectar-DHA.

3.1 Introduction

Environmental tolerance by *Leptospermum scoparium* to a wide range of environmental variables, both above and below ground, has been reported on numerous occasions. Plastic responses by plant species to environmental factors contribute to a greater habitat range and are an especially important adaptation in a successional scrubland species such as NZ Mānuka. In studying the intra-specific variation in Mānuka leaf size (Burrell, 1965; Ronghua et al., 1984), branching height (Gaynor, 1979), fitness (Price & Morgan, 2006), and growth characteristics (Lee et al., 1983; Ronghua et al., 1984), both genetic and environmental effects were shown to contribute to the observed phenotypic variation. Among the environmental factors reported, temperature seems an important determinant of growth in Mānuka. For example, in a study of seedlings from seventeen populations raised in a uniform environment, while variation in plant form (apical dominance, height, plant taper, erectness and branch angle) had a significant genetic component. Geographic and climatic factors (latitude, altitude, distance from the coast, and temperature variables) associated with the different ecotypes also contributed significantly (Ronghua et al., 1984). In particular, direct and indirect (latitude) temperature variables featured significantly. Direct temperature variables included mean winter and mean summer temperatures, and annual temperature range. In addition, in field trials carried out by the Massey University Manuka Research Team (MUMRT; formed in 2011 as part of a Primary Growth Partnership between the NZ Government and Industry), poor growth of Mānuka seedlings planted at higher altitudes was attributed in part to the cooler temperatures with increased altitude and exposure (*pers. comm.,* MUMRT*).*

In glasshouse experiments involving clones of various Mānuka cultivars differing in their nectar-DHA concentrations, both genotype and environment were shown to be involved in determining the growth characteristics (Nickless, 2015; Nickless et al., 2017). While in that study the relative growth rates were not correlated with nectar-DHA, Nickless (2015) emphasised the importance of assigning value to the cultivars not just by assessing their relative nectar DHA content but also the overall characteristics that contribute to greater nectar yields, of for example, plant growth and flowering phenology. At the time of undertaking this project and to our knowledge, there were no G x E studies on the growth and flowering traits across multiple environments in Mānuka of known genetic composition.

The objective of this chapter is to examine the effect of growing temperature on the growth and development of the three Mānuka clonal lines. The main effects of temperature and genotype, and their interactions will be quantified. This chapter addresses the question: Does temperature affect the growth and development of Mānuka clones varying in their relative nectar DHA content?

3.2 Materials and Methods (Experiment # 2)

3.2.1 Plant Material

Details of the experimental design were provided in Section 2.3, but to briefly recap, a factorial experiment (8 replicated individuals x 2 temperature treatments) was conducted in glasshouses using plants in their second year of growth and flowering from three clonal lines representing low, medium and high relative nectar-DHA (Green, Orange, and Black resp.). Based on appearance only and not a primary consideration in selection of clonal lines, Green was noted to have a semi-prostrate, weeping growth habit and to be of small stature. Orange and Black were visibly upright and strongly apically dominant. Orange appeared to have a relatively more compact and bushy habit than Black, which was more slender and 'whippy' in appearance (Figure 3.1). Cool and Warm temperature regimes (as described in Section 2.3) were administered to plants growing under conditions of natural radiation and day length, for a full 12-month period.

Figure 3.1 Images for the three clonal lines, Green (semi-prostrate growth habit), Orange (upright, bushy growth habit), and Black (upright, whippy growth habit) from left to right. Images not to scale.

3.2.2 Data Collection

Details of temperature recording and data collection have been provided in Section 2.3. Plant growth measurements were made at regular developmental intervals. Whole-plant linear growth measurements comprised a length component (mm main-stem length, L) and a radial component (mm main-stem basal diameter, BSD). Measures of length for the main-stem axis were used to account for visible differences in the apical dominance/plant form of the clones and thus represent measures of *real* or *extended height.* Also, In addition to the main stem (defined as 'order zero' using the nomenclature of Hallé et al., 1978), several (one or two) actively growing new-season side-shoots (1st order branches; Figure 3.2) on each plant were also selected for data collection. Selected shoots were tagged at regular developmental intervals i.e. *modules* of 10 nodes, with jewellers' tags. Tags were dated, and the measurements of shoot length were recorded at approximately monthly intervals. Single time-point brachyblast [the small branchlets of new growth on which flowers are borne, (Zieslin & Gottesman, 1986)] counts and total numbers of nodes on the primary shoot axes (Figure 3.2) were recorded at *quiescence* on the 16th June 2016 (Day 226) for Black; 18th June 2016 for Orange (Day 228); and 20th July 2016 for Green (Day 260). Quiescence is defined in this study as near-zero shoot extension as determined from repeat (monthly) measurements of shoot length. Lateral (second order) lengths were also recorded during the same quiescent period. Note that Green Warm shoots continued to elongate throughout the experimental period but were recorded on the same day as Green Cool for comparison.

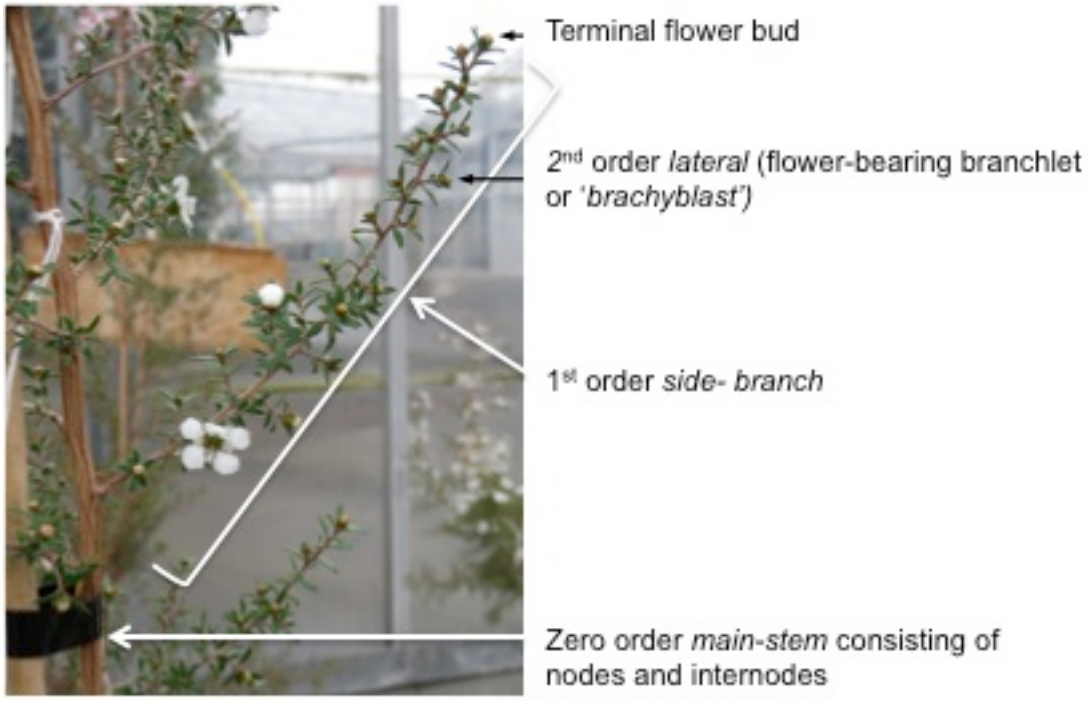

Figure 3.2 Branching hierarchy in the Mānuka clones. In this study, the main stem and side branches both represent 'shoots' with a primary axis and co-axial lower order laterals.

3.2.3 Statistical Approach

3.2.3.1 Temperature data

The collated means for average daily temperature in the Cool and Warm growing environments were analysed by ANOVA using days as 'Blocks' to generate mean annual temperature values (T_{MA}). The thermal time dependence of growth was tested using a Thermal Time Model (TTM), that is, cumulative growth plotted against accumulated growing degree-days (AGDD). AGDD was calculated by summing $T_m - T_b$, where T_m is the average of daily (24h) maximum and minimum temperatures [(Tmax. + Tmin.)/2] and T_b is base temperature (the threshold temperature below which plant growth is zero). A T_b of 10°C was used since Zieslin & Gottesman (1986) reported no growth in *L. scoparium* in long days (LD; 16h photoperiod) at constant temperatures of 8°C

3.2.3.2 Growth data

LMM or GLM were used for the growth parameters after the necessary data transformations to accommodate unequal variance and to satisfy the normality and homogeneity requirements of the model in use.

The whole-plant growth data were analysed by LMM after the necessary transformations, that is, the natural log of 'Final L', and the square root of 'Final BSD'. To account for any size differences in clones at the start of the experiment, 'Initial L' and 'Initial BSD' (measurements of L and BSD at Day zero) were used as covariates in the respective analyses. Together with the covariates, 'Clonal line' nested [11] in 'Treatment' was designated as a fixed term in the LMMs (GenStat®). 'Columns', 'Rows', and the 'Column by Row' interactions were designated as random terms. For the analysis of BSD, the term 'Column' was removed from the model to simplify it, and because it was shown to be negligible.

The shoot variables of shoot elongation rate (SER) and mean internode length (MIL) were also analysed by LMM. Log (natural) transformations of the MIL data were necessary to satisfy the normality and homogeneity assumptions of the model. 'Clonal line' nested in 'Treatment' and glasshouse 'Rows' were designated as fixed and random terms respectively in the models. Significance tests were conducted using pairwise comparisons of the *post hoc* Fisher's LSDs (least significant differences). The brachyblast counts were analysed by GLM using a Poisson distribution and logarithm link function. 'Block', 'Treatment', and 'Clone' were treated as fixed effects. One outlier (as determined by GenStat®) was removed from the standardised (per node or unit length of primary shoot) counts before statistical analysis to satisfy normality and homogeneity requirements of the model.

[11] A factor is *nested* within another factor when each category of the first factor co-occurs with *only one* category of the other

3.2.3.3 GEI

Multi- Environment Trial (MET) analyses were performed on the raw data values for 'Final L', and 'Final BSD' i.e. data values at the end of the experimental period.

3.2.3.4 Allometry

Dimensional relationships between the whole-plant parameters of L and BSD in the clones were examined by reduced major axis regression of $\log_e$ (L) on $\log_e$ (BSD). Data were grouped into clone-treatment combinations of Green Cool, Green Warm, Orange Cool, Orange Warm, Black Cool, and Black Warm (GC, GW, OC, OW, BKC, BKW resp.). Taking all of the data i.e. using all 495 data points across time (approximately monthly x 3 clones x 2 glasshouses x 8 replicates), temperature effects across the experimental period on the mathematical relationship between L and BSD were quantified. Equations of the form Ln (L) = α Ln (BSD) + β were obtained for each group from the reduced major axis regressions. The slope 'α' or the regression coefficient is the constant of differential growth between L and BSD. It describes the *ratio* of the respective specific growth rates i.e. 1/y (dy/dt) and 1/x (dx/dt). Such equations are mathematically equivalent to the allometric relationship $L = \beta\ BSD^{\alpha}$ for the untransformed data.

3.3 Results

3.3.1 A 4.8°C temperature differential in T_{MA} was achieved between the Cool and Warm

The temperature data for the experimental period in the Cool and Warm regimes are shown in Figure 3.3. The daily temperature averages in the two glasshouses were significantly different *(P* < 0.001; Table 3.8 in Appendix 3.3.1), with mean annual temperatures (T_{MA}) of 16.9°C in Cool and 21.7°C in Warm (Figure 3.3). That is, an achieved temperature differential in T_{MA} of 4.8°C. Temperature differences between the glasshouses were least (2.4°C) in mid-December 2015 and mid-January 2016, and greatest for the months of June and August 2016 (6.9°C & 7.0°C resp.). The temperature extremes were a low and high of 10.5°C and 34.5°C, respectively, in Warm and 5°C and 32.8°C, respectively, in Cool. The highest daily mean glasshouse temperatures for the 12-month experimental period were recorded on the 3rd of February 2016 (Day 92; i.e. average daily temperatures of 27.9 °C in Warm and 24.7°C in Cool). The coldest recorded glasshouse temperatures were from the 31st of May to the 17th of June 2016. That is, a temperature differential between glasshouses of > 8°C on 11 days out of the 18 days for this period, or 11 out of a total of 30 days for the month of June and 8 days for the month of August. The achieved minimum temperature thresholds in the Cool and Warm growing-environments were > 5°C and > 15°C year-round respectively.

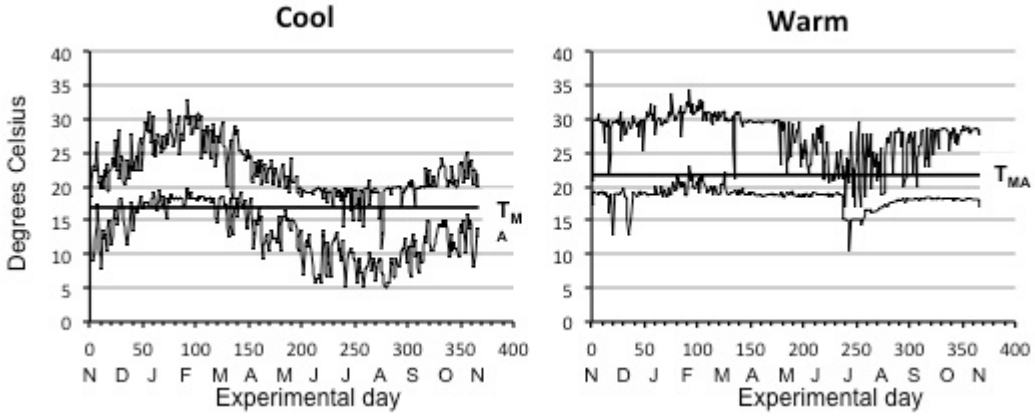

Figure 3.3 Daily temperature maxima and minima as recorded by data micro-loggers for the two glasshouse-growing environments, Cool and Warm, for the (12-month) experimental period. The mean annual temperatures (T_{MA}) of 16.9°C in Cool and 21.7°C in Warm are represented by the bold horizontal line on each plot.

3.3.2 The phenological stages of *growth* and *flowering* were temporally separated in the two-year old Mānuka clones

Whole-plant growth curves for clones growing in the Cool and Warm regimes are presented in Figure 3.4. The growth rates (GR) are averages of monthly growth increments of main-axis stem length (L) and basal stem diameter (BSD). Maximum GR (L) or main-axis extension (MAE) occurred *ca.* Day 80 in the Orange and Black clones, and Day 150 in the Green clones. That is, a time lag of approx. 70 days for the Green line (Figure 3.4 a, b, & c). Within the lines, MAE was delayed in GC compared with GW. The data typically show convergence of MAE for Cool and Warm treatments around Day 111 (see Discussion), however these values were left in the analyses to maintain a balanced data set. The maximum values were significantly higher in Warm compared with Cool for all three lines ($P \leq 0.05$), or increases of *ca*. 59 %, 47%, and 43% (1.6, 1.5, and 1.4-fold) for Green, Orange, and Black respectively. Near-zero GR (or near cessation of growth termed *quiescence)* occurred *ca.* Day 208 for all of the clone-temperature combinations except GW. Growth rates in GW were > 2.0 mm day^{-1} for the duration of the experiment and did not appear to have a quiescent phase.

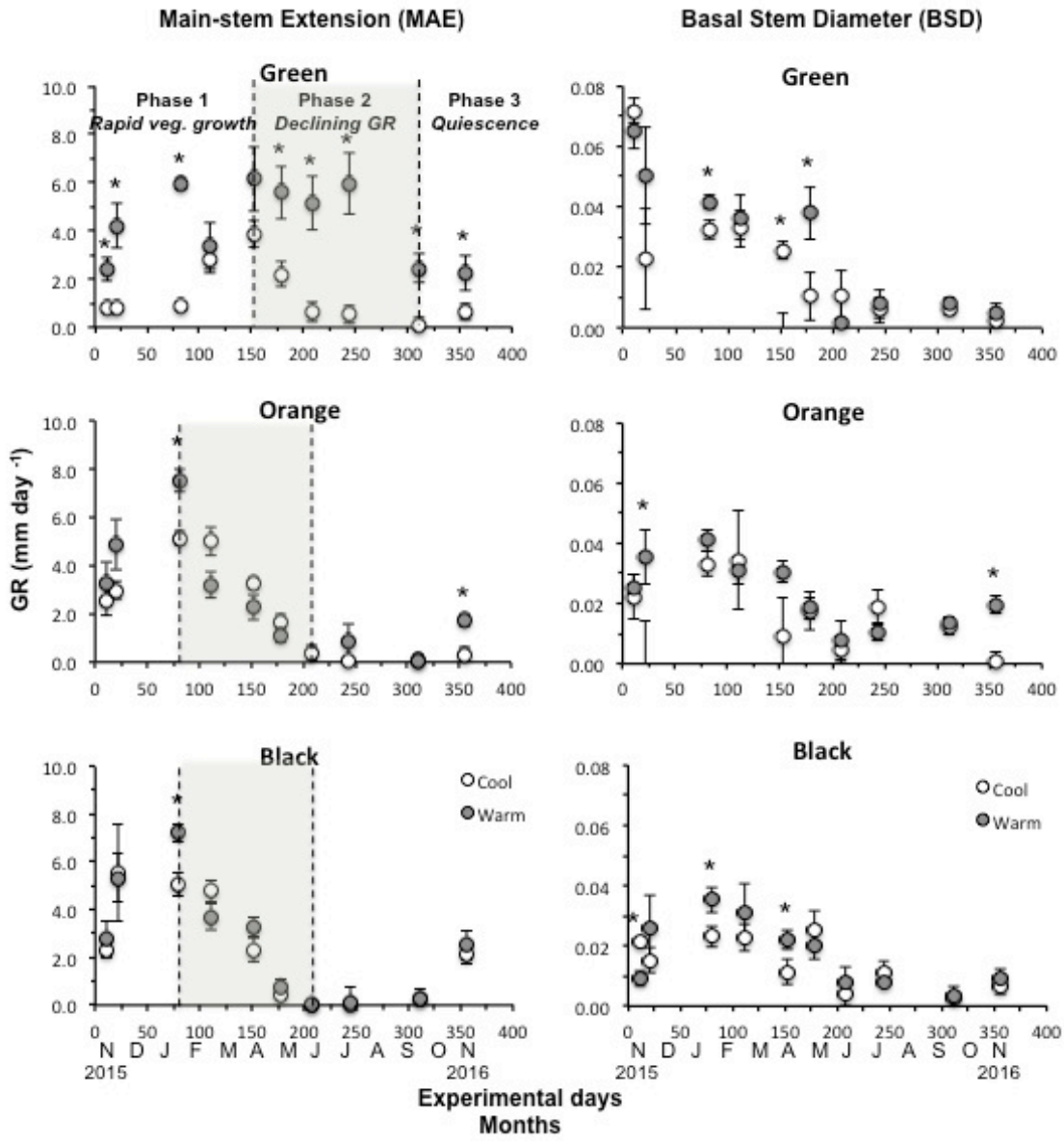

Figure 3.4 Empirical plots of mean monthly growth increments ± 1 s.e. for one complete cycle of growth and flowering, in the clones growing under Warm and Cool temperature regimes. Plots are for whole-plant main-axis extension (parameter L in mm day^{-1}; plots a, b, & c) and linear increases in basal stem diameter (parameter BSD (mm day $^{-1}$; plots d, e, & f). Units are as shown. * denotes significance at the 95% *CI* (*P* ≤ 0.05).

Table 3.1 The accompanying table shows the average day length in hours and minutes (h: m) and temperature (°C) for the three phases of MAE growth as indicated. Phase One occurred in long days (LD) and relatively high daily temperature averages; Phase Two (shaded area) occurred in short days (SD) conditions; and Phase Three occurred in LD at relatively low daily temperature averages.

Clonal line	Phase 1	Phase 2	Phase 3
Green (h: m °C Cool °C Warm)	13:57 20.2 23.8	10:08 16.2 21.2	11:12 16.2 21.2
Orange & Black (h: m °C Cool °C Warm)	14:44 19.5 23.3	11:22 14.1 19.7	11:12 14.1 19.7

Three major phenological stages were identified from the MAE curves in Figure 3.4 a, b, & c. Phase One was a period of rapid (*vegetative*) *extension growth*; Phase Two, *declining GRs*; and Phase Three, a period of *quiescence* (< 1.0 mm day^{-1}, except in GW). The phases were distinct and separated in time. For example, Phase One occurred from Day 0 to Day 80 in Orange and Black and Day 152 in Green; Phase Two from Day 80 to Day 208 in Orange and Black, and from Day 152 to 311 in Green; and Phase Three from Day 208 - 356 in Orange and Black, and from Day 311 - 356 in GC. The *growth and flowering* phases were less well defined in Green Warm. Reported results are for the second year of growth in the clones. Split-plot analyses of the log L to log BSD plots (data not presented) also showed a clear temporal separation of the *vegetative growth* and *flowering* phases. There was evidence going into year three (at the end of the experimental period) of growth and blossoming occurring simultaneously on shoots (see Discussion), however, no data were collected for this.

Additional information showing the absolute temperature minima and maxima for each of the three growth phases for Cool and Warm is provided in Table 3.19 of Appendix 3.3.7. The thermal time dependence of MAE growth was tested by a Thermal Time Model (TTM) using a base temperature (T_b) of 10°C (Figure 3.8 of Appendix 3.3.2). The rationale for using 10°C was explained in Section 3.2.3.1. From the plots, vegetative extension growth does not appear to be thermal time dependent as indicated by the curve plateaus which mark a slow down in MAE to near zero growth (described above as a 'quiescent' period of growth).

The growth curves describing linear increases in basal stem diameter (BSD) appear in Figure 3.4 d, e, & f. GR (BSD) was highest in Green (at Day 0 and declining thereafter). Near-cessation occurred *ca*. Day 311 in BC and BW, and *ca.* Day 356 in GC, GW, and OC.

3.3.3 Average whole-plant GRs across the experimental period were significantly higher in Warm than in Cool

The temperatures x clone interactions for absolute L and BSD at the end of the experimental period ('Final L' and 'Final BSD') were significant ($P < 0.001$ and $P \leq 0.05$ respectively; Tables 3.10 & 3.11 in Appendix 3.3.3). The significance of the initial measurements for L and BSD (at Day 0) was established from the respective analyses ($P < 0.001$), and the initial data were used as covariates. There was however, some evidence for position effects within the glasshouses i.e. fan effects ('Column' term in the model) for L and row effects for BSD (see heat maps; Figures 3.10 & 3.11 in Appendix 3.3.3).

At the clonal level i.e. within the lines, temperature effects for main-axis extension growth (MAE) were significant in the Green line only. That is, 3.7-fold increases in mean annual extension rates for GW compared with GC (4.4 ± 0.4 mm day $^{-1}$ and 1.2 ± 0.2 mm day $^{-1}$ resp.; $P \leq 0.05$; Figure 3.5a). Variability in MAE across the clone-temperature combinations was largest for GW ($CV = 0.23$). For GR (BSD), temperature effects were significant for the Orange in which average annual GR (BSD) was higher at the warmer temperatures ($P \leq 0.05$; Figure 3.5b). Variability across the clone-temperature combinations was least in BC ($CV = 0.05$) and similar in the others (see Figure 3.9 in Appendix 3.3.3 for distribution plots). There was evidence for row effects on BSD.

3.3.4 Genotype rank orders for main-stem extension (MAE) growth differed across the growing environments

Interaction plots *(syn. response norms)* for the three genotypes are presented in Figure 3.5. The observed changes in rank order across the growing-environments, for both MAE and BSD, are indicative of large GEI. Equations for the regression lines are given in Table 3.12 of Appendix 3.3.4

Genetic variance (total variation across the genotypes within an environment) for MAE differed in the two growing-environments, and was larger in Warm than in Cool (heterogeneous; standard deviations of 412.8 in Warm and 259.3 in Cool). That is, MAE was a poorly correlated trait across the temperature regimes (*F Corr*. = 0.0352; R^2 = 0.395, $P \leq 0.05$). For example, Green was ranked low (relative to Orange or Black) in Cool and high in Warm ($P \leq 0.05$; Figure 3.5). Low correlation was observed as changes in the genotype rank orders, for example, G = B = O in Cool and O = B < G in Warm ($P \leq 0.05$). In contrast, genetic variances for BSD were similar in the two growing environments (homogeneous; standard deviations of 2.24 in Warm and 2.03 in Cool). However, any correlations for phenotypic expression between Cool and Warm for the trait of BSD were not significant.

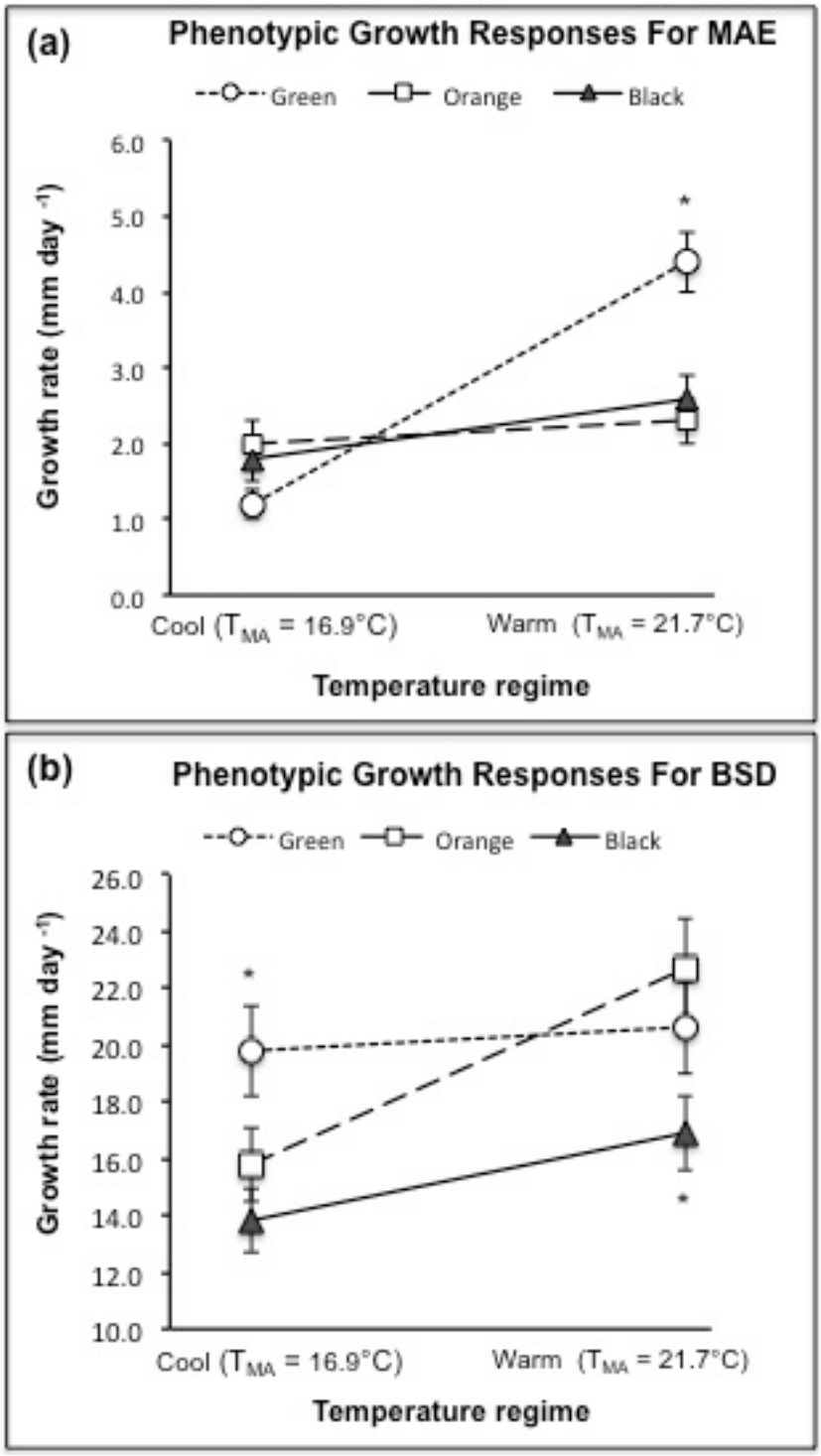

Figure 3.5 Interaction plots (means ± 1 s.e.) illustrating the effect of GEI on relative changes in the mean phenotypic growth responses for the linear measurements of whole-plant main-axis extension (MAE; graph (a)) and basal stem diameter (BSD; graph (b)), for the three clonal lines in the two growing environments for the 12-month experimental period. The temperature regimes were Cool and Warm, that is, mean annual temperatures (T_{MA}) of 16.9°C and 21.7°C respectively. * denotes a significant difference between the clonal lines within an environment at the 95% *CI* (*P* ≤ 0.05).

To further partition the L and BSD responses into their relative components of (genotype (G), environment (E), and GEI), simple Multi-Environment Trial (MET) analyses were performed using Finlay-Wilkinson regression (Section 3.2.7.3). The ANOVA outputs are presented in Tables 3.13 & 3.14 of Appendix 3.3.5. While G did not contribute significantly to the total variance of the L response (Table 3.2), the contribution of E was moderate and highly significant (33.9 %; $P < 0.001$). GEI (the *sensitivity* values *syn*. regression coefficients in Table 3.14) was also highly significant contributing 32.4 % to the total variance (as calculated from the sum of squares; $P < 0.001$). GEI was large in Green compared with Orange or Black (2.38, 0.26, and 0.36 resp.). The relative mean square deviations were largest in Green (140339) and smallest in Black (10100).

In contrast to the L response, both G and E contributed significantly to the total variance of the BSD response (43.6%, $P < 0.001$ and 9.74 %; $P \leq 0.05$ respectively (from sums of squares in Table 3.15 of Appendix 3.3.5). However, GEI (the sensitivity values or the relative regression slopes) was not significantly different between the genotypes, and any observed changes in rank orders between the growing-environments were not significant (output not presented).

Results for the relative influences of G, E, and GEI on the whole-plant growth traits are summarised in Table 3.2.

Table 3.2 Summary table for the relative contributions (%) of G, E, and GEI to the whole-plant MAE and BSD growth responses at the different growing temperatures by MET analyses. Significance is denoted by * ($P \leq 0.05$), ** ($P < 0.001$). ns denotes non-significance.

	MAE	BSD
G	ns	**44% ****
E	**33% ****	**10% ***
G x E	**32% ****	ns

3.3.5 Shoot elongation rates were increased 1.7-fold, on average, in the Warm compared with the Cool regime

At an individual shoot level, the temperature x clone interactions were significant for absolute shoot length such that mean shoot length was significantly greater in the Warm compared with the Cool regime for the Green and Black lines ($P \leq 0.05$; Table 3.3). Position effects within the glasshouses were not significant (statistical output not presented).

Mean shoot elongation rate (SER) was significantly higher in GW compared with GC ($P \leq 0.05$; Table 3.3). The statistical output is presented in Table 3.16 of Appendix 3.3.6. It is presumed that the marginal significance of Black (at the 10% level) in Table 3.3 may be biologically real based on the significance of all other measured shoot parameters for the Black clones. Variance was greatest under the Warm regime (*CV* = 0.34, 0.38, and 0.36 for GW, OW, and BW resp.; see distribution plot in Figure 3.12 of Appendix 3.3.6).

Table 3.3 Means for individual shoot growth parameters as shown (± 1 s.e.) for the three clonal lines in the Cool and Warm temperature regimes. ** denotes a significant difference within a clonal line at the 95% *CI* ($P \leq 0.05$); * denotes a marginal significance in Black of $P < 0.10$ (see text).

	Shoot Length (mm)		SER ($mmday^{-1}$)		MIL (mm)		No. Nodes	
	Cool	Warm	Cool	Warm	Cool	Warm	Cool	Warm
Green	301.0 ± 26.4	1006.3 ± 88.2 **	1.1 ± 0.1	4.1 ± 0.5 **	11.9 ± 0.6	14.2 ± 0.8 **	26 ± 2.8	66 ± 2.8 **
Orange	530.1 ± 46.4	539.7 ± 47.3	2.4 ± 0.3	2.3 ± 0.3	12.4 ± 0.7	12.21 ± 0.7	48 ± 2.8	48 ± 2.8
Black	372.0 ± 322.6	544.0 ± 47.7 **	1.9 ± 0.3	2.2 ± 0.3 *	10.4 ± 0.6	11.1 ± 0.6 **	36 ± 2.8	51 ± 2.8 **

Shoot length was a function of shoot elongation rate (*F Corr.* = 0.74; r^2 = 0.59; *P* < 0.001 and mean internode length (*F Corr.* = 0.68; r^2= 0.41; *P* < 0.001; Table 3.4).

Table 3.4 Correlation matrix for the primary shoot variables: mean internode length (MIL; mm), shoot elongation rate (SER; mm_day-1), number of nodes (Nod), shoot diameter (ShDi; mm), shoot length (ShL; mm). ** denotes significance at the 99.9% *CI* (*P* < 0.001).

MIL	-				
SER	0.3668	-			
Nod	0.0181	0.6178	-		
ShDi	0.4097	0.1964	0.2899	-	
ShL	**0.6807****	**0.7366****	0.6411	0.5639	-
	MIL	**SER**	**Nod**	**ShDi**	**ShL**

The temperature x clone interactions for mean internode length (MIL) were significant (*P* ≤ 0.05; output not presented) such that GW was greater than GC (14.2 ± 0.8 and 11.9 ± 0.6 mm resp.), and BW was greater than BC (11.1 ± 0.5 and 10.4 ± 0.5 mm resp.; *P* ≤ 0.05; Table 3.4). Overall, internode lengths were longest in the Green clones (*P* ≤ 0.05). Any position effects (row, column) within the glasshouses were not significant (by LMM; data and model not presented).

3.3.6 Standardised brachyblast numbers were greater in Warm than in Cool, in the absence of any GEI

The overall temperature effect on the total number of brachyblasts produced was significant (*P* < 0.001; ANOVA output not presented), that is, greater numbers in Warm than in Cool. Treatment differences within the lines for standardised brachyblast numbers (accounting for shoot length) were *ca.* 2.1, 1.6 and 1.4–fold for Green, Orange, and Black respectively, or increases of 106%, 63%, and 44% for Green, Orange, and Black respectively under Warm temperature treatment (*P* ≤ 0.05; Table 3.5). There were no detectable temperature x clone interactions i.e. an absence of GEI (Table 3.17 of Appendix 3.3.7).

Table 3.5 Numbers of brachyblasts per node of primary shoot axis ± 1 s.e. for the three clonal lines under the Cool and Warm temperature treatments. Values in brackets are the equivalent numbers of brachyblasts per 50 mm of primary shoot length. Counts were made at the developmental stage of shoot quiescence for *N* number of shoots as shown. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05).

	Cool	Warm	*N* (shoots)	
Green	1.6 ± 0.2 (6.4 ± 0.8) [c]	3.3 ± 0.4 (11.0 ± 1.2) [c]	18	13
Orange	5.9 ± 0.4 (23.7 ± 2.1) [b]	8.0 ± 0.6 (33.3 ± 3.4) [a]	11	9
Black	4.4 ± 0.6 (19.9 ± 1.5) [b]	7.2 ± 1.0 (31.4 ± 2.0) [a]	15	15

Brachyblast numbers were more highly correlated with 2nd order lateral length (*Pearson's corr.* = 0.93, r^2 = 0.86 for Orange and Black; *Pearson's corr.* = 0.82, r^2 = 0.68 for Green) than with primary stem length (*Pearson's corr*. = 0.74; r^2 = 0.55, data not presented).

3.3.7 Allometry

Allometry or *dimensional analysis* by reduced major axis (*rma*) regression (Section 3.2.7.4) was used to examine the relationship between L and BSD. The log-log plots for each of the clones are presented in (Figure 3.6). While temperature had no significant overall effect on the slope or intercept of the regression lines, temperature effects within the clonal lines were significant. Changes in both the slope *and* the intercept were significant for Green (χ^2 < 0.001), while slope changes only were significant for Black (χ^2 < 0.*10*), and intercept changes only for Orange (χ^2 < 0.001; Table 3.6). Trends were contrasting in Green and Black. For example, the slope was significantly larger in GW compared with GC (χ^2 < 0.001), and in BC compared with BW (χ^2 ≤ 0.05; Table 6). Slope differences between the Cool and Warm temperature treatments are significant for the Green and Black clones only. Overall, the slope (regression or scaling coefficient, *α*) was greatest in GW and least in GC (χ^2 <0.001)

Table 3.6 Regression coefficients (α or slope) and constants (β or y axis intercept) with standard errors by Reduced Major Axis Regression for Ln L vs. Ln BSD with 'Green Cool', 'Green Warm', 'Orange Cool', 'Orange Warm', 'Black Cool', and 'Black Warm' as groups (6 levels). Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05). ** and * denote significance within a clonal line (*P* < 0.001 and *P* ≤ 0.05 respectively).

	Green		Orange		Black	
Parameter	Warm	Cool	Warm	Cool	Warm	Cool
Slope (α)	1.76 ± 0.12 [a] **	0.94 ± 0.07 [c]	1.01 ± 0.04 [c]	1.09 ± 0.04 [c]	1.06 ± 0.06 [c]	1.24 ± 0.06 [b] *
Intercept (β)	2.88 ± 0.30	4.49 ± 0.19 **	4.79 ± 0.09 **	4.69 ± 0.10	4.86 ± 0.12	4.46 ± 0.13

Equations for each of the clonal lines were derived from the log-log plots in Figure 3.6 are listed below. Equations are of the form $y = \beta x^{\alpha}$ or, Ln L = β + α Ln (BSD) where, α is the slope or regression coefficient, and β is the (y-axis) interceptor or scaling coefficient i.e. the constant value when BSD is zero. The coefficients of determination (r^2) were lower in GC and GW compared with OC, BC, and BW.

GW: $L = 2.88\ BSD^{1.76;}$ $r^2 = 0.64$; GC: $L = 4.49\ BSD^{0.94;}$ $r^2 = 0.65$

OW: $L = 4.79\ BSD^{1.01}$; $r^2 = 0.69$; OC: $L = 4.69\ BSD^{1.09}$; $r^2 = 0.88$

BW: $L = 4.86\ BSD^{1.06;}$ $r^2 = 0.80$; BC: $L = 4.46\ BSD^{1.24;}$ $r^2 = 0.79$.

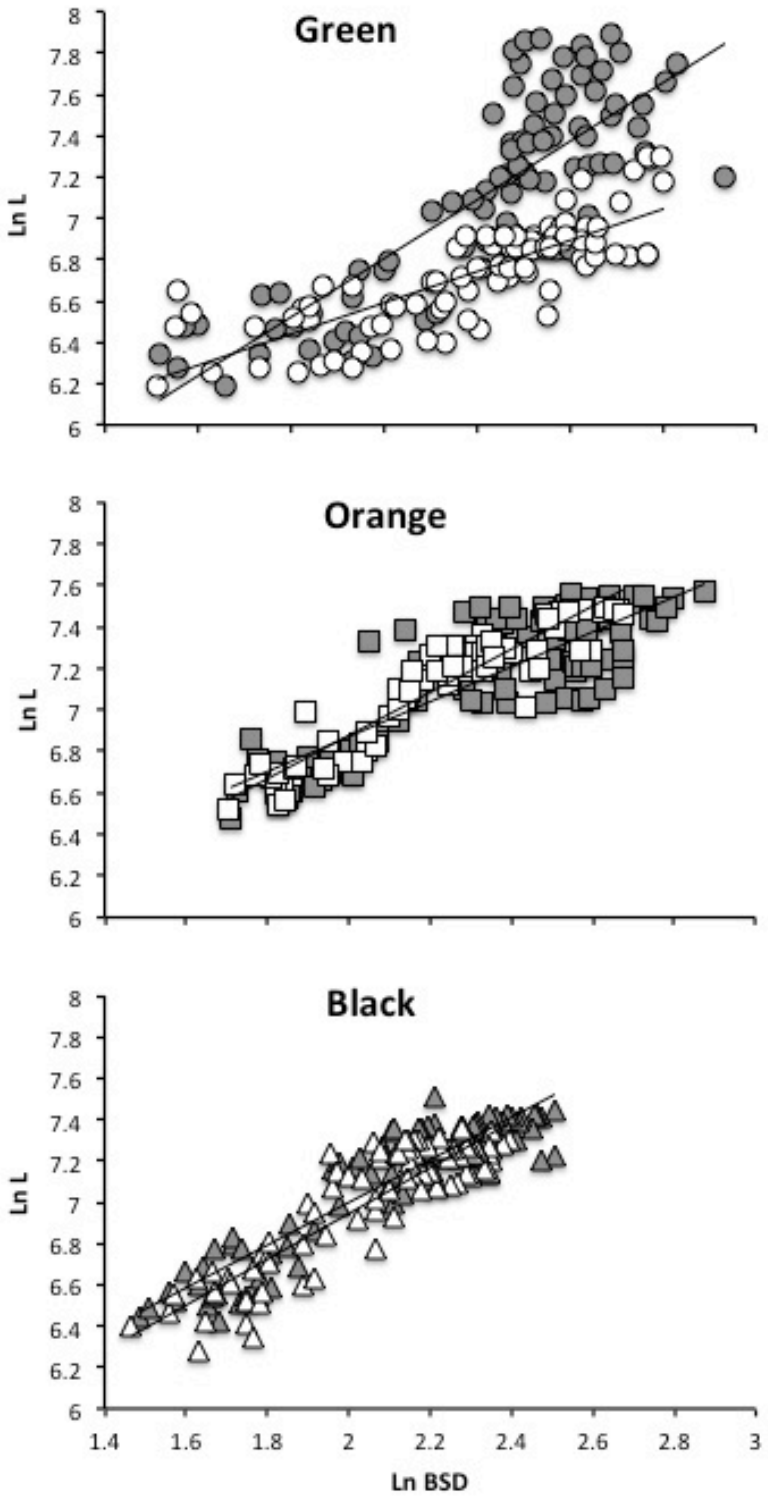

Figure 3.6 Log-Log plots showing the functional relationships between the (linear) growth parameters of main axis stem length (L) and basal stem diameter (BSD) across the experimental period for Green (top), Orange (centre), and Black (bottom) clones growing under the Cool (open symbols) and Warm (closed symbols) temperature regimes. Note the separation of Green data points for the two temperature regimes.

3.4 Discussion

In the three genotypes studied, growth temperature had a significant effect on the growth and development of the two-year-old clones growing in glasshouses under natural light and day length (DL). Temperature effects were genotype-specific. The clone-by-temperature interactions were significant for both the whole-plant and the shoot growth traits. At whole-plant level, effects were observed as changes in the magnitude of the growth response and shifts in phenology (developmental timing). At the shoot level, shoot elongation rates and brachyblast counts differed between the genotypes and between the temperature treatments.

Growth Phenology

Vegetative growth in the clones was promoted by long days (LD; averaging around 14 hours of day length; Phase One; Figure 3.4 a, b, & c). This is a well-known phenomenon in many species including woody plants (Thomas & Vince-Prue, 1996). In Mānuka, Zieslin & Gottesman (1986) also reported rapid vegetative growth in LD conditions together with high temperatures. BSD in the clones increased for several weeks after extension growth had almost ceased (Figure 3.4 d, e, & f). That is, photoperiod appeared to have an indirect influence on cambial activity in the clones. Indirect effects of photoperiod on cambial activity have been observed for many species. It is thought that DL acts indirectly on cambial activity by affecting the activity of the shoot apex since cambial activity is dependent on a supply of growth substances from the actively growing shoot (Thomas & Vince-Prue, 1996). Vegetative growth declined in SD conditions (averaging around 10 to 11.5 hours of day length; Phase Two; Figure 3.4 a, b, & c),) in all clones except Green Warm. SD do not typically induce complete dormancy in Mānuka affording small increases in shoot length (Zieslin and Gottesman, 1986), unlike many other temperate species. This response is similar to some evergreen tropical woody plants that do not experience winters of sufficient cold to prevent growth completely (Longman, 1978). A state of quiescence (near-

cessation of vegetative growth) occurred in Phase Three (Figure 3.4 a, b, & c) when the overnight temperatures were coldest i.e. the months of June through to September), with the exception of the Green Warm clones which appeared to have an indeterminate growth habit. This phenomenon is further discussed in Section 4.4 of Chapter 4.

The described phenological phases were less well defined in the Green line. That is, extension growth (both MAE and SER) in Green Warm was not typically slowed under SD conditions (Phase Two). In addition, there were shifts in phenology with changes in growth temperature evidenced as a later peak in vegetative growth (see also Allometry below) in the cooler conditions. In general across the clonal lines, Green appeared less sensitive to changes in DL and more responsive to changes in growing temperature. The implications of this on the floral traits are further discussed in Chapter 4.

Physiology

Vegetative extension growth in Orange and Black was relatively unaffected by the different growth temperatures. While extension growth in the (semi-prostrate) Green line was greater in the warmer growing conditions, lateral outgrowth was poor (Table 3.5). Across the clones, brachyblast numbers were more highly correlated with outgrowth of laterals than with primary shoot extension. Brachyblast counts reflect the *branching* process in Mānuka well since they describe the *functional relationship* between extension growth of first order (primary) shoots and that of subsequent second, third, fourth and fifth order laterals (using the notation of Hallé et al., 1978) that determine plant form or shape i.e. growth in both an upward (axial) and an outward (co-axial) direction. Brachyblast numbers may then provide a useful index for assessing the *branchiness i.e.* axial vs. co-axial growth*,* of Mānuka varieties of varying growth forms. Cultivar-specific *branchiness* may in turn be useful for predicting flower numbers to further estimate potential nectar production (discussed further in Chapter 4).

Evidence for brachyblast production as genotype-specific and environmentally-determined (temperature-sensitive) in the clones was provided by the absence of any G x E. Using the described statistical model of GLM, the standardised brachyblast counts were shown to be additive and may therefore be a useful physiological measure (index) of vigour or performance in Mānuka planted across multiple sites with varying environmental conditions. A limitation of GLM is that position effects ('Blocks') are necessarily treated as *fixed* rather than *random* terms. While more complex models (e.g. Hierarchical and Generalised Linear Models, HGLM and GLMM resp.) allow for 'Blocks' to be more suitably treated as random effects, these models were not further investigated due to time limitations. The expectation, however, would be for a shift in the mean values but no change in the non/significance of the interaction and main effects.

Overall (i.e. across the genotypes), standardised brachyblast numbers were 71% higher at the warmer temperatures, and this will have important consequences for the floral characteristics (see Chapter 4). Reported increases are for an entire growing season, that is, inclusive of both LD and SD growing conditions with an annual temperature differential of *ca.* 4.8°C. For comparison, Zieslin and Gottesman (1986) reported 50% increases in brachyblast numbers at the higher temperatures of 20/26°C (N/D) compared with 10/16°C, or a 10°C temperature difference, for an 8 week period in a 16 h photoperiod (LD).

GEI

The higher growth rates ($P < 0.10$ for MAE and $P \leq 0.05$ for BSD) in the warm-grown clones are typical of plants growing at warmer temperatures since plant growth, biomass production and allocation and their underlying metabolic processes are promoted by high temperature and restricted in cold (Franklin & Wigge, 2014). Indeed,

many species show marked temperature *acclimation*[12] when grown in different temperature regimes (Jones, 2014). All of the measured whole-plant and shoot growth traits were greater at the warmer growth temperatures, as is typical for temperate species. That is, a 4.8°C temperature differential in T_{MA} between the two growing-environments, within the temperature range 17°C – 22°C, resulted in 1.7- and 1.2-fold changes, on average, in extension and (linear) expansion growth of the main stem respectively. Data were also collected at an individual shoot level to determine any differences between the whole plant and lesser order shoot responses since differences might be expected in genotypes with strong apical dominance i.e. an inhibitory effect on lesser order or lower branch positions (*pers. comm.* Rod Thomas, Plant Physiologist). Also, replication of shoots rather than individuals has been shown to be a more efficient strategy in phenotyping studies (Kawamura et al., 2011).

Extension growth at the whole-plant and shoot levels were comparable (Figure 3.5a & Table 3.2). The observed growth responses were clone-specific i.e. the result of significant GEI (see below). Significant changes in rank order across the environments ($P \leq 0.05$) indicate that the temperature-induced growth responses of the clones are independent of their relative nectar-DHA concentrations. Rather, differences in the growth responses appear to be related to the different growth habits of the genotypes. That is, responses were shown to be similar in the upright Orange and Black clones compared with the more semi-prostrate Green clone. In general, growth habit is a heritable trait that can be modified by a plant's immediate environment (Bradshaw, 1965).

The G x E interactions in this study (Figure 3.4) for the whole plant growth traits of MAE and BSD are described as '*non-additive cross-over*' interaction patterns

[12] (by the definition of (Körner & Larcher, 1988)

(Malosetti et al., 2013). From a breeding perspective, this is the most important type of interaction since it implies that the choice of best genotype is determined by environment (Xu, 2010). Indeed, from the MET analyses, E and GEI contributed significantly to MAE ($P < 0.001$) while G did not (Table 3.2). For the BSD response, GEI was significant by LMM regression ($P \leq 0.05$) but not by Finlay-Wilkinson (FW) regression (Tables 3.10 & 3.13 resp.). The FW regression proved useful in partitioning the quantitatve data into genetic and environmental effects and their interactions. However, it does not take into account initial measurements (i.e. any covariates) or positonal effects within the glasshouse; LMM is able to accommodate both of these additional tems. The FW model is retained for presentation, because although it does not establish the statistical significance of the interaction, it does isolate the interaction and quantify it numerically (whereas LMM only establishes the statistical significance).

Usually, the aim of MET analyses is to find genotypes with large means and small sensitivities (GEI), to ensure a reliable crop under variable conditions. For the growth trait of MAE, the upright growing Orange and Black cultivars performed well in both growing-environments, that is, were more broadly adapted to the range of growth temperatures studied (T_{MA} of 16.9 °C to 21.7 °C)

Whether the temperature-induced phenotypes for MAE in the Green genotype are useful from a plantation perspective*,* is questionable. A reduced capacity for branching in Green relative to the upright growing cultivars suggests otherwise. Any limitations placed on branching (lateral outgrowth; see above) will result in fewer floral bud sites since sub/laterals (brachyblasts) are the flower-bearing shoots (see Chapter 4).

Similarly, for the growth trait of BSD, Orange performed ‘better’ at the warmer temperatures while Green and Black showed more stable rates of gain across the growing environments. Differences in the response norms for the genotypes appear to be related to differences in their growth habits and not to their relative DHA content (Figure 3.7).

Phenotypic Plasticity

The different phenotypes observed for the Green line growing in the two temperature-environments (the phenomenon of *phenotypic plasticity*[13]) were the result of altered (carbon) allocation patterns. That is, 1.8-fold higher specific growth rate ratios of main-stem length (L) to basal stem diameter (BSD) in the warmer growing conditions. In comparison, fractional growth in the Orange and Black lines was nearly isometric (approximately 1.0), and in both environments ($P < 0.001$; Table 3.6).

When comparing the relative performances of the genotypes across the growing-environments, ontogenetic shifts (size-related developmental changes across time) can be problematic (Weiner, 2004). By Weiner's definition (2004), changes in the slope/trajectory of Green for the growth trait of MAE then represent *true plasticity*. In comparison, intercept changes for Orange BSD represent *apparent plasticit*y that is, are size related and therefore not deemed biologically relevant in this context. Irreversible changes in phenotype (*developmental plasticity*) are associated with longer-term *acclimation*[14] processes and result in greater apparent acclimation of metabolism than phenotypic flexibility *(*reversible changes or *dynamic plasticity*) in for example photosynthetic and respiratory capacities (Athanasiou et al., 2010). Altered allometry in the Green line resulted in a greater allocation of resources to photosynthetic tissue (extension growth of shoot primary axes or leaves and twigs) over structural support (radial wood/trunk growth or secondary thickening) at the warmer growth temperatures. Temperatures may possibly have even been supra-optimal for this particular genotype since growth of primary shoots was exaggerated to such an extent that they were trailing across the floor of the glasshouse. Great adaptive significance has been given to the branching patterns of forest shrubs and understory trees. Minimisation of non-photosynthetic tissue in closed, shaded communities has adaptive function (Pickett and Kempf, 1980). However, assessing the

[13] The extent to which a phenotype is modified by the environment (Bradshaw, 1965).
[14] By the definition of Körner and Larcher (1988)

adaptive value of plastic responses in plants can be difficult (Bradshaw, 1965). In general, elongated phenotypes (taller growing varieties) are advantageous in dense swards, whereas bushier phenotypes have greater fitness in the absence of shading by neighbours (Sultan, 2000). To speculate then, it is likely that the lineage of the Green cultivar originates from a more open habitat. In an ecological sense, a greater capacity for *phenotypic plasticity* can facilitate range expansion of species (Price & Morgan, 2006) by enabling individuals to respond to varying environmental conditions across a diverse range of habitats (Williams & Black, 1993; Williams et al., 1995).

The more shrub-like habit of Mānuka is reflected in the functional (mathematical) relationship of $L \propto BSD^{1.15}$ by reduced major axis regression *(rma*), when averaged across the clone-temperature combinations (Table 3.5). 'α', is the constant of differential growth between L and BSD and describes the *ratio* of their specific growth rates i.e. 1/y (dy/dt) and 1/x (dx/dt), where the y variable is L and the x variable is BSD. An α of 1.15 is closer to published values for non-woody species than for trees. Shrubs have a reduced requirement for buckling (bending) limits compared with tall trees and this is reflected in a much-reduced α (Niklas, 1994). For example, Niklas (1994) lists regression coefficients determined by *rma* of 0.896 for 670 tree species; 1.29 for non-woody species (190 *spp*.); 1.26 for dicot herbs (117 *spp*.); 1.76 for palm; 0.474 for 375 dicotyledonous tree *spp*.); 0.430 for 105 conifer *spp*.; 1.1 for 40 *spp*. of moss; and 0.535 for gymnosperm-angiosperm trees (480 *spp*.).

3.5 Conclusions

In this study, growth responses in the clones to changes in long-term growing temperature were cultivar specific. The growth traits of stem length, stem diameter, node no., MIL, brachyblast number, and BSD were all positively correlated with temperature. While L, BSD, and MIL were subject to significant GEI, brachyblast numbers were shown to be additive. That is, in the absence of any detectable GEI, the effects of genotype and environment are independent of one another. In one of the genotypes (Green), shoot extension was shown to be a highly plastic trait and strongly determined by growth temperature ('Environment'; $P < 0.001$). From dimensional analyses, temperature-induced alterations in allocation patterns differed across the genotypes ($P < 0.001$). For example, ratios of specific L to BSD growth were greater at the warmer temperatures in the Green cultivar ($P \leq 0.05$). Altered allocation patterns in that line resulted in significant shifts in its phenology. In conclusion, differences in the temperature-induced allocation patterns between the genotypes appear to result from differences in plant form of the clones and do not appear to be correlated with their reported relative nectar-DHA.

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Appendix 3.2.1

Relative DHA content of the clonal lines

Table 3.7 Relative DHA content of the three clonal lines (developed and supplied by Comvita NZ Ltd.) used in the long-term Experiment #2. The Green, Orange and Black lines were chosen to represent low, medium and high DHA accumulators respectively. The low to high category labels for DHA content were arrived on from the best information available and were relative than definitive. Superscript 'a' is code relating to parental hybrid crosses (specific details confidential due to Plant Variety Rights).

Arbitrary Colour Code	Genotype Code [a]	Putative, relative DHA Content [b]
Black	28064-007	High
Orange	26011-820	Medium
Green	28076-029	Low

Appendix 3.3.1

Analysis of variance for the temperature data, Cool vs. Warm

Table 3.8 ANOVA table showing significant differences between the applied long-term Cool and Warm glasshouse temperature treatments.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Experimental Day stratum	366	6299.74	17.21	10.47	
Experimental Day x Units stratum Treatment	1	4263.55	4263.55	2593.98	<0.001
Residual	366	601.57	1.644		
Total	733	11164.86			

Day equivalents for the monthly growth increments

Table 3.9 Conversion of experimental days to date.

Experimental Day	Date
11	14-Nov-15
21	24-Nov-15
80	22-Jan-16
111	22-Feb-16
152	3-Apr-16
178	29-Apr-16
208	29-May-16
244	4-Jul-16
311	9-Sep-16
356	24-Oct-16

Appendix 3.3.2

The Thermal Time Model.

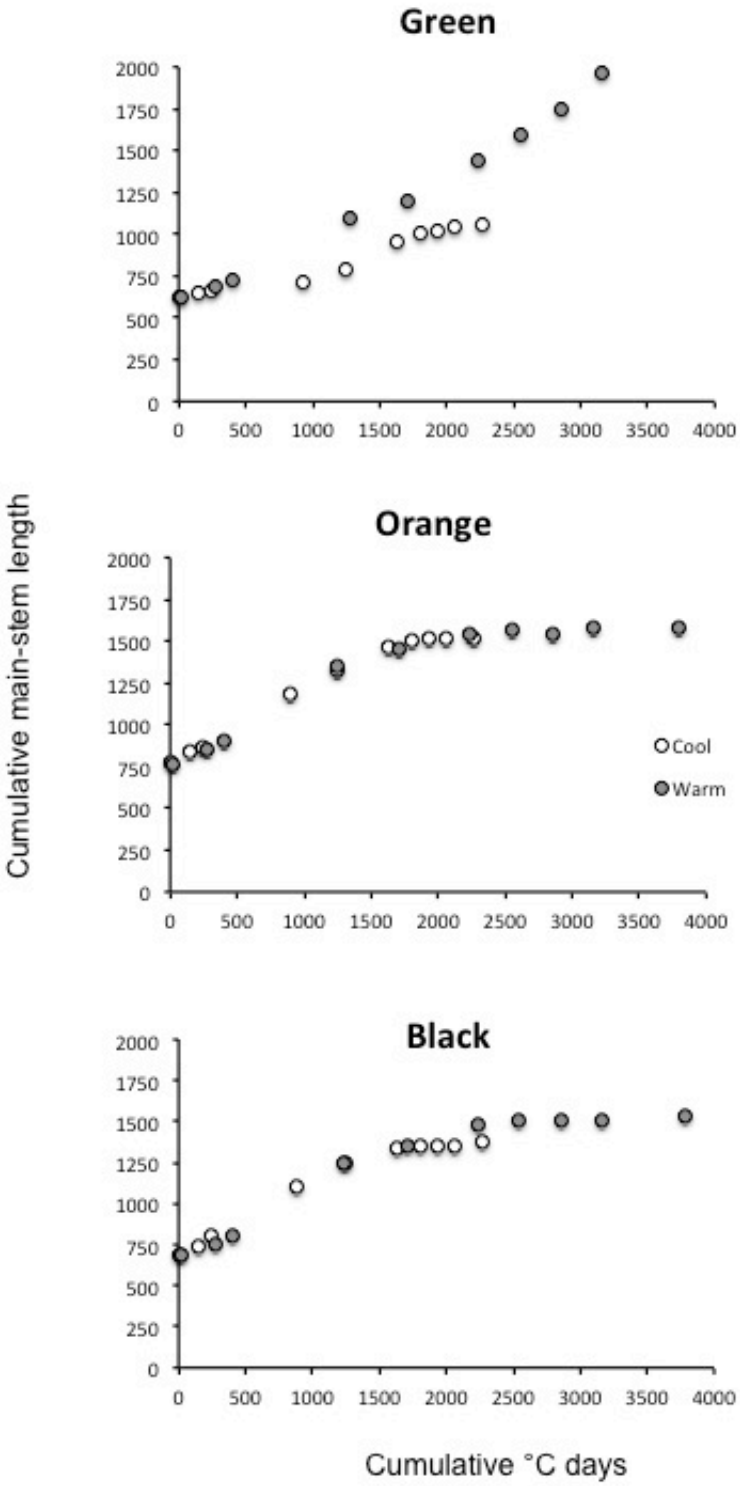

Figure 3.7 Cumulative main-axis stem length (mm) as a function of accumulated growing-degree-days (AGDD or cumulative °C days; The Thermal Time Model) for each of the three clonal lines. AGDD was calculated by summing Tm – Tb, where Tm is the average of daily (24h) maximum and minimum temperatures [(Tmax. + Tmin.)/2] and Tb is base temperature (10°C in this particular study). The point of inflexion indicates quiescence of extension growth.

Appendix 3.3.3

Distribution plots for the whole-plant growth parameters Final L and Final BSD

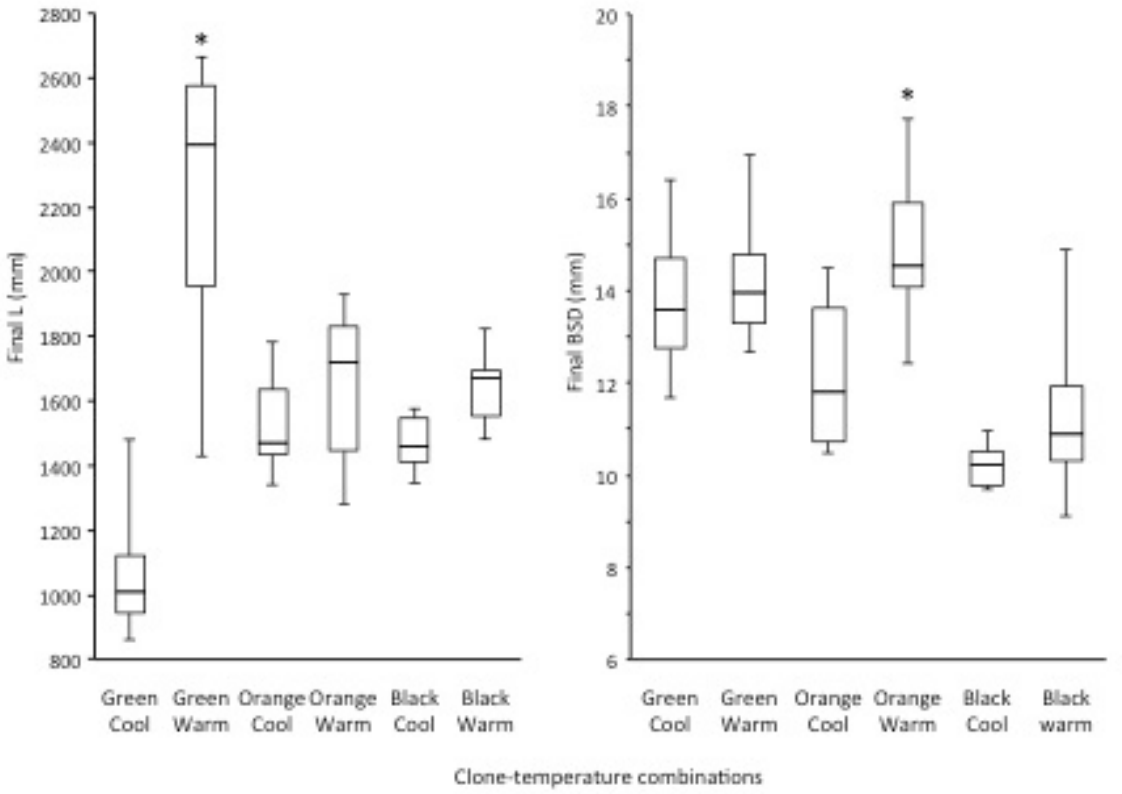

Figure 3.8 Box & whisker plots of the raw data (absolute values) for main-stem length (L; left plot) and basal stem diameter (BSD; right plot) at the end of the experimental period for the three clonal lines growing in the Cool and Warm temperature regimes. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Units are mm. * denotes significance within a line at the 95 % CI (P ≤ 0.05). N of 8, 7, 7, 8, 8, 7 for the clone-temperature combinations from left to right of plots.

Final L measurements

Table 3.10 Analysis of the variance for main-axis stem length (L) by a linear mixed model, for clones growing under the long-term Cool and Warm temperature regimes. Initial values at Day zero were used as covariates. Treatment and clonal line were fixed effects; columns and rows were used as random terms in the model.

Fixed Term	Wald stat	n.d.f.	F stat	d.d.f.	F pr.
Initial stem length	23.57	1	23.57	13.5	<0.001
Temperature treatment	18.79	1	18.79	2.0	0.051
Temperature x Clonal line	63.68	4	15.89	28.6	<0.001

Final BSD measurements

Table 3.11 Analysis of the variance for basal stem diameter (BSD) by a linear mixed model, for clones growing under the long-term Cool and Warm temperature regimes. Initial values at Day zero were used as covariates. Treatment and clonal line were fixed effects; columns and rows were used as random terms in the model.

Fixed Term	Wald stat	n.d.f.	F stat	d.d.f.	F pr.
Initial stem length	68.97	1	68.97	35.3	<0.001
Temperature treatment	11.85	1	11.85	35.1	0.002
Temperature x Clonal line	14.48	4	3.62	35.2	0.014

Glasshouse position effects for L

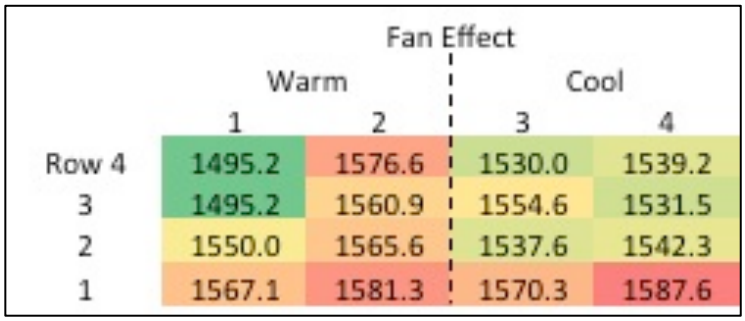

Figure 3.9 Heat map of position effects (row and column/fan) in the Cool and Warm glasshouses for main-axis stem length (L). Red cells represent larger values and green smaller, about the mean for each experimental unit (Block).

Glasshouse position effects for BSD

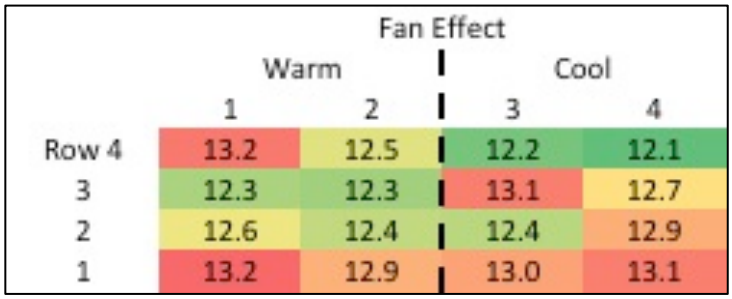

Figure 3.10 Heat map of position effects (row and column/fan) in the Cool and Warm glasshouses for basal stem diameter (BSD). Red cells represent larger values, green smaller about the mean for each experimental unit (Block).

Appendix 3.3.4

Regression equations for MAE and GR (BSD) by LMM

Table 3.12 Regression equations for whole-plant GR, main-axis extension (MAE; mm day^{-1}) and linear increases in basal stem diameter (BSD; mm day^{-1}).

Genotype	MAE	GR (BSD)
Green	y = 0.667 x − 10.0667	y = 0.167 x + 16.983
Orange	y = 0.0625 x + 0.9438	y = 1.438 x − 8.494
Black	y = 0.1667 x − 1.0167	y = 0.646 x + 2.885

Appendix 3.3.5

MET (Finlay-Wilkinson regression) analysis of variance for L and BSD

Table 3.13 Multi-Environment Trial (MET) analysis (employing Finlay-Wilkinson Regression) for main-axis stem length at the end of the experimental period (Final L) across the two growing environments, Cool and Warm.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Genotypes	2	33073.91	16536.96	0.25	0.777
Environment	1	2594001.67	2594001.67	39.75	<0.001
Sensitivities	2	2481097.16	1240548.58	19.01	<0.001
Residual	39	2544762.91	65250.33		
Total	44	7652935.64	173930.36		

Stability rankings (FW regression) for the L response

Table 3.14 Regression coefficients and genetic stability rankings for Final L across the two growing-environments, Cool and Warm, as determined by MET (Finlay-Wilkinson regression) analysis. A ranking of 1 is the most sensitive and 3 the least sensitive to changes in growing environment.

	Green	Orange	Black
Mean L (± 1 s.e)	1650 ± 66	1594 ± 66	1554 ± 66
Mean square deviation	140339	45312	10100
Regression coefficient	2.38	0.26	0.362
Rank	1	3	2

Stability rankings (FW regression) for the BSD response

Table 3.15 Multi-Environment Trial (MET) analysis (employing Finlay-Wilkinson Regression) for basal stem growth at the end of the experimental period (Final BSD) across the two growing environments, Cool and Warm.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Genotypes	2	95.95	47.98	20.02	<0.001
Environment	1	21.43	21.43	8.94	0.005
Sensitivities	2	9.23	4.61	1.93	0.159
Residual	39	93.47	2.40		
Total	44	220.08	5.00		

Appendix 3.3.6

Analysis of variance for shoot elongation rate by LMM

Table 3.16 Analysis of the variance for shoot elongation rates by a linear mixed model, for clones growing under long-term temperature regimes of Cool and Warm.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr.
Row	0.38	3	0.13	28.6	0.943
Temperature treatment	26.07	1	26.07	27.9	<0.001
Temperature x clonal line	37.00	4	9.24	29.8	<0.001

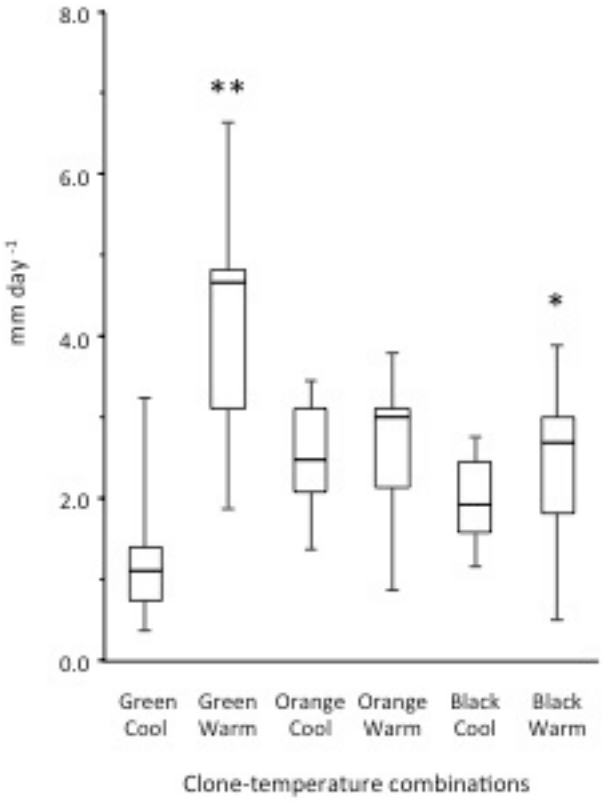

Figure 3.11 Box & whisker plots of the raw data values for shoot elongation rates for the three clonal lines growing in the Cool and Warm temperature regimes. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Units are mm/day. ** and * denote significance within a line at the 95 % and 90% CI respectively (P ≤ 0.05 and P < 0.10 resp.).

Appendix 3.3.7

GLM output for brachyblast numbers per node of primary shoot length

Table 3.17 Analysis of the variance for standardised brachyblast numbers (per primary shoot node), by a GLM, for clones growing under long-term Cool and Warm temperature regimes.

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Temperature	1	17.6021	17.6021	26.28	<0.001
+ Block x temperature	14	11.7146	0.8368	1.25	0.265
+ Line	2	58.2527	29.1263	43.49	<0.001
+ Temperature x line	2	1.2857	0.6429	0.96	0.389
Residual	61	40.8522	0.6697		
Total	80	129.7072	1.6213		

Temperature terms by phase, treatment and clone

Table 3.18 Temperature minima (Tmin.) and maxima (Tmax.) for the Cool and Warm long-term treatments by phase and clone (additional to Figure 3.4 & Table 3.1). Units are °C.

	Phase One		**Phase Two**		**Phase Three**	
	Cool	Warm	Cool	Warm	Cool	Warm
Green (Tmin. Tmax.)	7.8 32.8	12.8 34.5	5.0 24.5	10.5 30.0	7.3 25.3	17.8 29.0
Orange & Black (Tmin. Tmax.)	7.8 31.5	12.8 33.8	6.8 32.8	18.5 34.5	5.0 25.3	10.5 29.8

Chapter 4 Shifts in Flowering Phenology in Response to Temperature Changes

Abstract

Variation in flowering time both within and between populations of wild Mānuka (*Leptospermum scoparium)* is historically attributed to geographical and environmental gradients. Yet, variation in flowering times within populations, between individuals and between years, has also been attributed to genetics, environment or both (Primack, 1980). In this study, the flowering responses of three high performance Mānuka cultivars growing at two different temperatures were examined. Clones varying in their nectar dihydroxyacetone (DHA) concentration were grown under natural day length and radiation in overnight winter temperatures above and below 10°C in glasshouses (Cool and Warm regimes respectively). Open flower counts on shoots were modelled across time using Gaussian standard curves; the shapes of the curves were unique to each clone-treatment (r^2 = 63%; P < 0.001). The average time to peak flowering was significantly reduced, by 56 days on average, in clones growing at the warmer temperatures (P < 0.001). Genotype (G) accounted for 19%, Environment (E) 52%, and their interaction (GEI) 6% of the observed variation in timing (P < 0.001). Peak flower numbers also differed between genotypes and temperature regimes, and were strongly genetically determined (40% G and 28% GEI; P < 0.001). The bloom period duration was lengthened in one of the clones and shortened in another, at the higher temperatures, i.e. a strong GEI was observed. Flowering was protracted in the semi-prostrate line at the warmer temperatures, and the observed uni-modal flowering habit was associated with an indeterminate growth habit. By analogy from studies on roses, it is suggested that the differences in flowering behaviour observed in the Mānuka clones in response to temperature changes may be linked to differences in plant form.

4.1 Introduction

While *floral induction* in Mānuka is primarily photoperiod driven and temperature-modified, temperature is the main factor affecting *flower development,* such that flowering is constrained by low temperatures (Primack, 1980; Zieslin and Gottesman, 1986). For example, in glasshouse and growth room studies, Zieslin and Gottesman (1986) reported floral bud initiation at both low (20/10°C) and high (26/20°C) day/night (D/N) temperatures in photoperiods of shorter than 12 hours (h). Initiation was completely absent in 16 h photoperiods (long days, LD) compared with an 8 h (SD) photoperiod at similar temperatures of 26/20°C. Flowering was also absent for the same 16h photoperiod at the lower D/N temperatures of 16/10°C. In Israel, at natural day lengths of 13–15 h, floral bud initiation occurred at the lower temperatures (20/10°C) but not the higher temperatures (20/16°C). That is, low temperature had an inductive effect while flowering was prevented in high summer temperatures (described as 'Mediterranean-like conditions' in that study). Further evidence for photoperiod and temperature interactions was recently provided by Bicknell & Jaksons (2018) who reported a reduction in the inductive influence of a 9h photoperiod by lower temperatures i.e. 10°C but not 20°C.

Variation in *flowering time* among adjacent populations of wild NZ Mānuka was attributed to geographical and environmental gradients, in particular to changes in altitude (Primack, 1980). For example, in a single population located in the Upper Waimakariri River Basin, South Island, New Zealand, flowering occurred nine days earlier and was 17 days shorter in duration in a warm dry summer compared with the cool damp summer of the previous year. In addition, flower numbers were significantly reduced in the warmer year. The flowering rank-order of individuals in the different years was positively correlated (Primack, 1980). In the same population, plants at the base of the hill (at an altitude of 600m) flowered approx. three weeks earlier than plants at the top of the hill (850m). From Primack's work it is not possible to separate

differences due to G, E, or both. In unpublished work from field trials conducted by the Massey University Mānuka Research Team (MUMRT, formed in 2011 as part of a Primary Growth Partnership between the NZ Government and Industry), altitude and exposure had a significant effect on the flowering period of plantation Mānuka. Such was the effect of altitude at one particular site (Tutira, Hawkes Bay, NZ) that the rank order of flowering among the varieties was changed (*pers. comm.,* MUMRT*).* From herbarium specimens, flowering time also differs with latitude i.e. between the North and South Islands of NZ. *Peak flowering* is reported to occur in November–December in the North and in December-January in the South Island (Primack, 1980).

Glasshouse studies by Nickless et *al.* (2017) involving clones of cultivated varieties recently identified the floral characteristics of *flower density* (flower number/20cm of stem length at peak flowering) and *duration of the flowering period* as significant predictors of plant nectar yield.

At the time of starting this project, there was no published data on flowering phenology with respect to temperature changes in *L. scoparium* in controlled environments. In this study we attempt to quantify the relative contributions of G, E and GEI to the different phenophases. The objective of this chapter is to examine the effects of long-term growth temperature on flowering in three Mānuka clonal lines. The key question addressed is: Do floral characteristics differ in clones subjected to different growth temperatures.

4.2 Materials and Methods

4.2.1 Plant Material & Temperature Treatments

Plants were those reported on in Chapter 3 (Section 3.2.1). That is, Green, Orange, and Black clones respectively. The eight clonal replicates per treatment (Cool and Warm regimes) entering their second year of flowering were growing under natural radiation and day length. The experimental layout was a randomised block design. Blocks (the experimental units) were comprised of one clone from each of the three lines. Plants were initially assigned to the blocks on the basis of their initial size [measurements of plant height (mm) and cross-sectional area of the basal stem (mm^2)]. For further experimental details see Section 2.6.2.

4.2.2 Floral Traits

The measured floral parameters were 'total bud counts,' and 'open flower counts' across time.

4.2.2.1 Floral Bud Counts

Floral bud numbers on individual shoots were recorded around mid-June of 2016 in Orange and Black and late July 2016 for Green, when all shoots except those of Green Warm were at quiescence. Dates were chosen primarily for ease of counting at a stage when buds were clearly discernible as floral buds. Bud counts for Green Warm do not fully represent the total number of buds as shoots continued to elongate and form additional floral buds.

4.2.2.2 Open Flower Counts

Open flower numbers were recorded on individual shoots at regular intervals (every four to seven days), on new growth produced in the 2015/16 growing season i.e. in the spring and summer. Flower counts were recorded for two complete (from first to last open flower) floral flushes, termed FF1 and FF2. Subsequent floral flushes on growth produced in the 2016/17 growing season were noted but data was not collected for

these. A flower was considered 'open' when its status, hermaphrodite or staminate (Primack & Lloyd, 1980), was clearly determinable i.e. all flower parts visible. Flower counts were extended beyond the 12-month experimental period (up until the 6th of January, 2017; with continuation of the temperature treatments) to obtain *complete* floral curves since first and last occurrence dates are important to determine any shifts in phenology (Clark & Thompson, 2011).

4.2.3 Statistical Approach

Data for each of the flowering trait variables were analysed as a whole i.e. as single data sets (see Section 2.8) including all three clonal lines. All reported times (experimental days) to floral events are average event times for *N* number of replicated clonal plants, and *n* number of shoots per plant. This was explained in Section 2.8. The measured floral parameters are summarised in Table 4.1 together with units, developmental stage at sampling, data distribution patterns, and the statistical model used for the analysis of each trait. The most appropriate statistical model for each of the trait variables was determined by the distribution pattern of the data as explained in Sections 4.2.2.1 – 4.2.2.5 below.

Table 4.1 Summary table of the floral traits measured on individual shoots in the Long-term Temperature Experiment (Experiment # 2), together with units, developmental stage at sampling, data distribution patterns, and the statistical model used for each trait variable (LMM, Linear Mixed Model; GMM, Gaussian Mixed Model; GLM, Generalised Linear Model). G, O, and B represent the clonal lines Green, Orange and Black respectively.

Trait	Units	Developmental stage	Data Transformation	Data Distribution	Statistical Model
Floral bud no.	Counts	Quiescence (except Green Warm)	None	Poisson	GLM
Floral progression	Exp. Days	Blossoming		Normal (bimodal)	GMM
Start of blossoming	Exp. Days	Beginning of FF1	None	Exponential	GLM
Duration of blossoming	Exp. Days	FF1 &FF2	None	Exponential	GLM
Peak flower no.	Counts	Peak flowering	None	Poisson	GMM
Peak flowering time	Exp. Days	Peak flowering	Gaussian equations None	Normal (bimodal)	GMM

4.2.3.1 Floral Bud Counts

Floral bud counts were analysed by a GLM using a Poisson distribution and a logarithm link function algorithm. Shoot order, being main-stem or side-branch (Figure 4.1) did not significantly affect the total floral bud numbers in preliminary models and so was dropped from the terms in the final model.

4.2.3.2 Floral Curves

To model the progression of flowering, double Gaussian standard curves were fitted to the complete flowering data using the method of Proïa et al. (2016) who described Gaussian mixture models (GMM) as probabilistic models smoothing observations and highlighting hidden structures. They considered that the *waves* mechanism suited to statistical modelling also has a biological and genetic interpretation. Significance testing for differences in the timing of peak flowering for each of the curves was done using the method of Payton, Miller, and Raun (2000). 85% ($\alpha = 0.05$) confidence intervals were evaluated for the peaks by multiplying the standard error for each peak by 1.6. The null hypothesis, that the peak flowering times are similar, was rejected if the intervals failed to overlap. Non-linear, bimodal equations of the form:

$Y = A + B * PRNORMAL (X; M; S^2) + C * PRNORMAL (X; N; S^2)$ were fitted to the grouped data: Green Cool (GC), Green Warm (GW), Orange Cool (OC), Orange Warm (OW), Black Cool (BC), and Black Warm (BW), where Y variable is 'number of open flowers' on any given 'experimental day' (the X variable). A, B, and C are constants, M is mean of the first wave, N is mean of the second wave, and S is standard deviation.

4.2.3.3 Blossom Start and Bloom Period Duration

Blossom Start times were recorded as the number of Experimental Days to the appearance of the first flower on an individual shoot. A General Linear Model (GLM) was used to analyse the data, after removal of a single outlier (large residual identified by Genstat®) to satisfy normality requirements of the model, since the fitted Gaussian curves appeared inadequate for determination of an *actual* start date i.e. they

displayed a very long lead-in to floral progression. The GLM used an exponential distribution (as encountered in *flowering-time* data) with a reciprocal link function algorithm. Pairwise comparisons were carried out on the *post hoc* Fisher's LSDs to test for significance. A similar model was used to analyse duration of the blooming period. *Bloom Period Duration* was calculated as the number of experimental days between the recorded start and end dates for each clone-treatment.

4.2.3.4 Peak flower Numbers

The *Peak flowering* event is defined here as 'the maximum number of open flowers recorded on any day' and is synonymous with '*main flowering period'* used by other authors. Peak flower numbers for each clone-treatment were obtained from the GMM output (see above). For simplicity, standard errors and significant tests for peak flower numbers were calculated from a GLM performed on the raw (empirical) data. The model included 'Clonal Line' nested in 'Treatment', and 'Blocks' as fixed effects, and used a Poisson distribution with a logarithmic link function algorithm. 'Clonal Line' and 'Treatment' effects were predicted using the predict function. Any significant differences between the groups were tested by pairwise comparisons of the *post hoc* Fisher's LSDs. Floral density (open flower numbers at peak flowering standardised to 50mm of shoot length) was analysed by GLM with 'Groups' using a similar model.

4.2.3.5 MET Analyses

Simple Multi-Environment Trial (MET) analyses were performed on the variables of peak flowering time and peak flower number.

4.3 Results

4.3.1 Floral Induction

Flowering was induced in all clones as deduced from the appearance of floral buds on shoots (see below). The progression toward *flowering* in the Mānuka clones was not sufficiently explained by a thermal time model (from the MAE growth curves presented in Figure 3.8 of Appendix 3.3.2). This concept is further discussed in Sections 4.3.3 and 4.4 below.

4.3.2 Bud Formation

4.3.2.1 Floral buds formed on short shoots under short day (SD) conditions with declining growth rates

Floral bud formation occurred in SD conditions and coincided with declining GRs (Phase Two; Figure 3.4 of Section 3.3.2). Visual Bud Appearance (VBA) was first observed on the 14th of March, 2016 for Green Warm (GW), the 16th of March for Green Cool (GC), the 14th of April for Black Cool (BC), the 16th of April for Orange Warm (OW), the 10th of May for Black Warm (BW), and the 11th of May, 2016 for Orange Cool (OC). Open flowers were also noted on Green Cool in late April (20th April, 2016) on already established shoots i.e. older plant parts, but not on the selected new shoots. Floral buds formed on growth produced in the current growing season i.e. the spring and summer of the 2015/16 main growth period (Figure 3.2 in Chapter 3). Buds were predominantly terminal on short branchlets (termed *brachyblasts)*, and to a much lesser extent axillary buds. In this study, shoot apices [main-stem; and 1st, 2nd, and 3rd (where present) order laterals] always terminated in a floral bud in Black, almost always in Orange, and less often in Green.

Figure 4.1 Floral architecture of the Mānuka clones. Flower buds were predominantly terminal on short branchlets (termed brachyblasts) on new growth (primary shoot axes) produced in the 2015/16 growing season.

4.3.2.2 Ratios of brachyblast to floral bud numbers were significantly higher in Green Warm

Ratios of brachyblast to floral bud numbers describing the relationship between lateral growth (production of 2nd order and in some cases 3rd order branchlets) and flower production, were a little below 1.0 in all but GW ($P \leq 0.05$; Table 4.2). Ratios of higher than 1.0 in GW are an indication that some growing points were not reproductively competent, that is, they remained vegetative or *indeterminate*.

Table 4.2 Ratios for brachyblast to floral bud numbers in the three clonal lines under the Warm and Cool regimes. Ratios were calculated from the mean trait values as determined by LMM. * denotes significance within a clonal line at the 95% CI (P ≤ 0.05) by a student's t-test.

Clone	Cool	Warm
Green	0.87	1.33 *
Orange	0.92	0.92
Black	0.89	0.92

4.3.2.3 Floral bud numbers were greater in the Warm regime for Green and Black

Treatment, clonal line, and their interactions had a significant effect on the total floral bud numbers ($P < 0.001$ for the main effects and $P \leq 0.05$ for the interaction; Table 4.6 of Appendix 4.3.2). That is, there were significantly greater numbers of floral buds in GW compared with GC, and in BW compared with BC. Position effects within the glasshouses were not significant.

When standardised for shoot length, the temperature x clone interactions are no longer significant i.e. an absence of GEI. However, the effects of position in glasshouse, temperature, and clone are significant ($P \leq 0.05$; $P < 0.001$; $P< 0.001$ resp.; Table 4.7 of Appendix 4.3.2). That is, trends for greater floral bud numbers per 50 mm of (primary) shoot axis in Warm compared with Cool in all three clones. However, significance was established for Black only (by pairwise comparisons; $P \leq 0.05$; Table 4.3). Across the clonal lines, numbers were greatest in BW, and least in Green (GC and GW). Note that, bud counts in GW may not represent final numbers since shoots were continuing to grow and develop (elongate and form floral buds) at the time of data collection. Individuals of this particular clone-treatment combination appeared to have no quiescent growth period (Section 3.3.2).

Table 4.3 Mean standardised floral bud counts on shoots (buds per 50 mm of primary shoot length) ± 1 s.e. for the three clonal lines in the Cool and Warm regimes. Same letter indicates no-significant difference at the 95% *C*I ($P \leq 0.05$).

Clone	Cool	Warm
Green	7.8 ± 0.8 [c]	9.2 ± 1.1 [c]
Orange	24.3 ± 1.8 [b]	28.0 ± 2.0 [b]
Black	22.5 ± 1.4 [b]	34.7 ± 1.9 [a]

Total floral bud numbers in Orange and Black (Green not included) were highly correlated with shoot length (ShL) and shoot diameter (ShDi; $P < 0.001$; Table 4.4). Bud numbers were also correlated with average internode length (Int; $P \leq 0.05$). The data for the measured growth parameters was presented in Chapter 3.

Table 4.4 Correlation matrix for the growth and flowering parameters for the Orange and Black lines only: average internode length (Int; mm), shoot elongation rate (SER; mm_day^{-1}), number of nodes (Nod), shoot diameter (ShDi; mm), shoot length (ShL; mm), and total floral bud number (Bud). **denotes significance at the 99.9% *CI* (*P* < 0.001), and * at the 95% *CI* (*P* ≤ 0.05).

Int	-				
SER	0.3668	-			
Nod	0.0181	0.6178	-		
ShDi	0.4097	0.1964	0.2899	-	
ShL	0.6807 **	0.7366 **	0.6411	0.5639	-
Bud	**0.5314 ***	0.3323	0.4189	**0.7010 ****	**0.7499 ****
	Int	**SER**	**Nod**	**ShDi**	**ShL**

4.3.3 Floral Progression

Blossoming in the clones (except GW) occurred when the vegetative growth rates approached zero (GR < 1.0 mm day^{-1}) in GC, OC, OW, BC, and BW (in Phase Three of Figure 3.4).

4.3.3.1 Shapes of the phenological flowering curves are unique to each clone-treatment

Curves from modelling of flower numbers across time for the two complete floral flushes (FF1 & FF2) using double Gaussian standard curves (refer Section 4.2.2.2) are shown in Figure 4.2. The model explained 63% of the data variability (*P* < 0.001). The model ANOVA appears in Table 4.8 of Appendix 4.3.3. The 2- and 3-way interactions between clone, temperature, and experimental day are highly significant *(P* < 0.001), as are the shapes of the curves (the 'Separate non-linear 'term; *P* < 0.001). An example equation for the double Gaussian model (for Orange Warm) is given below:

$Y = 810 + 1194 * PRNORMAL [X, 228.6, (9.57)^2] + 509 * PRNORMAL [X, 249.4, (9.57)^2]$,

where Y is the number of open flowers and X is any given Experimental Day. PRNORMAL is the normal probability distribution for (the value X, the arithmetic mean, and the square of the standard deviation). Values in the model for A, B, C, M, N, and S were discrete for each clone-treatment (Group). A, B, and C are fitted constants, M and N are the mean number of open flowers for FF1 and FF2 respectively, S is the standard deviation. Fitted values for each Group appear in Table 4.9 of Appendix 4.3.3.

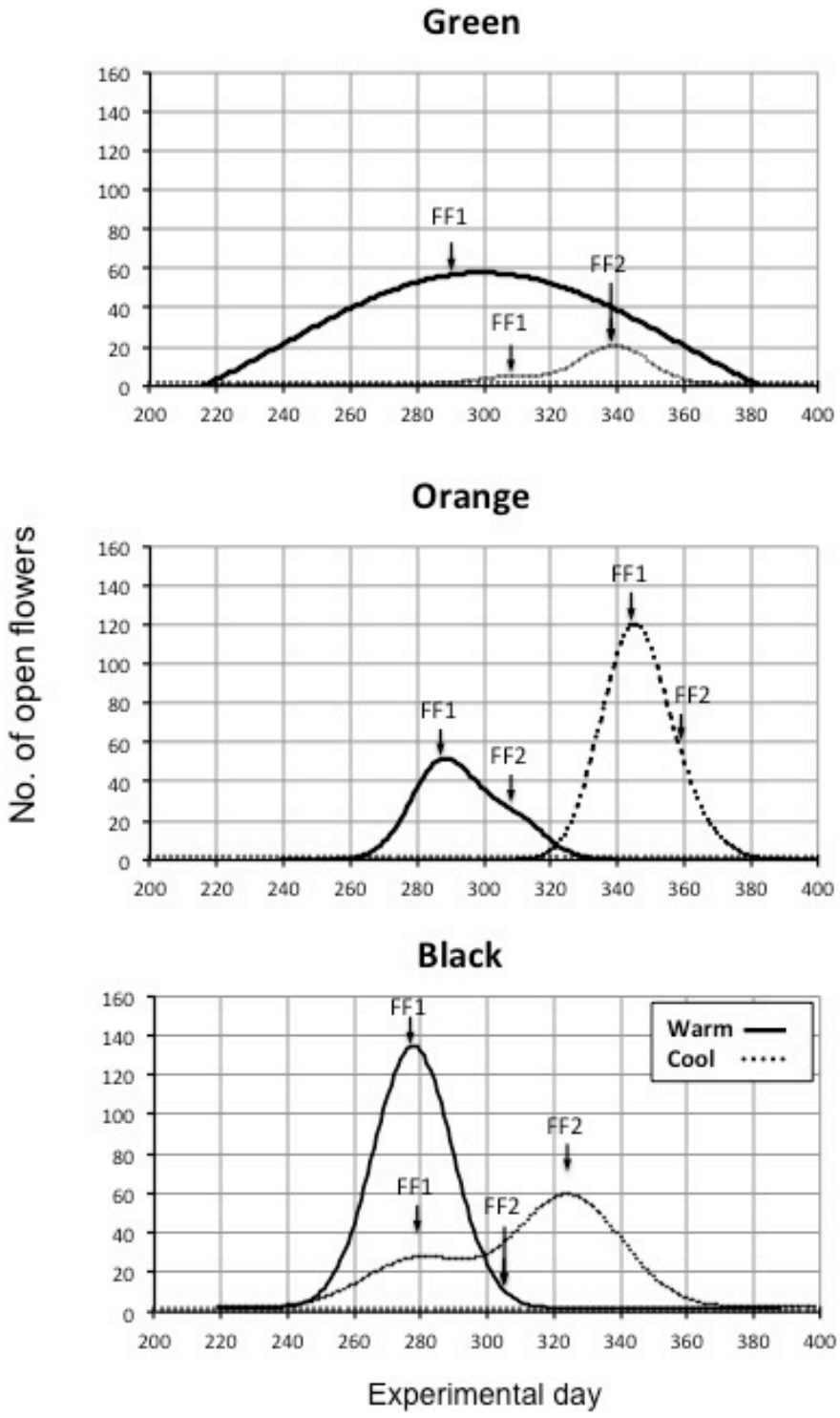

Figure 4.2 Complete phenological flowering curves for each of the clone-treatments as modelled by a Gaussian mixed model (GMM). Open flower numbers across time were recorded on shoots at four to seven day intervals. Experimental Day 200 equates to the 21st of May 2016; arrows mark the position of the respective floral flushes (FF1 & FF2). The predicted FF2 peak for Green Warm occurs at Day 407 (not marked on graph, see text). Actual values for curve parameters for each clone-treatment are given in Table 4.9 of Appendix 4.3.3).

4.3.3.2 Temperature affected both the magnitude and the timing of the different floral flushes

Temperature affected the timing of FF1 and FF2 within each of the clonal lines i.e. the first and second peaks or waves of flowering respectively for each clone-treatment combination (Figure 4.2). Dual peaks are distinguishable for GC, OW, and BC. In contrast, GW, OC, and BW appear to be uni-modal (single-peaked). Occurrence times for FF1 between the two temperature regimes were not significantly different for the Green or Black lines. However, FF2 was significantly earlier in the Cool regime for Green [339 ± 3 Experimental Days, and 407 ± 43 Experimental Days for GW (not marked on graph; see explanation below)], and in the Warm regime for Black (305 ± 2 Experimental Days, and 324 ± 2 Experimental Days for BC; $P < 0.16$). Significance testing is explained in Section 4.2.3.2. In the Orange line, FF1 was significantly earlier in OW (287 ± 2 Experimental Days, and 344 ± 9 Experimental Days in Cool) while the timing of FF2 was similar in OC and OW (359 ± 75 and 308 ± 7 Experimental Days resp.; $P < 0.16$). Variability in the timing of the peaks (as indicated by the standard errors for *M* and *N*; Table 4.9 of Appendix 4.3.3) was greatest for OC FF2, and for GW, FF1 & FF2.

FF1 was the larger of the two peaks in GW (56.7), OC (127.7), OW (63.2) and BW (59.6); FF2 was the larger peak in GC (20.8) and BC (134.5). Units are open flower numbers; an estimated average standard error of observations of ± 17.4 applies. A double Gaussian doesn't appear to fit the Green data as well as the other lines. For example, the predicted FF2 peak (*N* in the model) for GW on Experimental Day 407 falls outside of the standard curve (Figure 4.2) with a predicted value of -16.3 open flowers (see Discussion, Section 4.4 of this chapter).

4.3.4 Temperature Effects on the Major Phenophases

The timing of the major floral events (phenophases) of *Blossom start, Bloom period duration, and Peak flowering time, t*ogether with *peak flower numbers* and *floral density*

at peak flowering are summarised in Table 4.5. Refer to Section 4.2.3 for statistical approach for each of the variables. Significant trends only in each are discussed below.

Table 4.5 Averages for each of the floral events are as indicated, for the three clonal lines under Cool and Warm temperature regimes. Units for Blossom start, Bloom Period Duration, and Time to Peak Flowering are Experimental Days. Peak flower numbers are standardised to 50 mm of shoot length. Floral Density is number of open flowers/ 50 mm of primary shoot length at peak flowering. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05; see footnote). For the time parameters 'a' is earliest and 'c' latest.

	Green		Orange		Black		Section Discussed
Floral Event	Cool	Warm	Cool	Warm	Cool	Warm	
Blossom start	283 ± 7 [b]	237 ± 6 [a]	248 ± 7 [a, b]	263 ± 8 [b]	230 ± 6 [a]	241 ±6 [a]	4.3.4.1
Bloom duration	104 ± 6 [c]	184 ±11 [a]	157 ± 9 [a]	127 ± 10 [b]	116 ± 7 [c]	111 ± 6 [c]	4.3.4.2
Peak flowering time	339 ± 3 [c]	301 ± 3 [b]	345 ± 3 [c]	289 ± 3 [a]	324 ± 3 [b]	278 ± 3 [a]	4.3.4.3
Peak flower no.	21 ± 4 [c]	58 ± 6 [b]	128 ±14 [a]	60 ± 9 [b]	60 ± 7 [b]	135 ± 11 [a]	4.3.4.5
Floral density	4 ± 0.4 [d]	2 ± 0.4 [e]	14 ± 1 [a]	5 ± 0.7 [c, d]	7 ±0.6 [c]	10 ± 1 [b]	4.3.4.5

Footnote to Table: Standard errors for Time to Peak Flowering and Peak Flower No. were estimated from a GLM, while the significance tests of *P* < 0.16 used the method of Payton et al., (2000); refer to Section 4.2.3.2.

4.3.4.1 Blossom start times were significantly earlier in Green Warm

The temperature x clone interactions on the *Blossom Start* times i.e. the number of Experimental Days to 'First Open Flower' and position effects within glasshouses (Columns due to glasshouse fans) were both highly significant (*P* < 0.001, Table 4.10 and Figure 4.4 resp. in Appendix 4.3.4), by a GLM (Section 4.2.3.3). Within the clonal lines, temperature effects were significant for the Green line only, in which start dates were significantly earlier (by 28 days) in GW compared with GC (237 ± 6 days and 283 ± 7 days resp.; *P* ≤ 0.05; Table 4.5 above). Refer to Table 4.11 of Appendix 4.3.4 for Date and Day of Year equivalents.

Blossoming occurred progressively on shoots over windows of 121, 100, 61, 36, 42, and 29 days for GW, GC, OC, OW, BC, and BW respectively (see distribution plots; Figure 4.6 in Appendix 4.3.4). Variability was greatest among the Green clones (*CV* = 0.12, 0.17 in Cool and Warm resp.) and least in Black. Between the glasshouse growing-environments, variance tended to be greater in the Cool regime. Genotype rank orders differed within the two environments (B < O < G in Cool, and B = G < O in Warm.

4.3.4.2 *Duration of the bloom period was lengthened in Green and shortened in Orange, at the warmer temperatures*

Temperature and temperature x clone interactions had a significant effect on the duration of the bloom period ($P \leq 0.05$ and $P < 0.001$ respectively). Block effects were also significant ($P \leq 0.05$; Table 4.12 of Appendix 4.3.4). The bloom period durations were increased by 80 days at the warmer temperatures in Green (184 ± 11 days compared with 104 ± 6 days in GC; $P \leq 0.05$; Table 4.5), and by 30 days at the cooler temperatures in Orange (157 ± 9 days compared with 127 ± 10 days in OW; $P \leq 0.05$). The longest bloom periods occurred in GW and the shortest in BC and BW, which were similar. Variability was greatest in GC ($CV = 0.41$; refer to distribution plots, Figure 4.7 in Appendix 4.3.4).

4.3.4.3 *Peak flowering occurred earlier and during the first wave of blossoming (FF1) in Warm, in contrast to a later peak and during the second wave of blossoming (FF2) in Cool*

Temperature effects were significant for the timing of the *peak flowering* event in all three of the clonal lines (at the 84% *CI*; $P < 0.16$; Figure 4.2). Significance testing used the method of Payton et al. (2000) and was explained in Section 4.2.2.2. 'Day of Year' and 'Date' equivalents appear in Table 4.13 of Appendix 4.3.4. The p*eak flowering event* occurred during FF1 in GW, OC, OW, and BW and during FF2 in GC and BC (Figure 4.2 and Section 4.3.3.2 above). Overall, peak flowering occurred 56 days earlier, on average, in the Warm regime (means of 286 ± 2 Experimental Days in Warm and 344 ± 1 Experimental Days in Cool; $P < 0.16$). There was a general tendency in the warmer temperatures for blossoming to begin more abruptly and tail off more gradually compared with a more gradual beginning and an abrupt tailing off in the cooler growing conditions. In general, peak flowering occurred earlier and during the first wave of blossoming (FF1) in the warmer temperatures and peaked later and during the second wave of blossoming (FF2) in the cooler growing conditions

Within the clonal lines, the number of experimental days to peak flowering was reduced by 56 days in OW, 46 days in BW, and 38 days in GW ($P \leq 0.05$; Table 4.5 above). Within the individual growing environments, in the Cool regime, peak flowering time

was significantly earlier for BC (on Experimental Day 324) than GC or OC (Day 339 and 345 resp.). In the Warm regime, peak flowering occurred significantly later in GW (on Experimental Day 301) compared with OW and BW, which were similar (Day 289 and 278 resp. $P \leq 0.05$). That is, rank orders for peak flowering time of: B < G = O in Cool, and B = O < G in Warm, from earliest to latest.

4.3.4.4 Contributions of Genotype, Environment, and their interaction to the timing of the peak flowering event were significant in all three lines

For peak flowering time, genotype, environment, and their interaction were all highly significant by simple Multi-Environment Trial (MET) analysis of the raw data (Table 4.14 in Appendix 4.3.4; $P < 0.001$). G explained 19%, E 52%, and GEI 6% of the variation in timing (Experimental Days). GEI in Orange was twice as large i.e. a slope of 1.55 relative to an average regression slope of 1.0, as that of Green (0.78) or Black (0.78), which were similar ('Regression coefficient' term in Table 4.15 of Appendix 4.3.4). Peak flowering time was most sensitive to temperature differences across the growing environments in the Orange line (an 'Assigned rank' of 1) and the least sensitive in the Green line ('Assigned Rank' of 3; Table 4.15 of Appendix 4.3.4). Peak timing responses were also more predictable in Orange (mean square deviation of 24.8) compared with the Green (244.4) or Black (157.6) lines.

4.3.4.5 Peak flower numbers were significantly greater in Warm for Green and Black, and in Cool for Orange

Open flower numbers at peak flowering were significantly greater in the Warm regime for Green (58 ± 6, and 21 ± 4 in GC; Table 4.5 above) and Black (135 ± 11 in BW and 60 ± 7 in BC), and in direct contrast, in the Cool regime for Orange (128 ± 14 in OC and 60 ± 9 in OW). Mean values were generated from the fitted curves (by GMM), however the standard errors and significance tests were obtained from a simple GLM (explained in Section 4.2.3.4). The temperature x clone interactions were highly significant ($P < 0.001$ by GLM; Table 4.16 of Appendix 4.3.4).

Standardised values (accounting for shoot length) or a measure of floral density i.e. open flower numbers/50 mm of shoot length, were greater in the Warm regime for

Black (10.3 ± 1.0, and 6.7 ± 0.6 in BC; $P \leq 0.05$; Table 4.5), and in the Cool regime for both Green and Orange (4.0 ± 0.4 in GC and 2.0 ± 0.4 in GW; 14.0 ± 1 in OC and 5.5 ± 1.1 in OW; $P \leq 0.05$; Table 4.5). Values for GW are conservative and expected to be lower than those reported since all shoot lengths were measured pre-flowering, and unlike the other clone-treatments GW shoots were not quiescent at that time. The floral density patterns were strongly clonally differentiated. Values were lowest in Green in both environments, and highest in OC and in BW ($P \leq 0.05$; Figure 4.3). Significant changes in the rank orders indicate a significant GEI as illustrated by a crossover interaction pattern of the response norms.

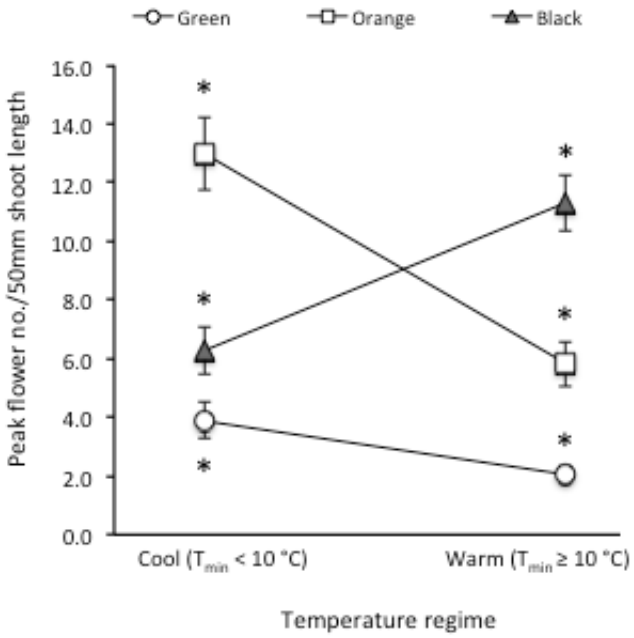

Figure 4.3 Response norms (means ± 1 s.e.) for floral density patterns at peak flowering (the number of open flowers per 50 mm of shoot length) for each of the three clonal lines across the two growing environments (Cool and Warm). * denotes significance at the 95% *C*I ($P \leq 0.05$) within a growing environment.

4.3.4.6 Genotype and GEI contributed significantly to peak flower numbers

By a simple MET analysis, genotype accounted for the majority of the variation in peak flower numbers across the growing environments (40%; $P < 0.001$), while GEI was significant and almost as large at 28% ($P < 0.001$; Table 4.17 of Appendix 4.3.4). Peak flower numbers in the Orange, were the most sensitive to temperature differences across the growing environments i.e. large *negative* GEI (the term 'regression coefficient' in Table 4.18 of appendix 4.3.4) and had low predictability as indicated by a relatively large mean square deviation. Black peak flower numbers were also subject to large *positive* GEI and low predictability, while genetic stability in Green was high i.e. the least sensitive genotype to temperature differences, with good predictability as indicated by a relatively small slope and low mean square deviation.

4.4 Discussion

The flowering responses to the imposed temperature treatments were specific to each of the clone-temperature combinations. While no attempts were made in this study to examine the effects of growth temperature on *floral induction per se,* the effects of genotype, temperature, and their interactions on the subsequent stages of *floral bud formation,* and *blossom initiation* and its *progression* across time, were examined and quantified.

Growth and Flowering

Floral bud formation occurred in SD conditions (at average DLs of between 10 and 11 h) and was marked by declining growth rates with the exception of Green Warm. Nyéki and Soltész (1996) consider a slowing of vegetative growth a pre-requisite for floral transition since slowed growth by lower temperatures or water stress in tropical and subtropical fruit species promotes floral initiation. The developmental processes of *growth (*main-stem extension and expansion*)* and *flowering* were temporally separated in the two year old clones (Figure 3.4). *Blossoming* was initiated only after growth had reached a state of winter quiescence (near-zero GR) in all but one (Green Warm) of the clone-temperature combinations. The unusual growth habit observed in the Green clones growing in the warmer temperatures had important consequences for its flowering phenology (see below). The respective timings of the phases are likely the combined effects of DL and temperature. Indeed, the simplest of the phenological models, a thermal time model, indicated that the *flowering process* in the clones was not *wholly* temperature-driven. Findings support the work of Zieslin & Gottesman (1986) and Bicknell & Jaksons (2018) in which day length more strongly influenced the transition to flowering (floral initiation) in several Mānuka varieties. Results indicate that there may not be a common base temperature among the clones and that base temperature (T_b) may indeed be different for each of the genotypes.

The relative timing of the *growth* and *blossoming* phases is typical of cold temperate species in which the growing season is relatively short, due to a long winter period. Flowers are typically initiated prior to winter dormancy (in summer or autumn). Growth and development are resumed ~~in~~ the following spring resulting in anthesis as early as possible in the season so that fruit development and seed dispersal can occur before next winter (Sedgley & Griffin, 1989).

Inflorescence architecture observed in this study (Section 4.3.2.1) is in direct contrast to published material in which flowers are reportedly '*mostly axillary and occasionally terminal on branchlets'* (Allan, 1961). In the clones, flowers were predominantly terminal on short shoots (termed 'brachyblasts*',* comparable with 'spurs' in apple), and were also to a lesser extent axillary on young (less than one-year-old) primary shoots. The behaviour observed here needs to be checked in a wider context for its universality in *L. scoparium.*

A strong correlation between the shoot floral bud numbers and growth parameters indicates a strong growth-flowering link in the clones ($P \leq 0.05$; Table 4.4). Floral capacity (bud number) in the Orange and Black lines was strongly correlated with absolute shoot length and diameter ($P < 0.001$) but not with shoot elongation rates (SER), suggesting that (high) temperature duration i.e. the length of the growing season and not intensity i.e. faster rates of growth with increasing temperatures, may be important in determining the flower-bearing capability in these particular genotypes. That is, their floral capacity will be determined by the *length* of the growing season, at least between the temperature range studied (T_{MA} of 16.9 and 21.7°C). In contrast, floral capacity in Green may be determined more by the size of the absolute temperature changes. However, any comparisons between GC and GW were not run (considered invalid) since shoots were indeterminate in GW and not a fair representation of *total* floral bud numbers. Further evidence for a strong growth-flowering link in the clones was provided by the ratios of brachyblast to floral bud

numbers. Ratios were close to 1.0 indicating a good linear correlation for all clone-temperature combinations but one (Green Warm; Table 4.2), the significance of which is discussed below. While the floral bud numbers increased in warm temperatures, increases were significant for one of the lines only (Black). In this particular line, increases in absolute shoot length of around 46% (1.5–fold; $P < 0.10$; Section 3.3.5) at the warmer growing temperatures resulted in 25% increases in (standardised) floral bud numbers ($P \leq 0.05$; Table 4.3).

Flower Development

Blossoming (Phase Three; Figure 3.4) occurred with lengthening days in the relatively cool temperatures of late winter and early spring. Blossoming coincided with a *quiescent* growth period (near-zero GRs) with the exception of the Green Warm clones. *Blossoming* was initiated in all clones in overnight temperatures of both above and below 10°C. Insensitivity of the flowering response in Mānuka to low-temperature treatment has been previously reported (Zieslin and Gottesman, 1986). Any (treatment) differences in bloom start times within the clonal lines in this study then, are attributable to differences in developmental processes i.e. rates of floral bud development. According to Lang (1952) variations in the onset of floral initiation frequently persist unchanged throughout the following stages of flower development. That is, variations in the later stages of flowering are simply 'projections of variations at initiation'. That the time of VBA (visible bud appearance) within the lines was similar in the two growing environments suggests that developmental differences in this study likely occurred at the stages of flower (bud) development and not initiation. This would be expected since initiation in Mānuka is asserted to be primarily day length and not temperature driven (Zieslin & Gottesman, 1986; Bicknell & Jaksons, 2018), and also since the (D/N) temperatures in the two growing environments were similar (close to ambient) at the expected time of induction i.e. soon after the longest day (Bicknell & Jaksons, 2018).

Phenological Flowering Curves

The flowering profiles (open flower numbers across time) were unique to each of the clone-temperature combinations (r^2 = 0.63; P < 0.001; Figure 4.2). The wave-like progression (in all, except the Green clones growing in the warmer conditions) is similar to *re-blooming of* modern-day (diploid) hybrid roses. The property of recurrent flowering also occurs in irises, hydrangeas, daylilies, strawberry and raspberry (Proïa et al., 2016). Re-blooming in *Rosa spp.* is described by Proia et al., (2016) as *'flowers arising from shoots that develop on axillary buds of shoots from the previous year, or new determinate shoots terminated by a flower emerging successively from older shoots i.e. much like biennial-bearing in apple'* (Durand et al., 2013). Proia et al., (2016) successfully used Gaussian mixed models (GMM) to quantify re-blooming in hybrid roses stating that the challenge of characterising re-blooming *i*s '*to distinguish a long unique flowering period from several partially overlapping ones, corresponding to successive initiations'*. The Gaussian curve is the classic bell-shape describing the normal distribution (density function). The double Gaussian then is the sum of two overlapping Gaussian curves with bimodal (dual) means or peaks. The induced waves of GMM are considered to be close to the environmental and genetic reality of the plant. The strength of the model is in being able to infer the underlying *process* of flowering over time and not just to describe the quantity of flowers over time, since re-blooming may be considered a semi-quantitative trait (Proïa et al., 2016). A double Gaussian does not appear to fit the Green data as well as the other lines, and it is suggested that a quadratic function may be a better fit for Green Warm.

Shapes of flowering curves for insect-pollinated species are reported as normal in some studies [Jeffree (1957) cited in Clark & Thompson (2011)] and skewed in others (Rabinowitz et al., 1981; Thomson, 1980). Skewing has also been observed in certain years (Tyler, 2001). In the clones, curves tended to be left-skewed at the warmer temperatures and right-skewed in the cooler growing conditions (Figure 4.2). That is, more rapid progression toward the *peak flowering event* (sometimes referred to as the

main flowering period by others) at the warmer temperatures, with the first wave of blossoming (FF1) being the larger of the two peaks i.e. corresponding to the main flowering period. At the cooler temperatures, the second peak (FF2) was the larger peak of the main flowering period as a result of a more gradual floral progression. The magnitudes of the FF1 and FF2 peaks within the clone-treatments appear to be correlated. For example, a smaller (minor) peak followed a major peak in Warm and vice versa in Cool (Figure 3). This may indicate that re-blooming and flower numbers (floral abundance) in the Mānuka clones are *inter-dependent*, unlike that of modern roses (Proïa et al., 2016). There is also some evidence to suggest that a late first flowering may produce a more abundant first flowering in the Mānuka clones (e.g. Orange Cool; Tables 6 & 7), as reported for rose bushes (Proïa et al., 2016).

Major Phenophases

While some authors report a 'few' out-of-season flowers on Mānuka plants (Thompson, 1989), in general, first flower appearance was a good indicator of the commencement of blossoming in the clones. The observed tendency for later blooming in the cooler conditions is common amongst temperate species. The tendency for larger variability in the blossom start times at the cooler temperatures is consistent with a two-year field study of a single population of wild Mānuka (Primack, 1980).

The average length of the bloom period in the glasshouses was around 19 weeks compared with reported durations of six to eight weeks in the field (Primack, 1980). Bloom periods are generally reported as shorter in warmer years [both in Mānuka (Primack, 1980) and in fruit trees (Nyéki & Soltész, 1996)]. However, the average length of blossoming in the clones was longer in Green (by 43%) and shorter in Orange (by 24%; $P \leq 0.05$) at the warmer temperatures.

The timing of peak flowering was significantly earlier in all three lines by approx. six weeks, on average, at the warmer temperatures (84% CI; $P < 0.16$; Figure 4.2). Findings agree with published work in which flower development in Mānuka is

reportedly constrained by cooler temperatures (Primack, 1980; Zieslin & Gottesman, 1986). Flowering time (the peak flowering event) in the clones was determined by genetics, growing environment, and their interactions. That is, genotype accounted for 19%, environment 52%, and their interaction 6% of the variation ($P < 0.001$). The effects of temperature are more than double those due to genotype. In contrast, numbers of flowers at peak flowering were strongly genotype-determined (40% of the variation) and subject to significant GEI (28% of the variation). That is, more flowers at peak flowering at the warmer temperatures in two of the lines (Green and Black) and fewer flowers at the cooler temperature*s* in the other *($P \leq 0.05$).*

Indeterminacy of Growth and the Once-flowering habit

There was a tendency among the clones for *determinate* growth in the Orange and Black lines and in Green Cool, and for *indeterminate* growth in Green clones growing at the warmer temperatures. Evidence for indeterminacy in Green Warm was provided by brachyblast to floral bud ratios of greater than 1.0 (Table 4.2), indicating that not all meristem apices were reproductively competent. Additional evidence is provided by floral density at peak flowering. While total flower numbers were greater at the warmer temperatures, bud numbers per unit of shoot length were low when compared with the cooler temperatures, indicating a clear preference toward vegetative over reproductive growth.

In studies on rosebushes *(Rosa spp.)* and woodland strawberry (*Fragaria vesca,* also a member of Rosaceae) flowering behaviour is highly correlated with growth habit (Kawamura, et al., 2015; Iwata et al., 2012). For example, in cross-hybridisation experiments in rose (*Rosa* 'The Fairy' X *R. wichuriana*), weeping or prostrate individuals displayed a once-flowering (OF; uni-modal) habit, while continuous flowering (CF; wave like) occurred in progeny with an erect growth habit (Kawamura, et al., 2015). CF in both species is associated with determinate growth, similar to that observed for the Orange and Black clones, while OF is associated with an

indeterminate growth habit, much like that observed in the Green clones. Interestingly, in *Rosa,* flowering time genes (FT1/FT) co-locate with genes controlling the architectural traits of plant height and plant form (Kawamura et al., 2015). FT/TFL1 is a multi-gene family in plants shown to regulate reproductive growth and flowering cycles in perennials. The FT gene is an integrator that promotes flowering while TF1 represses transition. In rose, a major QTL for flowering time was found to co-localise with *Ro*FT (FT gene in *Rosa spp.).* Several candidate genes involved in gibberellin (GA) biosynthesis and auxin signalling were identified in the vicinity of the QTLs, preventing independent selection of plant form and flowering behaviour in garden rose (Kawamura et al., 2011).

It is suggested that the differences in flowering behaviour observed in the Mānuka clones in response to temperature changes may be linked to differences in plant form, much like that of rose, i.e. upright vs. prostrate. However, this warrants further study.

4.5 Conclusions

Floral bud numbers, in general, were greater at the higher growth temperatures and not subject to any detectable GEI. That is, the effects of genotype and environment were independent. Open flower counts across time were subject to significant GEI (clone x temperature) resulting in unique flowering profiles for each of the clone-treatments ($P < 0.001$). Curves tended to be left skewed at the warmer temperatures and right skewed in the cooler temperatures. Peak flowering time was largely environmentally (temperature) determined in the genotypes, in contrast to peak flower number which was strongly genetically determined. The uni-modal flowering habit induced by the Warm regime in the semi-prostrate Green line was associated with an indeterminate growth habit. Parallels were drawn with the similar once-flowering habit of weeping (semi-prostrate) garden species roses. In conclusion, temperature-induced differences in the flowering responses among the genotypes suggest a strong link between growth form and flowering habit.

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Appendix 4.3.2

Total Floral Bud Numbers

Table 4.6 Analysis of variance by GLM for total floral bud numbers on shoots of clones in the Cool and Warm temperature regimes.

Term	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
Temperature treatment	1	2281.96	2281.96	55.36	<0.001
Treatment x block	14	608.42	43.46	1.05	0.414
Clonal line	2	2735.21	1367.61	33.18	<0.001
Treatment x line	2	574.2	287.10	6.97	0.002
Residual	64	2637.95	41.22		
Total	83	8837.74	106.48		

Standardised Floral Bud Numbers

Table 4.7 Analysis of variance by GLM for standardised floral bud numbers on shoots in the Cool in the Cool and Warm temperature regimes.

Term	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
Temperature treatment	1	58.573	58.573	41.81	<0.001
Treatment x block	14	37.018	2.644	1.89	0.045
Clonal line	2	343.754	171.877	122.69	<0.001
Treatment x line	2	7.927	3.964	2.83	0.067
Residual	62	86.857	1.401		
Total	81	534.13	6.594		

Appendix 4.3.3

Gaussian ANOVA table

Table 4.8 Accumulated analysis of variance for open flower counts as predicted by double Gaussian standard curves fitted to the floral flush 1 (FF1) and floral flush (FF2) data. Day is experimental day; Groups are the clone-temperature combinations of Green Cool, Green Warm, Orange Cool, Orange Warm, Black Cool, and Black Warm. The 'Separate nonlinear' term describes differences in the shapes of the curves.

Change	d.f.	s.s	m.s	v.r.	F pr.
+ Day	5	142159.5	28431.9	94.03	< 0.001
+ Group	5	117737.5	23547.5	77.88	< 0.001
+ Day x Group	10	289321.2	28932.1	95.69	< 0.001
+ Separate nonlinear	15	68328.2	4555.2	15.07	< 0.001
Residual	1144	345902.6	302.4		
Total	1179	963449.0	817.2		

Floral Progression Curves

Table 4.9 Standard curve parameters (by a double Gaussian model). Values are for 'Day of Year' data i.e. before converting to Experimental Days.

Parameter	Group	Estimate (Day of Year)	s.e.
S	**GC**	10.30	2.94
M	**GC**	249.0	13
N	**GC**	280.09	2.86
B	**GC**	101.2	90.4
C	**GC**	511.	143
A	**GC**	0.94	1.91
S	**GW**	58.7	54.6
M	**GW**	231.8	48.7
N	**GW**	348.8	43.1
B	**GW**	6751.	17740
C	**GW**	6899.	18202
A	**GW**	-34.	105
S	**OC**	9.74	2.92
M	**OC**	285.42	9.08
N	**OC**	300.7	75.1
B	**OC**	2800.	3091
C	**OC**	430.	2336
A	**OC**	8.27	1.37
S	**OW**	9.57	2.27
M	**OW**	228.59	2.33
N	**OW**	249.35	6.97
B	**OW**	1194.	305

C	**OW**	509.	200
A	**OW**	8.10	2.04
S	**BKC**	15.78	1.61
M	**BKC**	220.07	3.2
N	**BKC**	265.48	1.58
B	**BKC**	971.	194
C	**BKC**	2247.	281
A	**BKC**	2.42	3.12
S	**BKW**	11.79	1.08
M	**BKW**	218.713	0.927
N	**BKW**	246.02	2.02
B	**BKW**	2638.	200
C	**BKW**	1287.	166
A	**BKW**	1.90	2.27

Appendix 4.3.4

Time to Blossoming

Table 4.10 Accumulated analysis of deviance by a GLM for the number of Experimental Days to 'First Open Flower' (*syn.* Blossom Start) for the three clonal lines in the Cool and Warm temperature regimes.

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Temperature	1	0.0004	0.0004	0.06	0.812
+ Block x temperature	14	0.3248	0.0232	3.30	<0.001
+ Line	2	0.2310	0.1155	16.45	<0.001
+ Temperature x line	2	0.0854	0.04274	6.09	0.004
Residual	64	0.4495	0.0070		
Total	83	1.0913	0.0131		

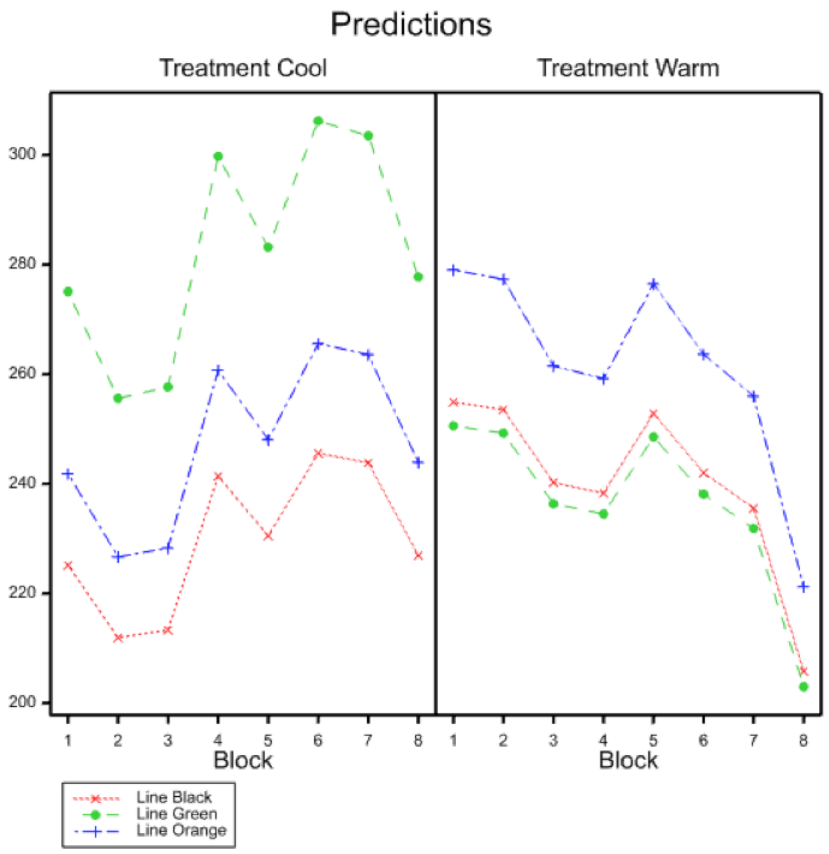


Figure 4.4 Block effects (1 to 8) for 'Blossom Start Times' within the two glasshouse growing-environments. Temperature regimes are 'Cool' (left plot) and 'Warm' (right plot).

Date equivalents for ‘First Open flower’

Table 4.11 Average equivalent Blossom Start Dates and Day of Year (from 1st January in the year of flowering, 2016) for the three clonal lines in the Cool and Warm temperature regimes.

Clone	Experimental Day (Day of year)		Date	
	Cool	Warm	Cool	Warm
Green	283 (224)	237 (178)	12-Aug-16	27-Jun-16
Orange	248 (189)	263 (204)	8-Jul-16	23-Jul-16
Black	230 (171)	241 (182)	20-Jun-16	1-Jul-16

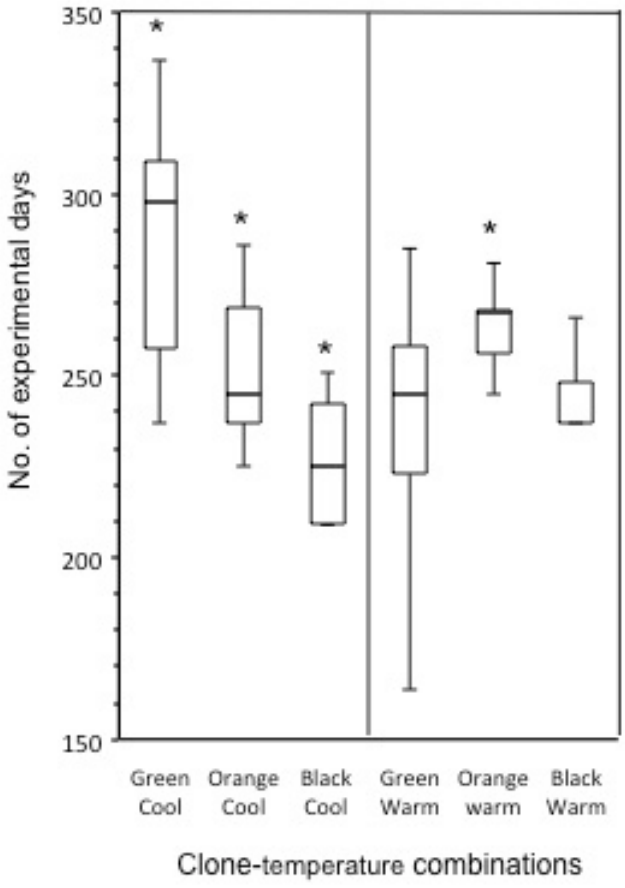


Figure 4.5 Distribution plot for the number of experimental days to the ‘Blossom Start’ on shoots for the three clonal lines in the Cool (on the left) and Warm (on the right) temperature regimes. Height of the boxes denotes the interquartile range i.e. the middle 50% of the data. Lines within the boxes mark medians. Whiskers are maximum and minimum values. * indicates a significant difference between the clonal lines within the growing environments at the 95% *CI* (*P* ≤ 0.05).

Bloom Period Duration

Table 4.12 Accumulated analysis of variance for 'Duration of the Bloom Period' by a GLM

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Block x Treatment	15	204.6	13.6	2.8	0.003
+ Treatment x Clonal Line	4	320.6	80.2	16.3	< 0.001
Residual	54	266.1	4.9		
Total	73	791.4	10.8		

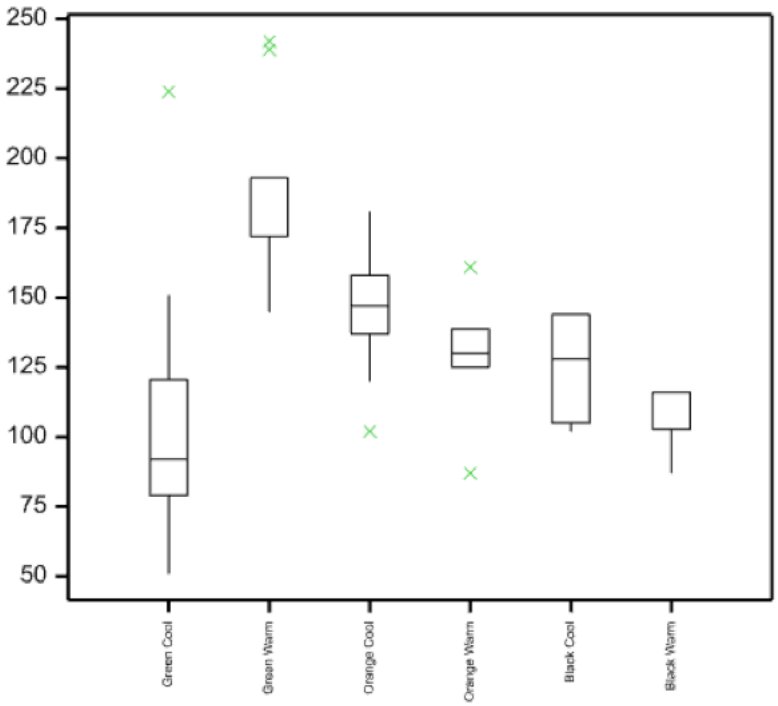


Figure 4.6 Distribution plot for 'Bloom Period Duration' (number of experimental days) for the three clonal lines in the 'Cool' and 'Warm' temperature regimes. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Green crosses have large residuals as identified by Genstat®.

Timing of the Peak Flowering Event

Table 4.13 'Day of Year 'and 'Date' Equivalents for the timing of peak flowering

Clones in Rank Order	Experimental Day	Date	Day of year (From 1st Jan 2016)
Black Warm	278	7-Oct-16	219
Orange Warm	289	18-Aug16	230
Green Warm	301	30-Aug-16	242
Black Cool	324	22-Sept-16	265
Green Cool	339	7-Oct-16	280
Orange Cool	345	13-Oct-16	286

MET Analysis Output Table For Peak Flowering Time

Table 4.14 Analysis of variance for the timing of the peak flowering event by a simple MET analysis (using Finlay-Wilkinson regression).

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Genotypes	2	9183.0386	4591.5193	30.07	<0.001
Environments	1	25436.4532	25436.4532	166.61	<0.001
Sensitivities	2	3086.362	1543.181	10.11	<0.001
Residual	73	11144.9056	152.6699		
Total	78	48850.7595	626.2918		

MET Analysis for Peak Flowering Time

Table 4.15 Sorted sensitivity estimates generated by MET analysis for peak flowering time in the three clonal lines, together with mean squared deviations, regression coefficients (Sensitivity), and genetic stability rankings (Assigned Rank) across the Cool and Warm growing-environments. A ranking of 1 is the most sensitive and 3 the least sensitive to temperature differences across the growing environments.

	Green	Orange	Black
Mean of peak timing	321.6 ± 2.3	317.1 ± 2.6	298.8 ± 2.4
Mean square deviation	244.4	24.8	157.6
Regression coefficient	0.775	1.548	0.782
Assigned Rank	3	1	2

Peak Flower Numbers

Table 4.16 Accumulated analysis of deviance for open flower numbers at peak flowering, by a GLM.

Term	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Row	3	0.1731	0.0577	0.14	0.936
+ Treatment	1	1.1670	1.1670	2.83	0.097
+ Treatment x Clone	4	61.4613	15.3653	37.33	<0.001
Residual	71	29.2273	0.4117		
Total	79	92.0287	1.1649		

MET Analysis for Peak Flower No.

Table 4.17 Analysis of variance for flower numbers at peak flowering, by a simple MET analysis (using Finlay-Wilkinson regression).

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Genotypes	2	78377.1074	39188.5537	46.73	<0.001
Environments	1	1381.141	1381.141	1.65	0.203
Sensitivities	2	55907.3354	27953.6677	33.33	<0.001
Residual	74	62057.9662	838.6212		
Total	79	197723.55	2502.8297		

Table 4.18 Sorted sensitivity estimates for peak flower numbers for the three clonal lines, generated by MET analysis, together with mean squared deviations and regression coefficients (Sensitivity) across the Cool and Warm growing-environments.

Clone	Green	Orange	Black
Mean	**32.2 ± 5.226**	**99.06 ± 6.09**	**86.04 ± 5.679**
Regression coefficient	**1.682 ± 1.252**	**- 8.115 ± 1.46**	**8.12 ± 1.361**
Mean square deviation	**326.4**	**1148.4**	**1186.4**

Chapter 5 Nectar Quantity and Quality Changes in Response to Temperature

Abstract

Floral nectar from the shrub, *Leptospermum scoparium (*Mānuka*)* is the key determinant of the high value manuka honey product. However, the response of Mānuka plants to changes in temperature, and the consequences for nectar quantity and quality, are poorly understood. No studies to date have examined the G x E interactions on the nectar properties of Mānuka. In controlled experiments conducted in temperature rooms and glasshouses, clones of Mānuka with varying levels of nectar dihydroxyacetone (DHA) were subjected to short-term (48 hour) and longer-term (12 months), Cool and Warm ambient temperature treatments. The average nectar volumes and total nectar sugars (TSugars) per flower were clone-specific and not significantly different across the temperature treatments. That is, the genotype rank orders across treatments (Cool and Warm) and treatment durations (short and long) were well correlated ($P \leq 0.05$). TSugars were highest in the morning after overnight low temperatures and in the afternoon with the warmer daytime temperatures. Increased ratios of DHA to TSugars (DHA/TS) at the warmer temperatures in the glasshouse environments for two of the clonal lines ($P \leq 0.05$) were the result of increased DHA in one of the lines ($P < 0.001$), and a tendency for reduced sugars in the other line. However in the former, in growth rooms, increased DHA/TS at the warmer temperatures were due to reductions in both DHA and TSugars ($P \leq 0.05$). Time of day was not a significant factor in the overall quality (DHA/TS) of the nectars. The different responses by the clones to the short and long-term temperature treatments indicate that both genotype and environment (temperature and light quality) influenced the quality of Mānuka nectar and potentially the honey derived from that nectar.

5.1 Introduction

Considerable variation in the quality of NZ manuka honeys is currently of high research interest. In monofloral manuka honey derived from the same floral source, much of the variation in the concentrations of the nectar-derived constituents (Wilkins et al., 1993) has been attributed directly to environmental or varietal influences on the floral nectar components (Stephens 2006). Intra-species variability in nectar characteristics is widely reported across plant species, and occurs at the level of individual flowers, between flowers on the same plant, between plants of a population, and between populations (Nicolson, Nepi, & Pacini, 2007). In Mānuka *(Leptospermum scoparium),* variability is attributed to plant age, flower age (developmental stage), genotype and environment (Clearwater et al., 2018; Nickless et al., 2017; Williams et al., 2014). While several studies report strong genetic influences for nectar yield in Mānuka (Clearwater et al., 2018; Nickless et al., 2017; Smallfield et al., 2018), none has examined the G x E interactions for nectar traits of interest. In general, the relative influences of genotype and environment on nectar composition and production are poorly understood in angiosperms e.g. Parachnowitsch et al., (2019). It is widely accepted amongst species that air temperature and plant age affect nectar production (secretion) whilst short-term microclimatic factors, especially relative humidity, affect concentration (Nicolson and Thornburg, 2007).

Inter-plant variation in the amount of the triose sugar, dihydroxyacetone (DHA) in Mānuka nectar, was first reported by Adams et al. (2009) who showed that nectar-derived DHA confers bioactivity to maturing honey by its non-enzymatic conversion to methylglyoxal (MGO). In a more comprehensive study (Williams et al., 2014), nectar was sampled from a wide range of locations across the North Island of NZ (Auckland, Coromandel, East Cape, Waikato, and Whanganui regions) and from the Nelson region of the South Island. Variation in the nectar DHA levels was assessed between cultivars (genotype variation), field sites (inter and intra-regional variation), and also

between years (seasonal). In one of the regions (East Cape) DHA/TS, a measure of nectar 'quality', went from a low (< 0.001 mg/ mg) to a high (> 0.002 mg/mg) arbitrary classification, and from low to moderate (0.001−0.002 mg/mg) between years (2009, 2010). Regional differences meant that DHA/TS was significantly higher in the Coromandel and Wairarapa regions in both of those years compared with the East Cape region. In addition, individual trees within a narrow radius (of 50 m) differed significantly in their nectar DHA/TS, ranging from high to low classifications in some cases. Soil composition (order and quantifiable components) was not a significant factor. There were also no apparent trends relating variety (*L. scoparium* var. *scoparium* vs. *L. scoparium* var. *incanum*) to high nectar DHA/TSugar. Results from that study suggest that both genetics and environment are influencing nectar DHA/TS.

Working with clonal cultivars of Manuka, Nickless (2015) showed variation in nectar yield to be influenced by both plant (genotype) and environmental factors (edaphic and climatic). In particular, temperature (minimum, maximum, and average) and solar radiation influenced DHA concentration and total sugar content, explaining a further 15 and 23 % of the variation respectively in addition to genotype (which explained 47 and 44 % resp.). It has been more recently reported that DHA/TSugar is dependent on daytime temperature of the previous day (Clearwater et al., 2018).

The objective of this chapter is to examine the effects of temperature on DHA accumulation in floral nectar of clones from three Mānuka genotypes representing low, medium and high nectar DHA accumulators. The relative contributions of G, E and GEI will be quantified. The key question is: Do the expected clone x temperature interactions affect the quantity and quality of nectar in clones of varying nectar DHA?

5.2 Materials and Methods

Eight replicated plants from three *elite* breeding lines representing low, medium and high DHA accumulators (Green, Orange, and Black respectively; Section 2.2.2) were subject to Cool and Warm temperature treatments. The treatment durations were for 48 hours (short-term duration; Experiment #1) at peak flowering and 12 months (one complete annual growth cycle; long-term duration; Experiment # 2). The short-term temperature treatments were carried out in growth rooms (*syn.* controlled temperature rooms, CTRs) at constant day/night (D/N) temperatures of 15/10°C and 25/15°C, in a 12 hour (h) photoperiod with artificial lighting (187 µmol m^{-2} s^{-1} at top of plants). The long-term temperature treatments were carried out in glasshouses under natural light (800 –1200 µmol m^{-2} s^{-1} at plant height) and day length with minimum overnight lows of < 10°C and ≥ 10° during the coldest months of the year. Daytime temperatures were close to ambient. A blocking structure was used in each experiment to account for any local position effects and to account for differences in initial plant size. For further Experiment #1 & 2 details, refer to Sections 2.2 and 2.3 respectively.

5.2.1 Nectar Sampling

All pollinators were excluded from glasshouses and growth rooms (Section 2.5.2).

Nectar collection started around 0900h when there was good (visual) nectar flow and continued until around 1730h. In CTRs (Experiment #1; see below) two operators worked side by side in the chamber anteroom, sampling nectar simultaneously from one CTR each, block by block, at the end of the 48-hour treatment period. In the glasshouses (Experiment #2; see below) nectar was collected on clear, sunny days by a single operator working block-by-block alternating between the two (Cool and Warm) glasshouses.

Nectar was collected using the pipette method (Section 2.7) and pooled from (ideally) fifteen (Stage IV; Section 2.6) flowers per clone into pre-weighed containers (1.5 mL Eppendorf tubes). The flower numbers sampled from each clone were recorded, along with the time of day. Each floral disc was rinsed with 5 µL of sterile water to recover all of the sugars from the surface of the floral disc. Flowers were sampled only once i.e. a single point in time from flowers that had not been previously sampled, by destructive sampling in growth rooms and non-destructive in glasshouses. Samples were immediately placed on ice for transferred to an –80°C freezer awaiting compositional analysis by high performance liquid chromatography (HPLC; Section 2.8).

5.2.1.1 Short-term (Experiment # 1)

In the short-term room crossover temperature experiment (Figure 5.1), nectar was sampled from all clones at the end of the 48 h treatment period on a single day, that is, the 2nd of October 2015 for Run 1 and the 12th of October 2015 for Run 2. Two persons sampled nectar side by side (the term 'Collector' in the statistical analyses) simultaneously from one of two CTRs.

Figure 5.1 The short-term (48 h) Cool (15/10°C D/N) and Warm (25/15°C D/N) temperature treatments were administered to clones in peak flower in controlled temperature rooms under artificial lighting (187 µmol m^{-2} s^{-1} output at flower height) and with a 12 h photoperiod. In Run 1 treatments are as labelled; in Run 2 the temperature treatments were switched i.e. a room-crossover experimental design.

5.2.1.2 Long-term (Experiment #2)

In the long-term temperature experiment, nectar was collected across a 9-day window, from tagged shoots only, on the 2nd of August 2016 (Experimental day 273) for Black, the 9th of August 2016 (Experimental day 280) for Orange, and the 11th of August 2016 (Experimental day 282) for Green. A single operator working block-by-block alternating between the two glasshouses collected nectar. The different sampling dates were taken into account in the statistical analyses (see below).

5.2.2 Statistical Approach

Nectar data from Experiments #1 and #2 were analysed as complete data sets where possible, to identify any possible block effects (between the experimental units), and to quantify the temperature x clone interactions. A linear mixed model approach (LMM via REML) using GenStat® was adopted since there were multiple error terms i.e. a multi-factor experiment, and the data set was unbalanced (Experiment #2 only) and non-orthogonal. Both fixed and random terms were included in the models (see below). All model outputs can be found in the Appendices as referenced.

5.2.3 Short-term Temperature Experiment #1

After any necessary data transformations (included in Results section) and removal of extreme outliers (values identified by GenStat® as having large residuals) to satisfy the model assumptions of normality and homogeneity, separate analyses were performed for each of the nectar variables (volume, total sugars, DHA, DHA/TSugars). A similar model was used for each variable. Run, Sequence, Treatment, Clone, Time of day, and Collector were fixed effects, while Block was a random term in the model (Table 5.1). Clone was nested in treatment. The sequences for the crossover treatments appear in Table 5.2. The terms Carryover and NoCarryover in the model refer to

possible Run and Sequence effects between the Cool and Warm temperature treatments. Treatments administered in Run 1 were deemed to have no residual effect, and so were logically assigned 'None' for the NoCarryover term, while treatments in Run 2 may have residual (pre-treatment) effects and so were assigned 'CO' for that term. The treatments in Runs 1 and 2 were accepted as equivalent only after it was established that they were not statistically different from each other (Carryover effects and NoCarryOver x Carryover interaction term). Time of day was converted to a factor and grouped into four (2 to 3 hour) categories of 9-11am, 11-1pm, 1-3pm, and 3-6pm. The term 'Collector' was dropped from the analysis (to simplify the model) since effects were found to be negligible.

Table 5.1 Short-term (Experiment #1) model term descriptions used in the analysis (linear mixed model, LMM) of the nectar variables. All terms except Collector were retained in the final model.

Model Term	Description	Fixed (F) or Random (R)
Run	1 (No pre-treatment) & 2 (pre-treated)	F
Sequence	1(Cool, Warm) & 2 (Warm, Cool)	F
Treatment	Cool or Warm	F
Clone	Green, Orange, Black	F
Time of day	Grouped (2– 3 h intervals)	F
Collector	Person sampling	F
Block	Position within each CTR	R

Table 5.2 Treatment structure (2 period/2 treatment) of the short-term crossover temperature experiment (Experiment #1) showing the sequences for applying the two treatments Cool (15/10°C, D/N) and Warm (25/15°C D/N) to experimental units in Runs 1 and 2. CTR 3, 5 were Controlled Temperature Rooms 3 and 5 respectively located at the Plant Growth Unit, Massey University, Palmerston North.

	Time/Run	
Sequence	1	2
1 (CTR 3)	Cool	Warm
2 (CTR 5)	Warm	Cool

5.2.3.1 Long-term Temperature Experiment #2

In the long-term temperature experiment, separate analyses for each of the nectar variables (volume, total sugars, DHA, DHA/TSugars) were performed after the necessary data transformations to meet normality and homogeneity requirements of the model. Row, Column, treatment, clone and time of day were designated as fixed terms in the model. Date was analysed as a random term since nectar was collected on different days for each of the clonal lines (Section 5.2.1.2). Clone was necessarily nested in treatment since glasshouse together with treatment was not replicated i.e. one glasshouse per treatment. Time of day was converted to a factor and grouped into two categories [morning (9am to midday) and afternoon (midday to 5pm)]. The means for clone and date were predicted using date as the classifier and averaging over clone and treatment (equal weights). A similar model was used for all of the long-term nectar variables.

Table 5.3 Long-term descriptions for the model terms (by a linear mixed model, LMM) used in the analysis of the nectar variables. All terms except Collector were retained in the final model. The term Column (describing possible fan effects) was dropped from the final model to simplify it since the heat maps showed no obvious column (position in glasshouses) effects in the data.

Model Term	Description	Fixed (F) or Random (R)
Row	Position effects (radiation)	F
Column	Position effects (fans)	F
Treatment	Cool or Warm	F
Clone	Green, Orange, Black	F
Time of day	Grouped (morning & afternoon)	F
Date	Day of sampling	R

5.3 Results

Analysis of the temperature data from the short-term experiment (Experiment #1) is presented in this section. Temperature data for the long-term experiment (Experiment #2) was presented earlier in Section 3.3.1, however for reader convenience, the raw data appears again in this chapter in Figure 5.18 of Appendix 5.3.1.

5.3.1 Temperature data

5.3.1.1 Short-term

The set Cool and Warm D/N temperatures (Section 5.2) were less stable in one of the CTRs. The effective (actual) treatments are shown in Figure 5.2.

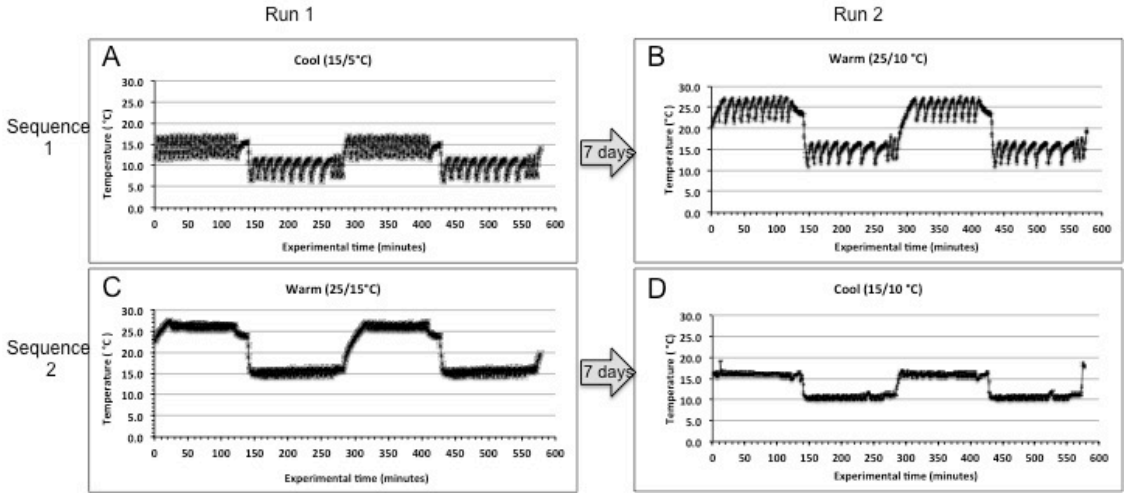


Figure 5.2 Temperature plots showing the actual (effective) temperature treatments for the short-term experiment. The Cool and Warm treatments in Run 1 are A & C resp., and D & B in Run 2. Temperatures were less stable in one of the CTRs (top set of graphs, A & B). Treatment durations were for 48 hours; temperature readings were taken at 5-minute intervals.

5.3.1.2 Long-term

At the time of nectar collection, overnight temperatures in the Warm and Cool glasshouses were above and below a 10°C threshold respectively (Section 2.3). Absolute minima and the mean maximal temperatures for the 24h period prior to nectar collection for each clone-treatment combination were: GC (5.5°C, 19.75°C), GW (17.5°C, 27.0°C); OC (5.0°C, 19.75°C), OW (17.75°C, 26.75°C); BC (8.8°C, 18.8°C), BW (16.8°C, 21.3°C). The sampling dates for each were given in Section 5.2.1.2.

5.3.2 Nectar data - Quantity

Results from both the short and the long-term temperature treatments are presented consecutively for each nectar variable. Nectar *quantities* are reported first (volumes; Section 5.3.2) followed by variables describing nectar *quality* (total sugars, DHA, and DHA to total sugar ratios, and in that order; Section 5.3.3). Generally for each, the statistical output (significance tests for main effects and their interactions) is presented firstly and then the actual data trends are reported on. Significant trends only (at the 5% level) are discussed. For clarity, a short summary comparing the short with long-term trends is provided at the end of each section. Tables providing an overall summary for each of the experiments are presented at the end of the Results in Section 5.3.6.

5.3.2.1 Nectar volumes were unaffected by the short-term temperature treatments but differed significantly between the clonal lines

Pre-treatment and room effects relating to the short-term experimental design (Section 2.6.1.1) had no effect on the single-point nectar volumes (Table 5.19 of Appendix 5.3). Clonal effects and the temperature x clone interactions were highly significant ($P < 0.001$). For example, trends within the clonal lines were for increased volumes at the warmer temperatures in Green and decreased volumes at the warmer temperatures in Orange and Black. Volumes differed significantly between the clonal lines. That is, the average volumes (combined treatment means ± 1 s.e.) were greatest in Orange (8.1 ± 0.5, µL flower $^{-}$1, $CV = 0.24$), intermediate in Green (6.1 ± 0.5 µL flower $^{-1}$; $CV = 0.39$), and least in the Black line (3.0 ± 0.5 µL flower $^{-1}$; $P \leq 0.05$; $CV = 0.59$; Figure 5.3). Intra-clonal variability was large as indicated by the reported coefficients of variance (*CV,* standard deviation as a proportion of the mean*).*

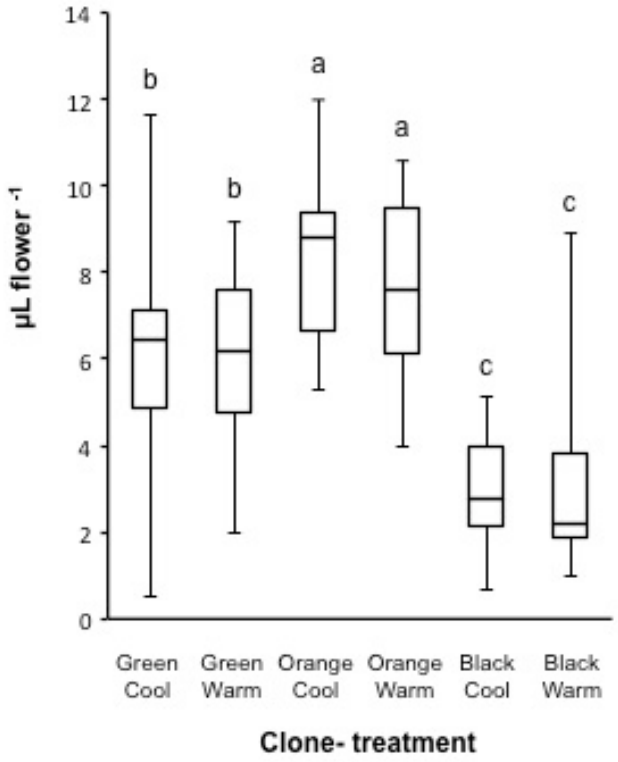


Figure 5.3 Distribution plot for nectar volumes (µL flower $^{-1}$) for the three clonal lines under short-term (48 h; 12 h photoperiod) Cool (15/10°C, D/N) and Warm (25/15°C, D/N) temperature treatments. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Data were collected from *n* = 240 flowers from 8 clonal replicates. Different letter indicates a significant difference at the 95% confidence interval (*CI*; *P* ≤ 0.05).

Time of day was highly significant ($P < 0.001$; Table 20 of Appendix 5.3.3), while the temperature x time interactions were not. That is, volumes decreased across the day for both temperature treatments. Volumes were highest in the morning (9 –11 am) and lowest at the end of the day (3 –6 pm), in all three clonal lines and in both treatments (overall averages of 7.4 ± 0.3 and 4.3 ± 0.3 µL flower $^{-1}$ resp.; $P \leq 0.05$). Volumes were moderately negatively correlated with time of day (*Corr.* = –0.41; $P < 0.001$).

The three-way interaction between temperature, clone and time was significant ($P \leq 0.05$; Figure 5.4). Volumes at the end of the day were significantly lower in GC than in GW, and in OW than in OC ($P \leq 0.05$).

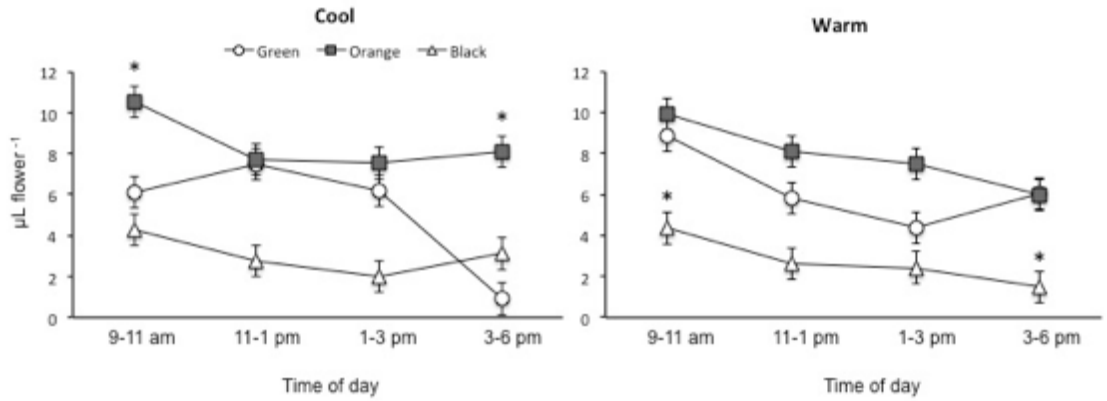


Figure 5.4 Average single-point nectar volumes (µL flower $^{-1}$) decreased across the day for all three of the clonal lines, under the short-term (48h; 12h photoperiod) Cool (15/10°C) and Warm (15/25°C) temperature treatments. Temperature effects and the temperature x time interactions are not significant. However, time and the temperature x clone x time interactions are significant ($P \leq 0.05$).

5.3.2.2 Long-term temperature effects on nectar volumes were significant for the Black line only

In the long-term temperature experiment, position (Row) effects within the glasshouses on nectar volumes were negligible (Table 5.20 in Appendix 5.3.3). The temperature x clone interactions were highly significant (P <0.001), such that temperature effects were significant for the Black line only. That is, greater volumes for BC compared with BW (7.4 ± 0.2 µL flower^{-1} and 4.6 ± 0.2 µL flower $^{-1}$ resp.; $P \leq 0.05$). Comparing across the clonal lines, average volumes (combined treatment means ± 1 s.e.) were greatest in Orange (13.8 ± 0.3 µL flower^{-1}), which were approximately double those of Green (6.4 ± 0.2 µL flower^{-1}) or Black (as reported above; $P \leq 0.05$). The variability was larger under Cool temperature treatment in Orange, and Warm temperature treatment in Green and Black (Figure 5.5).

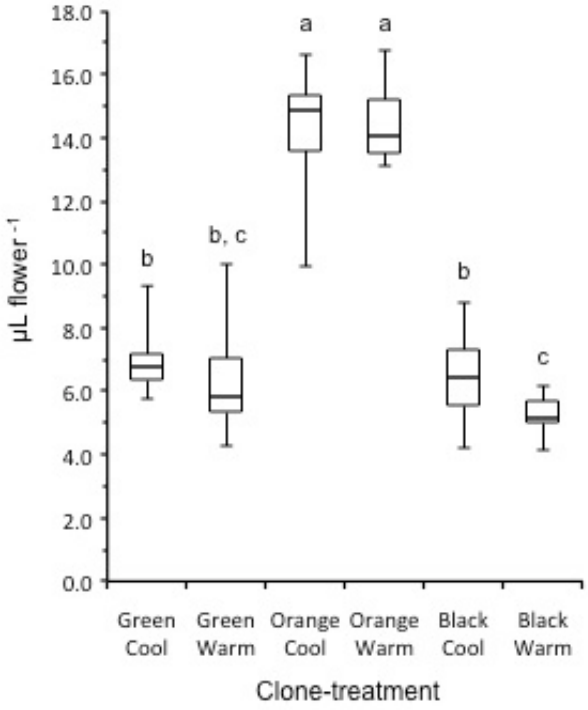
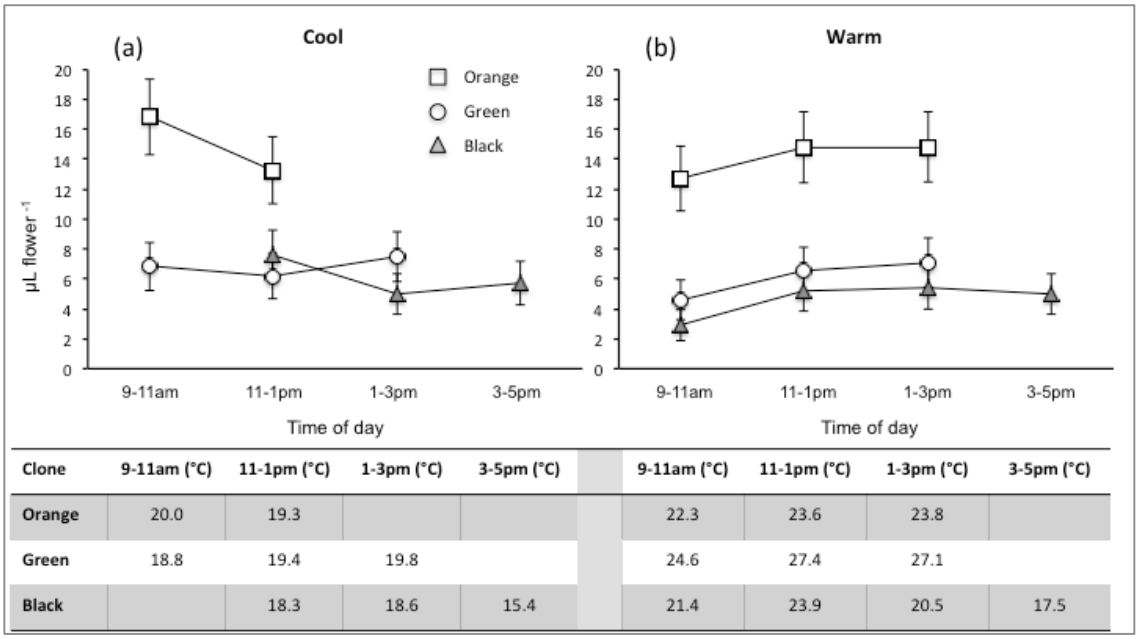


Figure 5.5 Distribution plot for nectar volumes (µL flower-1) for the three clonal lines under the long-term (12 month) Cool (Tmin. < 10°C) and Warm (Tmin. ≥ 10°C) temperature regimes. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values Sample sizes are for N = 8,8,4,7,8, & 8 clonal replicates and n = 96, 101, 54, 104, 116, & 120 flowers per clone respectively from left to right boxes. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05).

Time of day effects, and the temperature x time interactions are significant ($P \leq 0.05$; Table 5.20 in Appendix 5.3.3) i.e. time effects were *conditional* on treatment. However, any 3-way interactions between treatment, clone, and time of day are not significant. While models with time of day grouped into either two or four time intervals both ran; two groupings produced significance while four did not. The four-time-interval model was used to generate the 3-way interactions presented in Figure 5.6 (to allow for direct comparison with the short-term experimental results i.e. to identify any similar trends). Temperatures across the two sampling periods are also shown in Table 5.4. Volumes in the Cool regime were significantly higher in the morning (9am –12 midday) in contrast to the Warm regime in which volumes were lower in the morning ($P \leq 0.05$; Table 5.4). Afternoon (12 midday –5pm) volumes were similar in the two regimes.



Clone	9-11am (°C)	11-1pm (°C)	1-3pm (°C)	3-5pm (°C)	9-11am (°C)	11-1pm (°C)	1-3pm (°C)	3-5pm (°C)
Orange	20.0	19.3			22.3	23.6	23.8	
Green	18.8	19.4	19.8		24.6	27.4	27.1	
Black		18.3	18.6	15.4	21.4	23.9	20.5	17.5

Figure 5.6 Average single-point nectar volumes (µL flower $^{-1}$) across the day for the clonal lines, under the long-term Cool and Warm treatments. Time of day effects, and the temperature x time interactions are significant ($P \leq 0.05$). However, the treatment x clone x time of day interaction is not significant. The table at bottom of the plots is the average glasshouse temperature in units of °C recorded on the day of sampling for each of the clonal lines for the two time intervals as shown.

Table 5.4 Single-point nectar volumes (µL flower-1) across the day for the Cool and Warm long-term temperature regimes. Same letter indicates no significant difference at the 95% CI (P ≤ 0.05).

Treatment	9am to 12 Midday	Midday to 5pm
Cool	9.21 ± 0.03 [a]	8.82 ± 0.02 [a]
Warm	6.79 ± 0.03 [b]	8.66 ± 0.02 [a]

Nectar volumes collected from one of the lines (Green Cool) on the 20th of April and again on the 11th of August 2016 were significantly lower (10.6 ± 0.3 µL flower^{-1}) earlier in the season compared with the later flowering (12.0 ± 0.4 µL flower^{-1}; *P ≤ 0.05 by* a student's t-test). This is a 20% difference (data not presented).

Summary: Nectar Quantity (short vs. long-term treatment)

Overall, the nectar volumes were not significantly different between the Cool and the Warm temperature treatments for the short or the long-term experiments. There were however significant clone x temperature interactions, such that the clonal differences are significant. Volumes were consistently higher in the Orange line (approximately 2-fold) compared with Green or Black ($P \leq 0.05$). While volumes were not correlated with

real-time temperature or %RH at time of sampling, they were however *positively* correlated with time of day in the Warm glasshouse (long-term duration; $P < 0.05$), and *negatively* for both the Cool and the Warm treatments in CTRs (short-term duration; $P < 0.001$). Volumes were 1.5 to 2-fold higher in the glasshouses compared with the CTRs. Variability was large, both between and within the clonal lines. Importantly, rank orders of the genotypes across the experiments (short vs. long-term) were well-correlated, that is, BK ≤ G < O.

5.3.3 Nectar Data – Quality

5.3.3.1 Total sugars per flower were significantly different between the short-term temperature treatments in the Orange line only

While the overall effects of the short-term temperature treatments (after square-root transformation of data) on the relative total sugar (TSugar) concentrations (mg mL^{-1}) of the *dilute* nectar samples (unadjusted for water used at sampling) were not significant, the clone x temperature interactions were significant ($P < 0.001$; statistical output not presented). That is, temperature effects were significant in the Orange line only, with higher TSugar concentrations (approx. 1.2-fold) in OC compared with OW (325.4 ± 14.4 and 277.6 ± 13.3 mg mL^{-1} resp.; $P \leq 0.05$; Table 5.5). Time effects ($P < 0.001$), the treatment x time ($P < 0.001$), and the treatment x clone x time interactions ($P \leq 0.05$) were significant. Average TSugar concentrations at the start of the day (0900 – 1100h) were significantly higher in Cool compared with Warm (245.9 ± 10.8 and 196.0 ± 9.7 mg_mL^{-1}; resp. $P \leq 0.05$). Concentrations increased across the day under Warm temperature treatment, such that concentrations were significantly higher (1.2-fold) at the end of the day in Warm compared with Cool (307.7 ± 12.1 and 222.6 ± 10.3 mg mL^{-1} resp.; $P \leq 0.05$).

Table 5.5 Means for the relative total sugar (TSugar) concentrations (in units of mg mL^{-1}) ± 1 s.e. for diluted nectar samples (refer to text) in the three clonal lines after 48 hour temperature treatment at 15/10°C and 25/15°C D/N with a 12 hour photoperiod. Sample sizes are for 240 flowers (8 clone replicates, 15 flowers per clone). Same letter indicates no significant difference at the 95% *CI* ($P \leq 0.05$).

Clone	TSugar Concentration (mg mL^{-1})	
	Cool	Warm
Green	254.4 ± 12.8 [b]	267.6 ± 13.1 [b]
Orange	325.4 ± 14.4 [a]	277.6 ± 13.3 [b]
Black	195.4 ± 11.2 [c]	193.5 ± 11.1 [c]

On a standardised or per flower basis, the temperature x clone interactions for the TSugars (mg flower $^{-1}$ by Equation 5.1 below, where TSugar is abbreviated to TS) were significant (the ANOVA output appears in Table 5.17 of Appendix 5.3.3). Any pre-treatment or room effects were not significant.

$$TS\ (mg\ flower^{-1}) = \frac{Sample\ TS\ concentration\ (mg\ mL^{-1}) * total\ (pooled)\ volume\ (mL)}{No.\ of\ flowers\ sampled}$$

Eqn. 5.1

Temperature effects were significant for the Orange line only. That is, TSugar content was *ca*. 20% higher than under Cool temperature treatment ($P \leq 0.05$; Table 5.6). Comparing between the lines, the TSugars were highest in the Orange line compared with the Green or Black lines, which were similar to each other and in both treatments ($P \leq 0.05$).

Table 5.6 Mean standardised total sugars (mg flower $^{-1}$) ± 1 average s.e. for the three clonal lines, after 48 hour temperature treatment at 15/10°C or 25/15°C (D/N) with a 12 hour photoperiod. Sample size is for 240 flowers. Same letter indicates no significant difference at the 95% *CI* ($P \leq 0.05$).

Clone	TSugar Content (mg flower $^{-1}$)	
	Cool	Warm
Green	2.51 ± 0.21 ^{c}	3.04 ± 0.21 ^{c}
Orange	4.37 ± 0.21 ^{a}	3.63 ± 0.21 ^{b}
Black	1.62 ± 0.21 ^{c}	1.58 ± 0.21 ^{c}

While the overall time of day effects were not significant, that is, the TSugars were not correlated with time of day, the two- and three-way interactions between treatment, time and clone were significant ($P \leq 0.05$; Table 5.17 in Appendix 5.3.3). That is, TSugars were highest in the morning under Cool temperature treatment (overnight 12 h dark period of constant effective 5 –10°C) and in the late afternoon in the Warm regime (overnight 12 h dark period constant effective 10 –15°C; $P \leq 0.05$; Table 5.7).

Table 5.7 The 2-way interactions between time of day and treatment are significant such that TSugars were highest in the morning in Cool (overnight 12 h dark period of constant 15/10°C), and in the late afternoon in Warm (overnight 12 h dark period constant 25/15°C). Units are mg flower $^{-1}$. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05).

Treatment	9-11am	3-6pm
Cool (10/15°C)	3.3 ± [a]	2.2 ± [b]
Warm (15/25°C)	2.6± [b]	3.1 ± [a]

TSugars decreased across the day, in Cool (12 h light periods of constant 15°C) and increased in Warm (12 h light periods of constant 25°C; *P* ≤ 0.05; Figure 5.7). The Low values for GC 3-6pm are due to much reduced nectar volumes in Run 2 (COW; refer to Section 5.3.5.1) in the two particular clones sampled at that time of the day i.e. small sample sizes of two clones for each of the time categories. The rank orders of the genotypes were maintained in the two environments such that, in general, O > G > B (Figure 5.7).

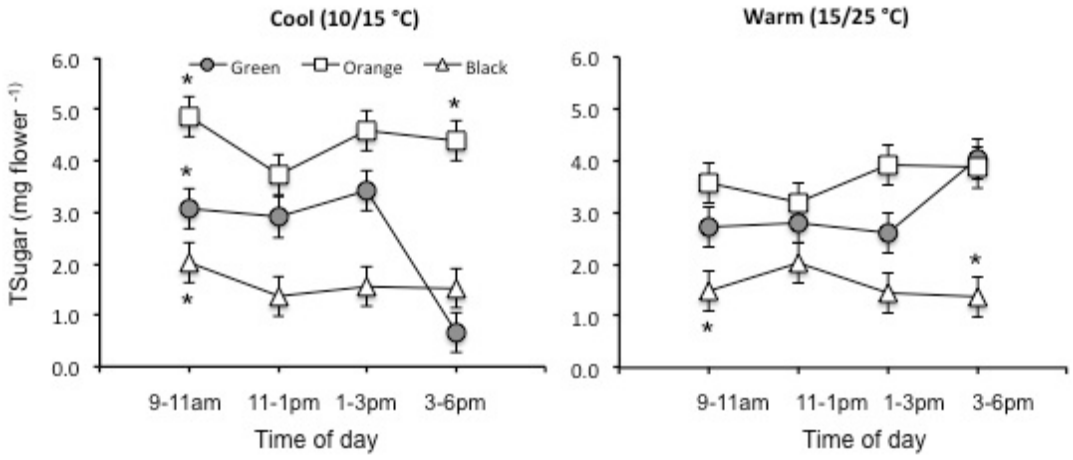


Figure 5.7 Mean total sugars (mg flower $^{-1}$) ± 1 s.e. in nectar sampled across the day under the short-term (48 h) temperature treatments, decreased in Cool (15/10°C; D/N) and increased in Warm (25/15°C) with time of day (*P* ≤ 0.05). The interactions between clone, temperature, and time are significant (*P* ≤ 0.05).

Under short-term temperature treatment, the standardised nectar TSugars and volumes were highly correlated (*Corr.* = 0.8526; *P* < 0.001).

5.3.3.2 Total nectar sugars in the long-term temperature regimes were clonally differentiated, and treatment effects for standardised sugars were significant for the Black line only

In the long-term temperature experiment, while the overall TSugar concentrations (mg mL^{-1}) of *diluted* nectar samples tended to be higher in the Cool compared with the Warm regime (Table 5.8; for variable *n* flower numbers as shown in Summary Table 5.13), any treatment effects were not statistically significant. Total sugars (% w/v) were higher in the Orange line (*ca.* 60 % sucrose equivalents) compared with the Green and Black lines, which were similar to each other (*ca.* 40% sucrose equivalents ($P \leq 0.05$) While time of day and any 2- or 3-way interactions involving time were not significant, there was a general trend for decreasing concentrations across the day in both temperature regimes (data not presented).

Table 5.8 Mean relative total sugar (TSugar) concentrations ± 1 s.e. for diluted nectar samples in the three clonal lines growing in glasshouses under long-term (12 month) Cool (Tmin. < 10°C) or Warm (Tmin. ≥ 10°C) temperature regimes. Sample sizes varied as shown in Table 5.13 Same letter indicates no significant difference at the 95% *CI* ($P \leq 0.05$).

Clone	TSugar Concentration (mg mL^{-1})	
	Cool	Warm
Green	436.1 ± 25.1 [b]	371.6 ± 25.1 [b]
Orange	649.4 ± 35.2 [a]	628.3 ± 29.1 [a]
Black	428.0 ± 37.7 [b]	379.2 ± 28.3 [b]

When standardised to a per flower basis, the temperature x clone interactions were significant ($P \leq 0.05$; Table 5.21 of Appendix 5.3.3). That is, treatment differences were significant for Black only, in which TSugars were 1.5-fold higher in BC compared with BW ($P \leq 0.05$; Table 9). Between the clonal lines, TSugars were highest (more than double) in Orange and similar in Green and Black ($P \leq 0.05$).

Table 5.9 Mean standardised total sugars (mg flower $^{-1}$) ± 1 s.e for nectar sampled from the three clonal lines growing under long-term Cool (Tmin. < 10°C) and Warm (Tmin. ≥ 10°C) temperature regimes. Same letter indicates no significant difference at the 95% *CI* ($P \leq 0.05$).

Clone	Cool	Warm
Green	5.1 ± 1.2 [b]	4.2 ± 1.1 [b, c]
Orange	12.2 ± 1.9 [a]	11.9 ± 1.9 [a]
Black	5.3 ± 1.3 [b]	3.5 ± 1.0 [c]

Time of day effects, and the 2- and 3-way interactions involving time (treatment x time, and treatment x clone x time) were not significant. However, there was a trend for higher TSugars in early morning Cool compared with early morning Warm, in the Green and Black lines. In BC, there was a negative trend for TSugars across the day (in contrast to all other clone-treatment combinations in which trends were positive) at the lower daytime temperature averages of 16 –17°C compared with common averages of between 19°C and 27°C (Table 5.10). Temperature data for the previous 24 hours prior to nectar collection were presented in Section 5.3.1.2.

While not correlated with the real-time (at time of sampling) temperatures or %RH, the TSugars (mg flower $^{-1}$) were highly correlated with nectar volumes (µL flower $^{-1}$; *Corr*. = 0.9702; $P < 0.001$).

Table 5.10 Mean TSugars (mg flower $^{-1}$) ± 1 s.e. for nectar sampled across the day (morning and afternoon) in the long-term Cool and Warm temperature treatments. The average glasshouse temperatures for each of the sampling periods are shown in brackets.

	Cool		Warm	
	0900 – 1200h	**1200 – 1700h**	**0900 – 1200h**	**1200 – 1700h**
Green (Day 282)	4.9 ± 1.3 (*19.2°C*)	5.4 ± 1.4 *(19.6°C*)	3.9 ±1.2 *(26.0°C)*	4.4 ± 1.2 *(27.1°C)*
Orange (Day 280)	11.6 ± 2.0 *(19.8°C)*	12.8 ± 2.1 *(19.2°C)*	11.3 ± 2.0 *(22.4°C)*	12.5 ± 2.1 *(23.9°C)*
Black (Day 273)	6.7 ± 1.5 *(17.8°C)*	4.2 ± 1.2 *(16.5°C)*	3.8 ± 1.2 *(23.1°C)*	3.3 ±1.1 *(19.8°C)*

Summary: (short vs. long-term TSugars)

Volumes and TSugars were highly correlated in both the short (0.85) and the long-term (0.97) experiments ($P < 0.001$). In the glasshouses, treatment and time of day effects on TSugar concentrations were not significantly different between Cool and Warm. In the CTRs, while concentrations were similar between treatments, TSugars per flower increased across the day in Warm ($P \leq 0.05$).

In the CTRs, TSugars (mg flower $^{-1}$) were highest in the morning in Cool and in the afternoon in Warm. Trends across the day were for decreasing TSugars in Cool and increasing in Warm ($P \leq 0.05$; Table 5.7). Trends were somewhat similar in the

glasshouses with morning TSugars appearing higher in Green and Black Cool compared with Warm. Trends across the day were for increasing and decreasing TSugars above and below daytime averages of around 19°C resp. (Table 5.10).

5.3.3.3 DHA per flower under the short term treatments was clonally differentiated with temperature effects significant in Orange only

Under short-term temperature treatment, after standardising the DHA concentrations of the samples (using Equation 5.3, i.e. accounting for water used at sampling) and after square-root transformation of the data (Section 5.2.3), the temperature x clone interactions were highly significant ($P < 0.001$).

$$\text{DHA (mg)} = \frac{\text{Sample DHA concentration (mg mL}^{-1}\text{)} * \text{total nectar volume (mL)}}{\text{No. of flowers sampled}}$$

Eqn. 5.3

Treatment differences were significant for Orange only, in which the DHA content was 1.4-fold higher under the Cool temperature regime (OC of 26.2 ± 1.8 and OW of 18.8 ± 1.5 µg flower $^{-1}$ respectively; $P \leq 0.05$; Figure 5.8). Between the clonal lines, DHA content was significantly higher in Orange under both temperature treatments (*ca.* 2 to 2.5-fold those of Green or Black), and lowest in Black ($P \leq 0.05$). Time of day effects and the time x temperature, and time x temperature x clone interactions were not significant. Block effects within the CTRs were negligible.

DHA content was highly correlated with TSugar content (*Corr.* = 0.8953; $P < 0.001$) and with nectar volume (*Corr.* = 0.8050; $P < 0.001$), on a per flower basis.

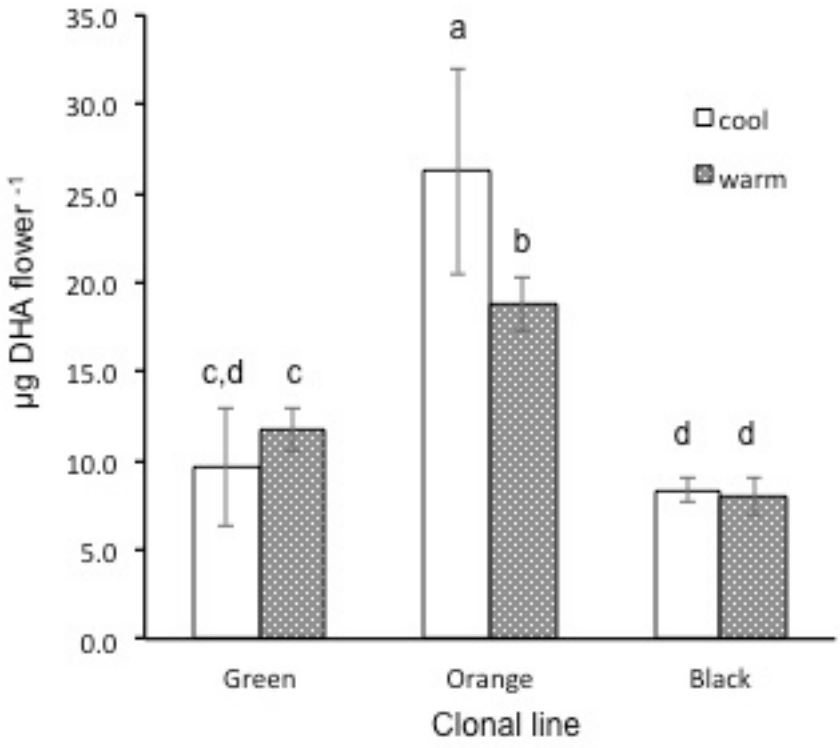


Figure 5.8 Mean DHA content (ug flower $^{-1}$) ± 1 s.e. in nectars from the three clonal lines under the short-term (48 h) Cool (15/10°C D/N) and Warm (25/15°C D/N) temperature treatments in growth rooms (12 h photoperiod;). *n* is for 240 flowers. Different letter indicates a significant difference at the 95% *CI* (*P* ≤ 0.05).

5.3.3.4 DHA content in the long-term treatments was clonally differentiated in Warm and any temperature effects were significant in the Orange and Black lines

The overall temperature effects on DHA content (µg flower $^{-1}$) under long-term temperature treatment were significant ($P \leq 0.05$; Table 5.24 of Appendix 5.3.4). Position effects within the glasshouses were not significant. Time of day effects, treatment x time, and treatment x clone x time interactions were also not significant. The temperature x clone interactions were highly significant ($P < 0.001$), such that the temperature effects were significant for the Orange and Black lines ($P \leq 0.05$; Figure 5.9). DHA content was increased at the warmer temperatures in Orange (85.2 ± 2.2 µg flower $^{-1}$, and 54.1 ± 3.9 µg flower $^{-1}$ in Cool) and at the cooler temperatures in Black (39.9 ± 4.2 µg flower $^{-1}$, and 26.6 ± 3.1 µg flower $^{-1}$ in Warm). Changes were 1.6-fold in Orange, and 1.3-fold in Black. Any differences between the clonal lines were significant

in the Warm regime only, in which the DHA contents were highest in Orange and similar in Green and Black ($P \leq 0.05$).

DHA content was highly correlated with both volume (*Corr.* = 0.92; $P < 0.001$) and TSugars (*Corr.* = 0.90; $P < 0.001$), on a per flower basis. DHA content was not correlated with plant size or growth rate (data not presented).

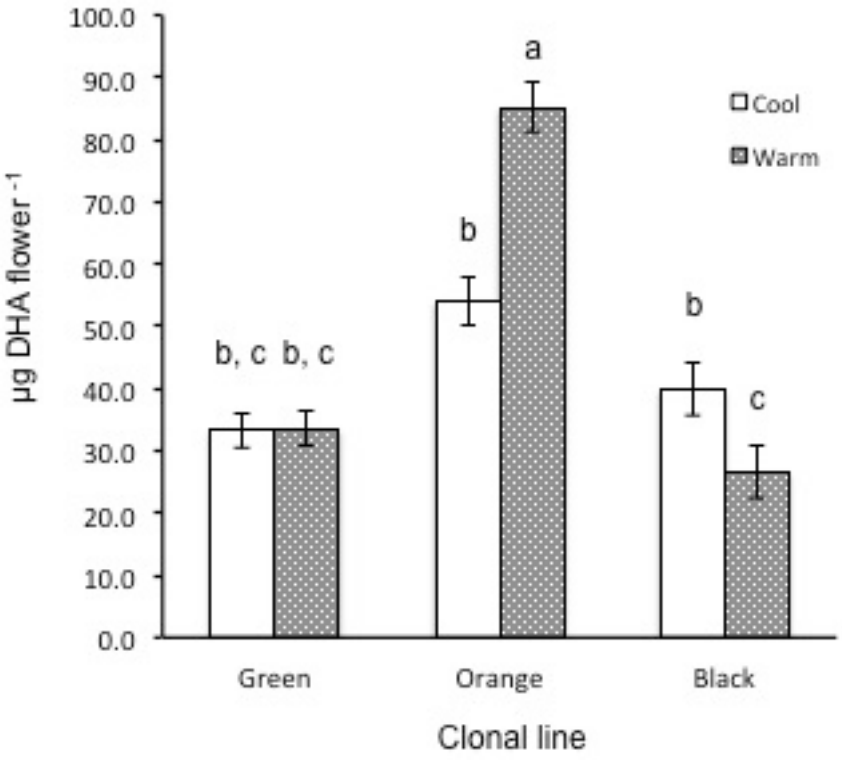


Figure 5.9 Mean DHA content (µg flower $^{-1}$) ± 1 s.e. for the three clonal lines growing in glasshouses under two different temperature regimes, Cool (Tmin. < 10°C) and Warm (Tmin. ≥ 10°C). Sample sizes are for 101, 96, 54, 104, 116, and 120 flowers for respective bars from left to right. Different letter indicates a significant difference at the 95% *CI* ($P \leq 0.05$).

Summary: (short vs. long-term DHA)

The temperature x clone interactions were significant for both experiments, such that temperature effects were significant in Orange in CTRs, and in Orange and Black in glasshouses. Any diurnal effects were absent.

Mean DHA in growth rooms were around 35% lower, on average, than in glasshouses.

DHA and TSugars were highly correlated within the experiments.

5.3.4 Compositional Changes

The compositional changes for the long-term glasshouse experiment (Experiment #2) are presented only (Experiment #1 data not collated due to time limitations).

5.3.4.1 Changes in sugar composition (% w/v) under the long-term temperature treatments are significantly different

The relative proportions of sucrose, glucose, and fructose differed between the clonal lines (Table 5.11). Fructose was the dominant sugar in all three of the lines and under both temperature regimes with levels ranging from 55 to 59% w/v (sucrose equivalents) of the total sugars. Sucrose was present in relatively small amounts ranging from between 0.6 and 2.7% w/v (sucrose equivalents), and tended to be higher in Green (significance tests between lines not done; within line treatment means were tested for significance by Student's t-tests).

Long-term temperature treatment altered the relative abundance of the sugars. Sucrose and glucose were significantly higher in the Cool compared with the Warm regime, in all clonal lines ($P \leq 0.05$ & $P < 0.001$ resp.). In contrast, fructose was significantly reduced in Cool ($P < 0.001$) and in all lines. The sucrose (S) to hexose (H; combined fructose and glucose) ratios were significantly higher under Cool temperature treatment ($P \leq 0.05$; Figure 5.10).

Table 5.11 Mean proportions of sucrose, glucose and fructose as a percentage of total sugar in nectar, on a weight by volume basis (% w/v based on sucrose equivalents; mg mL $^{-1}$) ± 1 s.e. for the clones. ** denotes significance within a clonal line at the 99.9% *CI* (*P* < 0.001); * at the 95% *CI* (*P* ≤ 0.05) by student's t-tests.

Clone	Sucrose		Glucose		Fructose	
	Cool	Warm	Cool	Warm	Cool	Warm
Green	**2.7 ± 0.1 ****	1.2 ± 0.1	**42.1 ± 0.2 ****	39.5 ± 0.3	55.2 ± 0.2	**59.3 ± 0.4 ****
Orange	**1.0 ± 0.01****	0.7 ± 0.01	**42.5 ± 0.1 ****	41.2 ± 0.1	56.5 ± 0.1	**58.1 ± 0.1 ****
Black	**0.8 ± 0.02 ***	0.6 ± 0.03	**42.8 ± 0.2 ****	41.1 ± 0.1	56.5 ± 0.2	**58.3 ± 0.1****

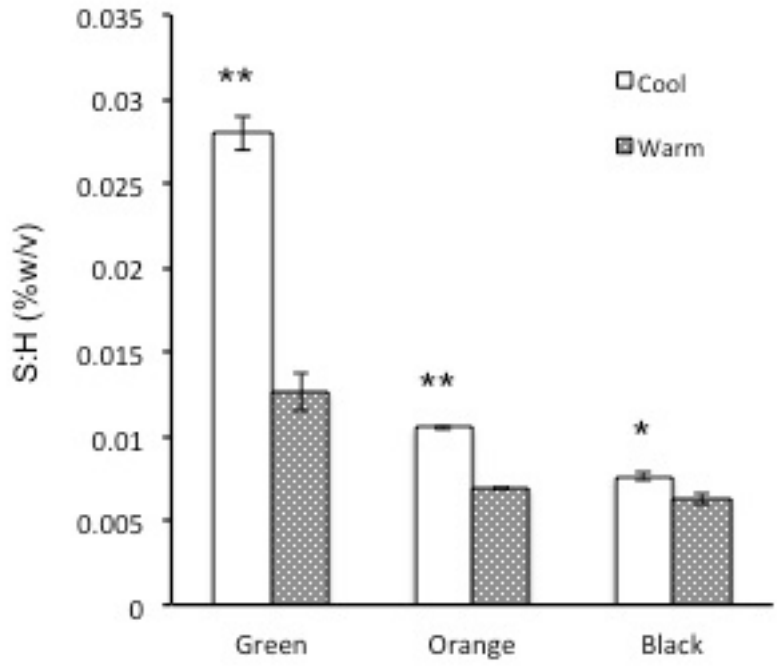


Figure 5.10 Sucrose (S) to hexose (H) ratios (%w/v) for the three clonal lines under long-term temperature treatment. ** denotes significance within a clonal line at the 99% *CI* (*P* < 0.001); * at the 95% *CI* (*P* ≤ 0.05) by student's t-tests.

5.3.5 DHA to Total Sugar Ratios (DHA/TS)

5.3.5.1 DHA/TS was significantly different between the Cool and Warm short-term temperature treatments for the Orange line only

Under short-term temperature treatment, the temperature x clone interactions on the ratios of DHA to total sugars (DHA/TS) were highly significant ($P < 0.001$; Table 5.21 in Appendix 5.3.5). There were also significant carryover (pre-treatment) effects (Table 5.21 in Appendix 5.3.5), that is, statistical non-equivalence of the Sequences (the NoCarryover x Carryover term in the model resp.; $P \leq 0.05$) but not Runs (refer to Table 5.1 in Section 5.2.3 for term descriptions). Temperature effects for DHA/TS were significant for the Orange line only, in which ratios were higher in the Cool than in the Warm temperature treatment (0.0061 ± 0.0003 and 0.0052 ± 0.0003 resp.; $P \leq 0.05$; Figure 5.11). At a clonal level, DHA/TS was highest in Orange and Black and lowest in Green, in both treatments ($P \leq 0.05$). Time of day and any 2- and 3-way interactions involving time were not significant.

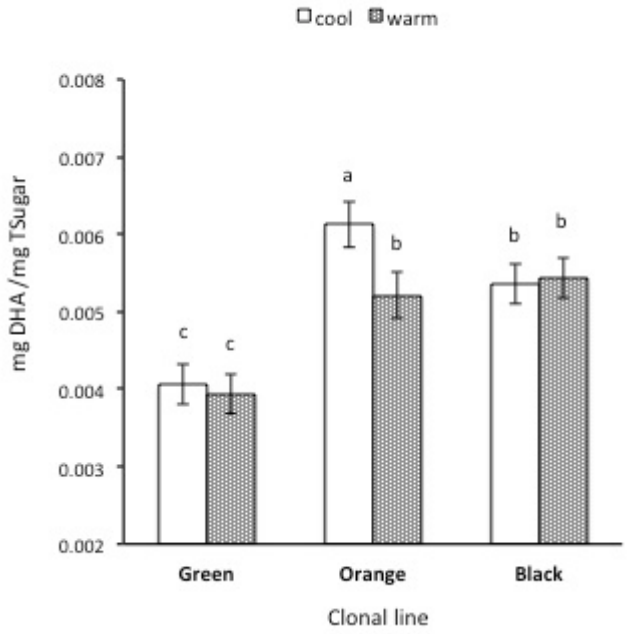
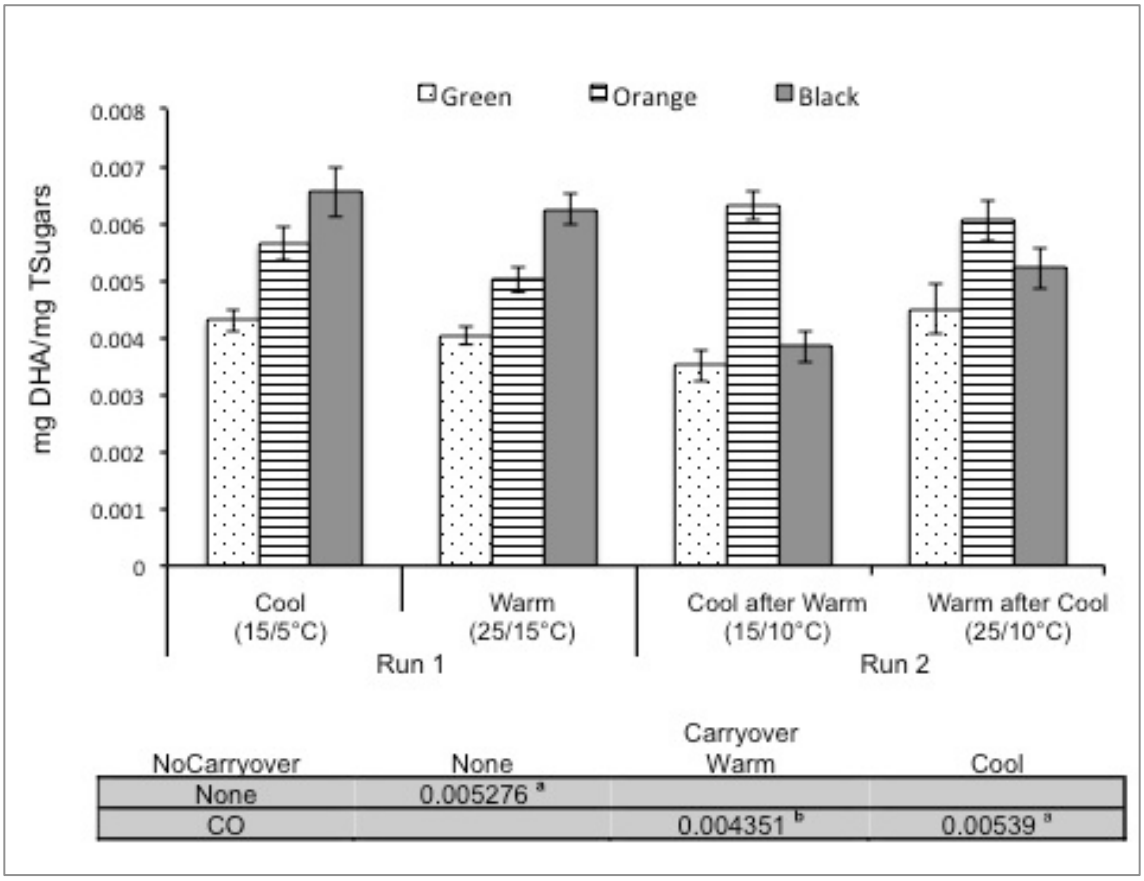


Figure 5.11 Mean nectar DHA to total sugar ratios (DHA/TS) ± 1 s.e. for the three clonal lines (Green, Orange, and Black) under short-term (48 h) Cool (15/10°C D/N) and Warm (25/15°C D/N) temperature treatments in growth rooms (constant D/N temperatures; artificial lighting; 12 h photoperiod). Different letter indicates a significant difference at the 95% *CI* (*P* ≤ 0.05).

Further analysis of the pre-treatment effects revealed that while there were no treatment effects in Run 1 (Cool = Warm), there were in Run 2 (Cool ≠ Warm). That is, the treatment means for Warm followed by Cool or 'Carryover Warm' (COW; Sequence 2) were significantly lower (0.0044 ± 0.0002; Figure 5.12) than for Cool followed by Warm or 'Carryover Cool' (COC; Sequence 1; 0.0054 ± 0.0002; $P \leq 0.05$;). It cannot be confirmed however whether the observed differences are the result of *carryover* effects or *room* effects since the two factors are statistically confounded. However looking at the actual temperature data (Section 5.3.1) the author considers it more likely to be room effects.

The normalised DHA equivalents (total sugars standardised to 80 °BRIX or 800g L^{-1} to equate with that of honey), as determined in the absence of any pre-treatment effects i.e. using the means from Run 1 only, were confirmed as significantly different between the clonal lines. That is, normalised DHA (when averaged across the treatment means) of 3333 ± 147 mg kg^{-1}, 4257 ± 217 mg kg^{-1}, and 5100 ±283 mg kg^{-1} in Green, Orange, and Black respectively; P ≤ 0.05). Refer to Table 5.22 of Appendix 5.3.5 for the actual clone-treatment values.



NoCarryover	Carryover None	Carryover Warm	Carryover Cool
None	0.005276 [a]		
CO		0.004351 [b]	0.00539 [a]

Figure 5.12 Mean DHA/TS ± 1 s.e. for the three clonal lines, Green, Orange, and Black, in the short-term temperature crossover experiment. Runs 1 & 2 (the NoCarryover terms of None and CO resp.) were statistically equivalent. However, non-equivalence of the crossover treatments, Sequence 1 (Cool after Warm or CO Warm) and Sequence 2 (Warm after Cool or CO Cool) was established ($P \leq 0.05$). Temperatures shown are 'effective' as explained in Section 5.3.1.1.

5.3.5.2 DHA/TS was significantly different between the Cool and Warm long-term temperature treatments for the Green and Orange lines

Under the long-term temperature regimes, there were significant temperature effects and temperature x clone interactions for DHA/TS ($P < 0.001$), but an absence of time of day effects or their interactions (Table 5.23 of Appendix 5.3.5). That is, overall means for DHA/TS of 0.0075 and 0.0061 in the Warm and Cool regimes resp., or an average 1.2-fold increase in DHA/TS under the Warm temperature treatment ($P \leq 0.05$). While mean DHA/TS was similar in all three lines in the Warm regime (a convergent interaction pattern), in the Cool regime DHA/TS was significantly higher in Black than in Orange. Within the clonal lines, temperature effects were significant for the Green and Orange lines only, that is, 1.2- and 1.6-fold increases respectively under Warm temperature treatment ($P \leq 0.05$; Figure 5.13).

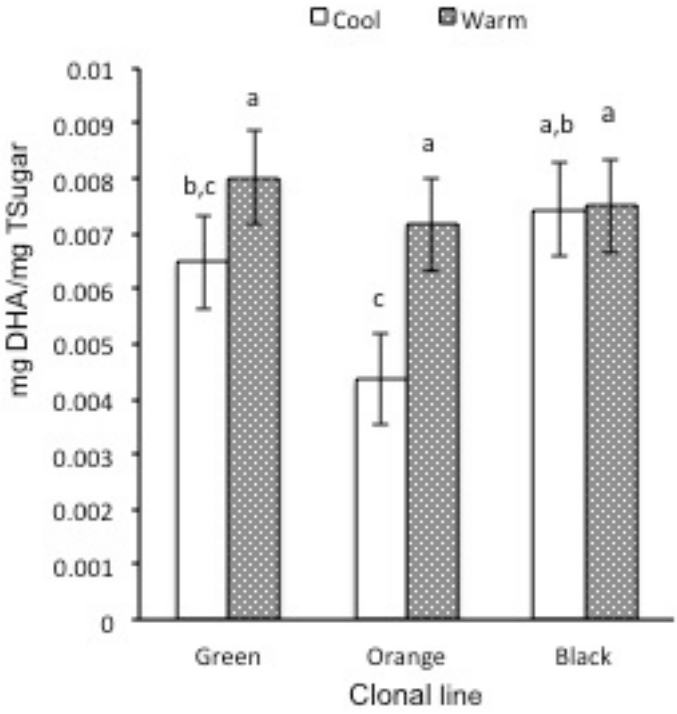


Figure 5.13 Means for nectar DHA to total sugar ratios (DHA/TS) in the three clonal lines, Green, Orange, and Black, under the long-term (in glasshouses) Cool (Tmin. < 10°C) and Warm (°Tmin. ≥ 10°C) temperature treatments. Standard errors are average standard errors. Different letter indicates a significant difference at the 95% *CI* (*P* ≤ 0.05).

The normalised DHA equivalents (at 80° BRIX) appear in Table 5.24 of Appendix 5.3.5. Maximum values were highest in GW (6414 ± 677 mg kg^{-1}), and lowest in OC (3491 ± 677 mg kg^{-1}; $P \leq 0.05$).

Summary:

DHA/TS of clones at peak flowering was not significantly altered by short-term temperature treatment, and any temperature x clone interaction effects were significant for the Orange line only ($P < 0.001$). The DHA/TS was clone-specific, with the rank orders well correlated across the Cool and Warm regimes (G < B ≤ O; $P \leq 0.05$). The normalised DHA equivalents in the growth rooms (in the absence of pre-treatment of temperature effects) were highest in Black (4942 ± 191 mg kg^{-1}), intermediate in Orange (4188 ± 150 mg kg^{-1}) and lowest in Green (3271 ± 105 mg kg^{-1}; $P \leq 0.05$).

Values were lower than in the glasshouses, in which maximum values were 6139 mg kg^{-1} in Black Warm, 6385 mg kg^{-1} in Orange Warm, and 5705 mg kg^{-1} in Green Warm.

5.3.5.3 MET Analyses of DHA/TS

By separate (short- and long-term) MET analyses (using FW regression; output not presented), G, E, and GEI accounted for 20%, 25%, and 23% of the variation respectively in the DHA to TSugar ratios in the long-term glasshouse experiment (P < 0.001). Under the short-term temperature treatments in growth rooms, the contribution of G to the observed variation in DHA/TS was 31% (P < 0.001). E and GEI did not contribute significantly.

5.3.6 Overall Summary

Tables summarising trends for all of the presented nectar variables (quantity and quality with the exception of normalised DHA) in each of the two experiments are presented below for comparison.

Table 5.12 Means ± 1 s.e. for the short-term (48 h; 15/10°C; 25/15°C Day/Night constant, 12 h photoperiod, artificial lighting) nectar variables. Data is for *N* number of clones and *n* number of flowers per clone. * denotes significance within a clonal line at the 95% *CI* (*P* ≤ 0.05).

Clone	Volume (µL_flower^{-1})		DHA (µg flower^{-1})		TSugars (mg flower^{-1})		DHA/TS		*N, n*	
	Cool	Warm	Cool	Warm	Cool	Warm	Cool	Warm	Cool	Warm
Green	5.2 ± 0.4	6.3 ± 0.4	9.6 ± 1.1	11.8 ± 1.2	2.5 ± 0.2	3.0 ± 0.2	0.0041 ± 0.0003	0.0039 ± 0.0003	8,240	8,240
Orange	8.5 ± 0.4	7.9 ± 0.4	**26.2 ± 1.8** *	18.8 ± 1.5	**4.4 ± 0.2** *	3.6 ± 0.2	**0.0061 ± 0.0003** *	0.0052 ± 0.0003	8,240	8,240
Black	3.0 ± 0.4	2.7 ± 0.4	8.3 ± 1.0	8.0 ± 1.0	1.6 ± 0.2	1.6 ± 0.2	0.0053 ± 0.0003	0.0053 ± 0.003	8,240	8,240

Table 5.13 Means ± 1 s.e. for the long-term (12 month; Tmin. < 10°C or Tmin. ≥ 10°C; fluctuating day time temperatures; natural light & day length) for the nectar variables. Data is for *N* number of clones and *n* number of flowers per clone. * denotes significance within a clonal line at the 95% *CI* (*P* ≤ 0.05),

Clone	Volume (µL flower^{-1})		DHA (µg flower^{-1})		TSugars (mg flower^{-1})		DHA/TS		*N*, n	
	Cool	Warm	Cool	Warm	Cool	Warm	Cool	Warm	Cool	Warm
Green	6.9 ± 0.4	6.4 ± 0.6	33.3 ± 2.8	33.5 ± 2.8	5.1 ± 1.2	4.2 ± 1.1	0.0065 ± 0.0008	**0.0080 ± 0.0008** *	8,101	8,96
Orange	14.1 ± 1.4	14.5 ± 0.5	54.1 ± 3.9	**85.2 ± 2.2** *	12.2 ± 1.9	11.9 ± 1.9	0.0044 ± 0.0008	**0.0071 ± 0.0008** *	4,54	7,104
Black	**6.4 ± 0.5***	5.28 ± 0.2	**39.9 ± 4.2** *	26.6 ± 3.1	**5.3 ± 1.3** *	3.5 ± 1.0	0.0073 ± 0.0008	0.0075 ± 0.0008	8,116	8,120

5.3.7 Other Environmental Variables in Brief

5.3.7.1 Nectar And Water Balance

In the Autumn Dry-down Trial (Experiment # 3, Section 2.6.3), average soil moisture content on the day of nectar sampling, as recorded by TDR (Time Domain Reflectometry) technology was *ca.* 32% and 10.4% in the sufficiently-watered and water-deficient Yellow line respectively, *ca.* 31% and 13% in Orange respectively, and *ca.* 24% and 13% respectively in the White line. Mean DHA/TS was unaffected by watering regime in two of the clonal lines (Orange and White). In a third line (Yellow) means were significantly different between the two treatments. For example, DHA/TS was reduced 40% in the Water-Deficient clones e.g. 0.0022 ± 0.0002 compared with 0.0034 ± 0.0003 in the Water-Sufficient clones ($P < 0.05$; Figure 5.14). Altered ratios were due to significantly higher DHA concentrations (1.7-fold) under the well-watered conditions (1.21 ± 0.07 mg mL^{-1} compared with 0.71 ± 0.11 mg mL^{-1} in the water-deficient conditions; Table 5.16). Clone-specific trends in DHA/TSugars were similar for nectar collected early and late in the season i.e. in early July and late October of 2015; data for July only is presented. Overall trends were for reduced concentrations of DHA/TS under limited soil water (Table 5.14). Nectar quality (DHA/TSugar) was maintained in the Orange line under conditions of limited soil moisture (70% FC). Root to shoot ratios were significantly higher in this line under the low water compared with the water sufficient treatment. In Yellow, the reduced DHA/TS under limited water treatment were associated with no significant change in root to shoot allocations. However, the root: shoot ratios in Yellow were significantly higher than those of Orange. No data was collected for the White clones.

Table 5.14 Mean with standard errors for DHA and total sugar concentrations for nectar collected in July, 2015 under the two soil moisture treatments, 100% FC (water-sufficient) and 70% FC (water-deficient). Treatments were administered in the autumn of 2015 to clones from the three clonal lines Yellow, Orange, and White.

Clonal line	DHA (mg mL^{-1})		Total Sugars (%w/v)	
	Deficit	Sufficient	Deficit	Sufficient
Yellow	0.71 ± 0.11	1.21 ± 0.07	32.3 ± 1.5	36.0 ± 3.0
Orange	2.43 ± 0.12	2.64 ± 0.07	28.3 ± 3.2	33.9 ± 4.7
White	1.99 ± 0.06	2.09 ± 0.26	30.5 ± 2.7	33.1 ± 2.0

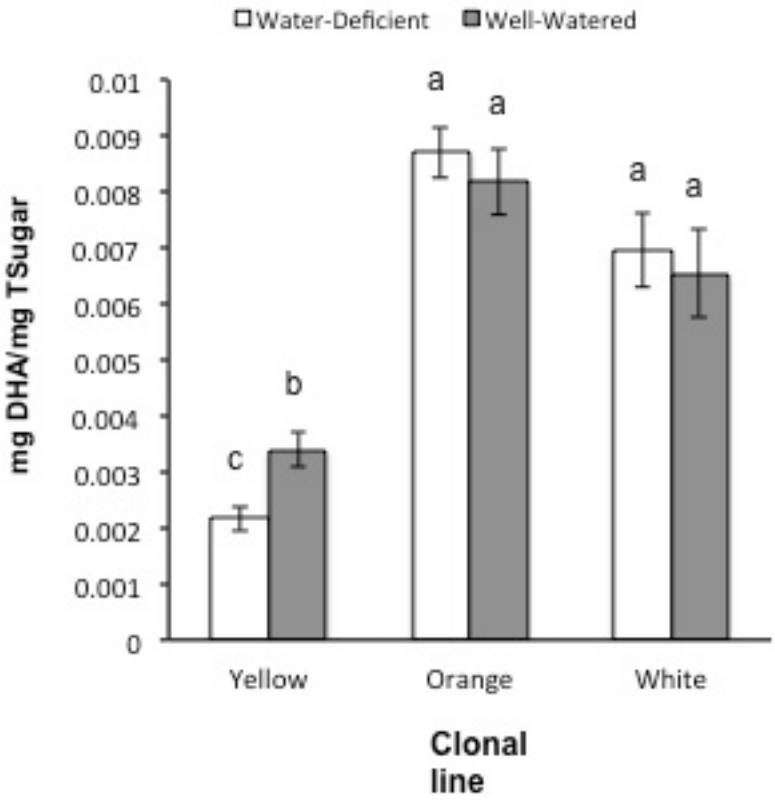


Figure 5.14 Mean DHA to total sugar ratios (DHA/TS) together with standard errors for the three clonal lines, Yellow, Orange, and White under mildly water-deficient (open bars; ~ 70% FC) and water-sufficient (coloured bars; ~ 100% FC) soil-water treatments. Sample sizes are *N* = 3, 4, 10, 9, 8, & 6 replicated clones from left to right. Sampling dates were the 2nd, the 7th, and the 6th of July, 2015 for Yellow, Orange, and White resp. Different letters denote significantly different means at the 95% *CI* (*P* ≤ 0.05).

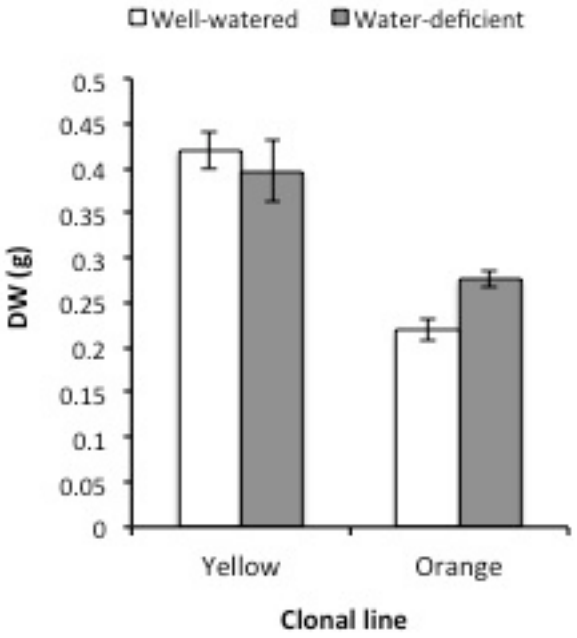


Figure 5.15 Mean root to shoot ratios (± 1 s.e.) in two of the clonal lines, Yellow and Orange for plants harvested (November 2014) from the Autumn Dry-down Experiment #4.

5.4 Discussion

In the controlled environments (glasshouses and growth rooms), traits describing the *quantity* and *quality* of nectar in the three Mānuka clonal lines (representing low, medium, and high accumulators of nectar DHA) were subject to significant GEI (clone x temperature x time of day). Secretion (nectar volumes and TSugars) was relatively unaffected by the temperature treatments. However, genotype and time of day (diurnal patterns) contributed significantly. In contrast, nectar composition (concentrations, DHA, DHA/TS, sucrose: hexose) was more strongly influenced by the temperature treatments.

Key to this chapter was establishing the relative nectar DHA content of the three clonal lines. Importantly, the relative rankings of the clones based on ratios of DHA/TS averaged across the Cool and Warm treatments in growth rooms in the absence of any pre-treatment or phenological or day effects, were reported as low in Green (0.0042 ± 0.0001 mg kg^{-1}), medium in Orange (0.0053 ± 0.0002 mg kg^{-1}), and high in Black (0.0064 ± 0.0002 mg kg^{-1}; Figure 5.12 in Section 5.3.5.1). That is, the equivalent normalised DHA values (total sugars standardised to 80 °BRIX) of 3333 ± 147 mg kg^{-1}; 4257 ± 217 mg kg^{-1}; and 5100 ± 283 mg kg^{-1} respectively. For comparison, the normalised DHA values for the same clones (Green and Orange), growing in a glasshouse under mild temperatures described as 'close to ambient', were previously variously reported as 6153 ± 583 and 4116 ± 521 for Green and 5177 ± 510 and 5981 ± 442 for Orange in respective publications (Nickless, 2015; Nickless et al., 2016). In our study, ratios were higher in the glasshouses (ranging from 0.0044 to 0.0080) than in growth rooms (0.0039 –0.0061).

Genetic determinacy for Nectar Yield Traits in Mānuka

Substantial amounts of nectar were visible on floral discs on early morning arrival in the glasshouses (0800h; Figure 5.16). Comparing the trends in volumes across the day for the growth rooms and glasshouses (Figures 5.4 and 5.6), daytime temperatures of around 20°C (and above) together with sunlight appeared to support active nectar flow/secretion. This agrees with visual observations that temperatures at or above around 19°C together with incident sunlight on flowers supported good nectar flow.

Figure 5.16 There were substantial amounts of nectar on floral discs (marked by arrows) on early morning arrival in glasshouses for 'Green', 'Orange' and 'Black' clones (from left to right respectively). Images not to scale.

In this study, the yield traits of both nectar volumes and TSugars (since these were shown to be highly correlated, $P < 0.001$) were strongly determined by genotype (Figures 5.4 & 5.6 (short-term); Figure 5.7 & Table 5.10 (long-term)). For example, in the Orange line (medium DHA; *L. scoparium* var. *scoparium* X *L.* 'Nicolsonii') yields were consistently higher (approx. 2-fold; $P \leq 0.05$) than those of Green (low DHA line; open cross *L. scoparium* var. *incanum*) or Black (high DHA line; *L. scoparium* var. *incanum X L. scoparium* var. *scoparium*). Whilst nectar production (volume and TSugars per flower) was variable between the glasshouse and growth room environments (in conditions of fluctuating temperatures and sunlight, or constant temperatures and reduced PAR, afforded by each respectively), the genotype rank orders were highly correlated. That nectar production is a strongly genetically determined trait in Mānuka has also been inferred by others (Clearwater et al., 2018; Nickless et al., 2017; Williams et al., 2014). It is generally accepted that nectar

production in plant species, whilst variable at many levels (population, plant, individual flower), is a highly heritable trait (Pedersen, 1953; Percival, 1965; Teuber & Barnes, 1979; Mitchell, 2004 and references therein; Hawkins, 2009).

Slightly earlier flower stages for Black Warm may have contributed to the lower yields. Recent publications report the importance of flower developmental stage for robust analysis and comparisons of nectar yield and composition (Clearwater et al., 2018; Smallfield et al., 2018). While all attempts were made to sample flowers at a similar stage (Stage IV), flower development was not well synchronised across the glasshouses and it was often challenging to sample enough flowers at the same stage on the same day.

High Variability in Single-point Manuka Nectar Volumes

The recovered single-point nectar volumes were highly variable, both between and within the clonal lines. Large inter-clonal variation (at least as large as that contributed by genotype) has been observed by others working with clones of other cultivated *L.scoparium* varieties (Clearwater et al., 2018). In the absence of insect foragers, the single-point volumes reported here are more indicative of *nectar secretion rates* than of *nectar standing crop* (NSC). The strength of controlled environment studies to observe temporal variability by eliminating the spatial patterning of NSC observed in the field has been recognised. The volumes reported in this study then, reflect plant-generated variability.

Volumes compare well with those published for other plant species pollinated by medium to large sized bees, that is, maximum nectar volumes of around 10 microlitres per flower (Nicolson et al., 2007). The one- to two-fold differences between growth rooms and glasshouses (Figures 5.3 & 5.5) may be due to a variety of factors including plant age, light quality or phenological stage (see below). While visually, quantities

appeared greater at the warmer temperatures, this was not reflected in the data. Discrepancies could be due to lowered viscosity since viscosity is inversely (linearly) proportional to temperature (Nicolson et al., 2007), allowing nectar to flow more freely onto the sides of the floral disc. It is possible that not all of the nectar was recovered from floral discs by the pipette sampling method used. Perhaps, floral washing (of the entire flower) may be the more appropriate method to have used in this situation.

Time of Day Influences on Yield

Total nectar sugars changed with time of day i.e. there were strong diurnal effects. In general, TSugar production increased across the day with the warmer daytime temperatures and decreased with the cooler daytime temperatures, in both glasshouses and growth rooms (Tables 5.7 & 5.10). While trends in the glasshouses were not significant, patterns in the data presented in Table 5.10, suggest that daytime temperatures of around 20°C and above, supported active sugar secretion. At time of sampling, temperature differences between the Cool and Warm glasshouses may not have been large enough to observe any significant temperature effects, hence patterns in the data can be useful for speculation. The results reported here agree with the generally reported trend in other temperate species of higher secretion rates at higher temperatures, for example, in white clover (*Trifolium repens*; Jakobsen & Kritjansson, 1994), *Thymus* spp. (Petanidou & Smets, 1996), and red clover (*Trifolium pratense*; Jakobsen & Kritjansson, 1994; Petanidou & Smets, 1996; Shuel, 1952).

TSugar concentrations in the glasshouses (under natural day light) were relatively stable across the temperature treatments (Table 5.8), and the commonly observed inverse relationship between nectar volume and concentration with rising daytime temperatures was not observed (Nicolson et al., 2007). That is, a*ctive secretion* (observed here as increasing volumes *and* TSugars) appeared to be counterbalancing the effects of evaporation. Similar findings in Mānuka were previously reported by Nickless (2015). This could be an example of the universally understood separate

regulation of water and sugar in nectar (Nicholson et al., 2007). In contrast, in growth rooms, the more highly concentrated nectar at the higher temperatures (constant 25°C, artificial lighting; ($P \leq 0.05$; Table 5.5) was presumed due to secretion rates being insufficient to counteract the effects of evaporation. While no photosynthetic data was collected, reduced secretion is presumed due to reduced photosynthesis under the reduced light intensity of CTRs i.e. 20 to 25% PAR of sunlight (*ca*. 800 - 1200 µmol m^{-2} s^{-1} as measured in the glasshouses on a typical sunny day with some cloud). Under the cooler growth room conditions (constant 15°C), concentrations were comparatively well maintained across the day observable as both decreasing volumes *and* decreasing sugars. It is suggested that this is evidence for *reabsorption* of sugars under conditions of low light and low temperature (145 µmol m^{-2} s^{-1} and constant 15°C). Reabsorption occurs in several *Eucalyptus* species (Davis, 1997), and has also very recently been inferred in aging Mānuka flowers (Clearwater et al., 2018).

The TSugar concentrations (20 –30% w/v;) under artificial lighting of growth rooms (25% PAR of sunlight) were well below typically reported averages for bee pollinated (open-type) flowers of around 42% w/v sucrose equivalent (Pyke & Waser, 1981), and also for the glasshouse experiment (65% w/v). However, the contribution of plant age, size, or seasonally related differences (since experiments were carried out in consecutive years) to the observed differences cannot be ruled out.

The Positive Influence of Overnight Low Temperatures on Secretion

In addition to the positive influence of high daytime temperatures on sugar production, low overnight temperatures also appeared to increase nectar production in this study. That is, both volume and TSugars were highest in the morning after cold overnight temperatures (Tables 5.7 & 5.10). While this has been reported elsewhere for two other (leguminous crop species, alfalfa (Walker et al., 1974) and white clover (Jakobsen & Kritjansson, 1994), it has not been previously reported for Mānuka.

TSugar increases in those species were presumed due to decreased night respiration. TSugar content of floral nectar has sometimes been correlated with maximum daytime temperatures of the previous day (24h period), mostly in Mediterranean species (Perez-Banon 2000, cited in Petanidou, 2007), in red clover (Shuel, 1952), and also in a very recent study of Mānuka (Clearwater et al., 2018). We suggest that the amounts of nectar sugars produced are the combined effects of both night time and daytime temperature lows and highs resp. in the previous 24 hour period. This may well explain the often observed [Clearwater et al., 2018, and the Massey Mānuka team (*unpubl.*)] lack of correlation between real-time temperatures and RH, and nectar TSugars at time of sampling. We suggest the reported 'lag effect' (Clearwater et al., 2108) is best explained by the influence of low overnight temperatures that often follow clear hot days. While a regime of overnight lows together with daytime high temperatures was not one of the treatments tested in this study, the possibility of this combination producing elevated TSugars (due to reduced respiration in cool night temperatures) could be investigated in a future experiment.

Other Environmental Effects

While secretion rates are reported to be higher in plants growing under sufficient soil moisture (O'Brien, Loveys, & Grant, 1996; Petanidou & Smets, 1996; Wyatt et al., 1992), in this study the average nectar sugar concentrations per flower were not supressed by drying soil. However under conditions of low soil moisture (~70% FC; Experiment #4), nectar DHA/TS in one of three clones (Yellow, low DHA producing line) was significantly lower than in clones at ~100% FC (Table 5.14).

Compositional Changes (S, F, G balance; DHA, DHA/TS)

In the clones growing in glasshouses, nectar sucrose content and the sucrose to hexose (fructose and glucose) ratios were higher at the cooler temperatures ($P \leq 0.05$). This has also been reported for white clover (*T. repens)* and alfalfa (*Medicago sativa*),

(Jakobsen & Kritjansson, 1994; Walker et al., 1974). In those studies, the higher sucrose levels were attributed to low night temperatures.

DHA, DHA/TS

The DHA content of nectar was largely genetically determined in agreement with Nickless, (2015) and Nickless et al., (2017). That is, the genotype rank orders for DHA were fairly well correlated both between treatments and across the experiments. The variables of DHA and TSugars were highly correlated within experiments. There was an absence of diurnal effects on DHA. Mean DHA contents in growth rooms were around 35% lower, on average, than in glasshouses, and also when compared with a previous (glasshouse) study using some of the same clones (Nickless, 2015). Rank orders for DHA/TS agreed fairly well across the treatments but were poorly correlated across the growth rooms and glasshouse experiments). For example, Orange was ranked high in CTRs and low in the glasshouses. The clonal means for DHA/TS tended to converge under warm temperature treatment in both experiments (reduced genetic variance; $P \leq 0.05$; Figure 5.17). Both DHA and TSugars were plastic across the treatments, that is, strongly influenced by environment in Orange in the CTRs, and in Orange and Green in the glasshouses (Figures 5.11 & 5.13). While cold nights acted to increase the amount of nectar sugars (see above), warm nights acted to decrease them leading to increased ratios of DHA/ to TSugars. Time of day was not a significant factor, and DHA and TSugars were highly correlated within the treatments.

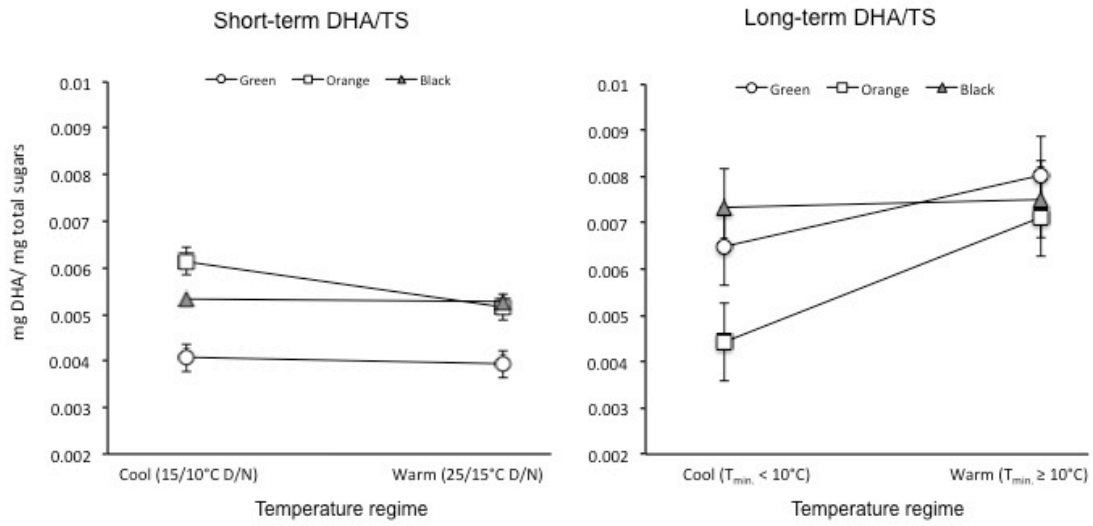


Figure 5.17 Interaction plots for nectar quality (mean DHA to TSugar ratios i.e. mg DHA to mg TSugars, ± 1 average s.e.) in the three clonal lines, Green, Orange, and Black, in the Cool and Warm temperature environments under short- and long-term treatment durations (left and right images respectively).

5.5 Conclusions

DHA (µg flower $^{-1}$) and TSugars (mg flower $^{-1}$) were plastic across both the temperature treatments and environments. Given the strong environmental influence on these two variables, it is not surprising then, that the assigned (relative) categories of 'low', 'medium', and 'high' for nectar DHA in this chapter do not necessarily agree with those of Nickless (2015) presented in Table 2.2. The ratio of DHA to TSugars was driven by changes in DHA, TSugars, or both, and responses varied between the clonal lines. While traits for nectar *quantity* (volumes) appear to be under strong genetic control and were little affected by differences in temperature, traits describing *quality* (total sugars, DHA) were strongly influenced by environment. However, whether the observed plasticity in glasshouses arises from phenological, temperature, or day effects, or their combinations, is unclear. Certainly from the growth room experiments, in which phenology and day effects were eliminated, it would appear that temperature and light quality (both wavelength and photon flux density), and their likely interactions were major determinants of nectar quality in the clones.

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Appendix 5.3.1

Long-term Temperature data

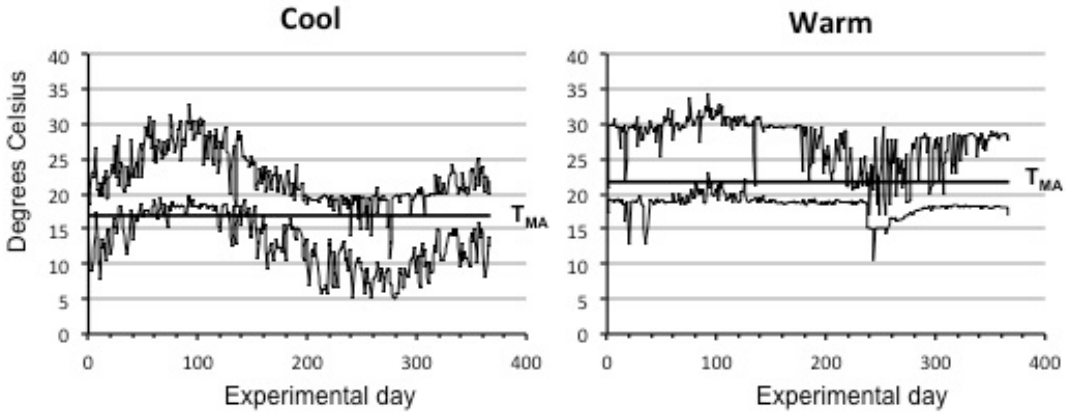

Figure 5.18 Temperature data from the long-term temperature treatments (Experiment #2). Further analysis was reported in Section 3.3.1.

Appendix 5.3.2

Short-term nectar volumes

Table 5.15 Analysis of variance by LMM for the single-point nectar volumes in the short-term experiment. n.d.f. is numerator degrees of freedom, d.f.f is denominator degrees of freedom.

Fixed term	n.d.f.	*F* statistic	d.d.f	*P* value
NoCarryover (Run effects)	1	1.48	61.7	0.228
NoCarryover x Carryover (Sequence effects)	1	0.15	62.3	0.700
Treatment	1	0.75	31.5	0.394
Time	3	14.13	40.0	<0.001
Treatment × Clone	4	57.69	58.2	<0.001
Treatment × Time	3	1.13	42.3	0.350
Treatment × Line × Time	12	2.92	61.6	0.003

Long-term nectar volumes

Table 5.16 Analysis of variance by LMM for the single-point nectar volumes in the long-term temperature experiment. n.d.f. is numerator degrees of freedom, d.f.f is denominator degrees of freedom.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Row	7.95	3	2.65	28.0	0.068
Treatment	3.29	1	3.29	28.0	0.081
Time_Grouped	7.09	1	7.09	28.0	0.013
Treatment.Line	25.33	4	6.33	28.0	<0.001
Treatment.Time_Grouped	4.21	1	4.21	28.0	0.050
Treatment.Line.Time_Grouped	6.47	4	1.62	28.0	0.197

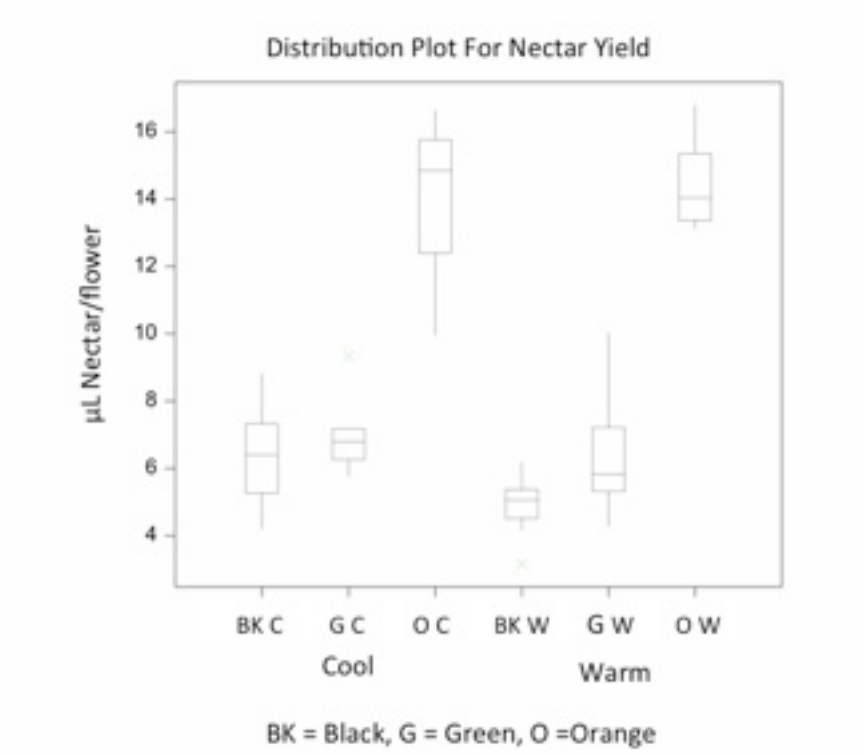

Figure 5.19 Distribution plot of mean nectar volumes with s.e. for the three clonal lines in the long-term (one complete annual cycle) temperature treatments 'Warm' and 'Cool' in the. Height of the boxes denotes the interquartile range i.e. the middle 50% of the data. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Data were collected from 158, 197, and 236 flowers for the Green, Orange, and Black clones respectively from left to right of plot.

Appendix 5.3.3

Short-term Standardised TSugars

Table 5.17 Short-term TSugar ANOVA (mg flower $^{-1}$) by LMM with four time points for time of day of sampling, 9-11am, 11-1pm, 1-3pm, and 3-6pm. n.d.f. is numerator degrees of freedom; d.f.f is denominator degrees of freedom.

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f.	F pr.
NoCarryover	2.05	1	2.05	64.3	0.157
NoCarryover x Carryover	0.05	1	0.05	64.6	0.818
Treatment	3.37	1	3.37	52.9	0.072
Time	5.94	3	1.98	12.1	0.171
Treatment x Clone	141.82	4	35.45	60.1	<0.001
Treatment x Time	17.76	3	5.91	14.2	0.008
Treatment x Clone x Time	24.21	12	2.01	65.8	0.037

Long-term Standardised TSugars

Table 5.18 Long-term TSugar ANOVA Table (mg flower $^{-1}$) by LMM with two time points for time of day of sampling, morning (9am –midday) and afternoon (midday –5pm). n.d.f. is numerator degrees of freedom; d.f.f is denominator degrees of freedom.

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f.	F pr.
Row	5.56	3	1.88	28	0.155
Treatment	5.27	1	5.27	28	0.029
Time	0.15	1	0.15	28	0.702
Treatment x Line	22.17	4	5.54	28	0.002
Treatment x Time	0.25	1	0.25	28	0.622
Treatment x Line x Time	5.47	4	1.37	28	0.271

Distribution Plot for Long-term TSugars flower $^{-1}$

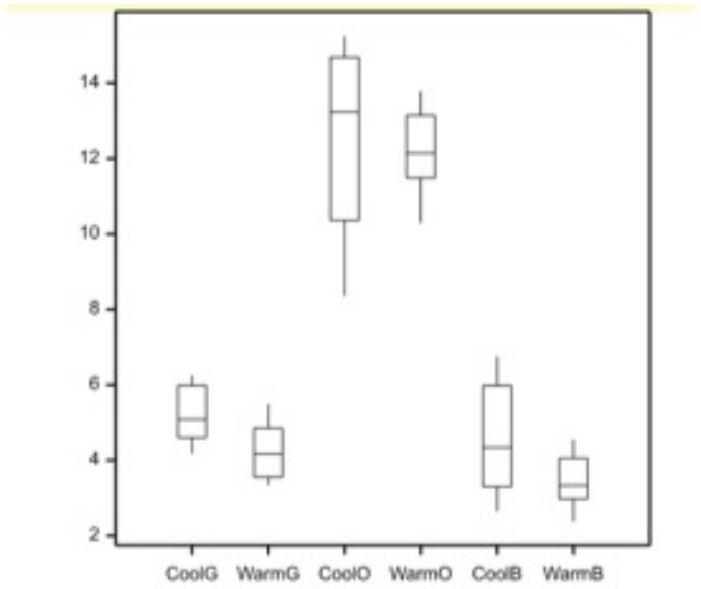

Figure 5.20 Distribution plot for total nectar sugar (TSugar) in the three clonal lines (Green, G; Orange, O; and Black, B) under the long-term temperature treatments. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Units are mm.

Appendix 5.3.4

Short-term DHA Model Output

Table 5.19 ANOVA Table for DHA content (µg flower $^{-1}$) for the three clonal lines under short-term (48 h) Cool (10/15 °C N/D) and Warm (15/25 °C N/D) temperature treatments. Time intervals across the day were, 9-11am, 11-1pm, 1-3pm, and 3-6pm.

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f.	F pr.
NoCarryover	3.44	1	3.44	63.6	0.068
Nocarryover x Carryover	0.68	1	0.68	63.8	0.414
Treatment	4.45	1	4.45	26.1	0.040
Time	3.36	3	1.12	11.4	0.382
Treatment x Clone	132.80	4	33.2	58.3	<0.001
Treatment x Time	5.35	3	1.78	13.5	0.199
Treatment x Clone x Time	16.34	12	1.36	64.5	0.209

Short-term DHA Content (µg flower $^{-1}$)

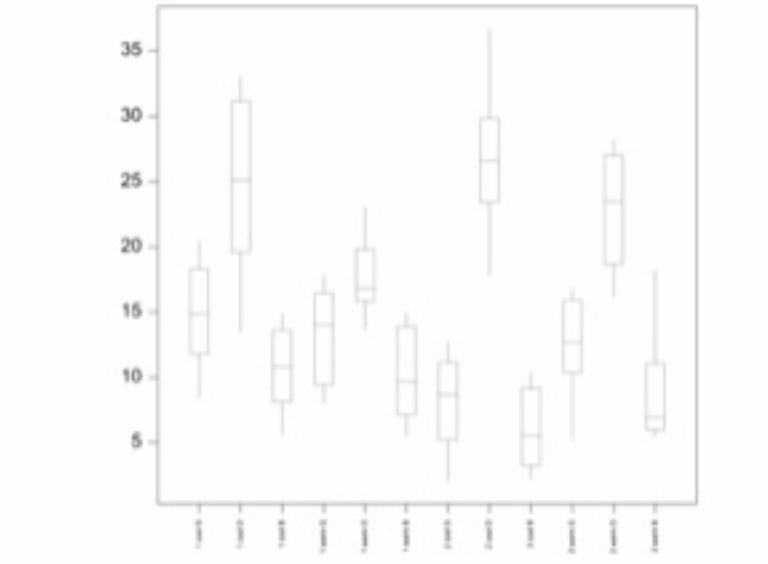

Figure 5.21 Distribution plot for mean DHA (µg flower $^{-1}$) under short-term the temperature treatments in the three clonal lines, B = Black, G =Green, and O = Orange. The height of the boxes denotes the interquartile range. Lines within the boxes mark medians. Whiskers are maximum and minimum values. Units are mm.

Long-term DHA Statistical Output by LMM

Table 5.20 ANOVA table for DHA content (µg flower $^{-1}$) for the three clonal lines under the long-term (12 month) Cool and Warm temperature regimes. Time intervals were 9am –12 midday and 12 midday – 5pm.

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f	F pr.
Row	2.49	3	0.83	28	0.489
Treatment	4.79	1	4.79	28	0.037
Time Grouped	2.02	1	2.02	28	0.167
Treatment x Clone	67.31	4	16.83	28	<0.001
Treatment x Time	2.05	1	2.05	28	0.164
Treatment x Clone x Time	7.10	4	1.78	28	0.162

Appendix 5.3.5

Short-term DHA: TSugar Ratios

Table 5.21 ANOVA Table for DHA/TS under the short-term temperature treatments.

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f.	F pr.
NoCarryover	3.25	1	3.25	63.9	0.076
NoCarryover x Carryover	4.45	1	4.45	64.2	0.039
Treatment	2.16	1	2.16	44.1	0.149
Time	14.34	3	4.77	14.7	0.016
Treatment x Line	41.52	4	10.38	59.0	<0.001
Treatment x Time	3.80	3	1.26	17.3	0.318
Treatment x Line x Time	7.16	12	0.60	64.7	0.838

Short-term Normalised DHA (to 80° BRIX)

Table 5.22 Normalised DHA values (means across the Runs and Sequences ± 1 average s.e.) for the three clones under the short-term temperature treatments. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05).

Clone	Cool	Warm
Green	3249 ± 206 [c]	3159 ± 206 [c]
Orange	4897 ± 206 [a]	4172 ± 233 [b]
Black	4273 ± 206 [b]	4347 ± 206 [b]

Long-term DHA/TSugar Model Output

Table 5.23 ANOVA Table for DHA/TS under the long-term temperature treatments

Fixed Term	Wald Statistic	n.d.f.	F Statistic	d.d.f.	F pr.
Row	1.47	3	0.49	28	0.691
Treatment	25.83	1	25.83	28	<0.001
Time	0.93	1	0.93	28	0.344
Treatment x Line	26.11	4	6.53	28	<0.001
Treatment x Time	0.14	1	0.14	28	0.710
Treatment x Line x Time	2.66	4	0.66	28	0.622

Long-term Normalised DHA (to 80° BRIX)

Table 5.24 Normalised DHA values (means ± 1 average s.e.) for the clones under the long-term temperature treatments. Same letter indicates no significant difference at the 95% *CI* (*P* ≤ 0.05).

Clone	Cool	Warm
Green	5186 ± 677 [b, c]	6414 ± 677 [a]
Orange	3491 ± 677 [c]	5737 ± 677 [a, b]
Black	5945 ± 677 [a, b]	6002 ± 677 [a, b]

Chapter 6 General Discussion

The Importance of Understanding GEI

In this small-scale study involving clones from three *elite* Mānuka cultivars varying in their relative nectar DHA concentrations, growth temperature had a significant effect on the physiological component traits of the growth and flowering processes, and on the subsequent physico-chemical properties of nectar. Phenotypic responses of the genotypes to the different temperature treatments, for the majority of the traits studied, displayed strong GEI. The only exceptions were brachyblast counts and floral bud numbers.

The final nectar yields reflect the cumulative environmental effects interacting with genotypically determined developmental sequences over a plant's entire growth cycle. In crop species, in addition to GEI, perhaps the greatest difficulty in defining an ideal plant (ideotype) has been the capacity for 'yield compensation.' That is, alternative 'strategies' by plants in response to limited resources or environmental pressure that result in similar yields (Jones, 2014). This study attempted to uncover some of the physiological processes underpinning changes, if any, in the observed end-point *nectar yields* i.e. both quantity (production) and quality (composition), in response to the imposed temperature treatments.

Results from this study will contribute to a better understanding of trait stability in the clones across different temperature environments, and will help in determining clone suitability. Findings may also be useful in future breeding programmes of Mānuka.

Temperature Acclimation

The high degree of thermal plasticity observed in the Green cultivar indicates greater 'apparent' acclimation since *irreversible* changes in phenotype (developmental plasticity) reportedly involve acclimation of metabolism compared with *reversible* changes (phenotypic flexibility) in, for example, photosynthetic and respiratory capacities (Atkin et al., 2005). Indeed, phenotype is the result of complex interactions between phenotypic plasticity, epigenetics, and allometry. That is, respectively, genetically mediated responses to both external and internal environmental changes, and the way in which these responses are coordinated to produce a coherent whole (Schlichting & Smith, 2002). Phenotypic plasticity has often been reported for Mānuka, and is believed to explain some of the intra-specific diversity of form reported in early texts (Allan, 1961; Cockayne, 1910). Our study aligns with the work of Ronghua et al. (1984), in which variation in flowering phenology and plant form between widely spread geographical ecotypes was reported to have both a plastic and a genotypic basis. The genetic mechanisms underlying plastic responses in plants are poorly understood. However it is thought that several different mechanisms might be involved in different aspects of plasticity. For example, environmentally dependent regulatory loci as well as non-epistatic loci at which allelic expression varies with the environment (Sultan, 2000).

The Growth-Flowering Link

Floral capacity was shown to be tightly linked to the growth process. That is, the flowering traits of *total floral bud numbers* and *floral density* were dependent on the amount of vegetative growth made. This is similar to crop species in which the amount of growth during the vegetative phase sets the achievable limit for grain yield (Jones, 2014). Floral capacity was strongly determined by the outgrowth of laterals (branching) and the subsequent production of brachyblasts. That is, the combined coaxial outgrowth of second and third order (in this study) laterals on primary shoot axes. Brachyblast production was temperature dependent establishing *floral capacity* as a plastic trait in the clones. The observed growth - flowering link is not an unusual one. In

apple and pear for example, in which floral bud formation has been extensively studied, conditions for flower formation in young trees are set by modifications of growth correlations (between *apical dominance* and *acrotony*[15]*)* to establish a hierarchy among the branches that form the canopy of the tree. Moderation of the two, leads to the development of a large number of meristematic growing points, among which the growth capacity is distributed (Nyéki & Soltész, 1996). Indeed similarities exist between the flower bearing *shoot shorts* of Mānuka and apple (*syn. brachyblasts* and *spurs* resp.). Furthermore, floral architecture and branching traits in garden rose have been shown to be genetically linked (Kawamura et al., 2011).

Altered growth and flowering habits in the Green cultivar provide further evidence for a strong growth-flowering link in Manuka. For example, indeterminate shoot growth at the warmer temperatures was associated with a uniquely protracted, uni-modal flowering curve. In garden rose (*Rosa spp.)* flowering time genes (FT1/FT) have been shown to co-locate with genes controlling the architectural traits of plant height, and plant form (Kawamura et al., 2015). Linkage groups (co-located alleles) containing genes for both were identified as candidate genes preventing independent segregation (or selection) of plant form and flowering behaviour in garden rose (Kawamura et al., 2015). The temperature-induced once flowering-like habit observed in the semi-prostrate Green cultivar is analogous to that seen in older rose varieties with weeping growth form.

Nectar Production

Nectar production in the clones was established as a strongly genetically determined trait that was influenced by temperature, radiation, and soil moisture i.e. the microenvironment. Importantly, in this study, the average amount of DHA (in micrograms) per flower was not correlated with plant size or growth rate in agreement with Nickless (2017), who also reported a similar result for relative growth rates in

[15] *Acrotony or basitony* are frequently considered as two fundamental phenomena underlying *arborescent or bushy* growth habits respectively *(Troll, 1937 cited in Barthelemy & Caraglio, (2007).*

Mānuka clonal cultivars. Our study aligns with the implicitly held view that nectar production is, at least partially, genetically fixed, and that variation among individuals is due to environmental factors (Teuber & Barnes, 1979; Walker, Barnes, & Furgala, 1974). We suggest that simple source-sink relationships, such as for example flowering and fruiting, which require considerable amounts of energy and are significant carbohydrate sinks (Burquez & Corbet, 1998) may explain quality differences between nectars collected early and late in the season. It is also quite possible then, that the plasticity observed in the DHA and TSugar levels in the glasshouses in this study may be better explained by phenological rather than temperature effects. While nectar was collected on the same day within the clonal lines, phenology differed between the glasshouse environments.

Variable DHA to TSugar ratios across the environments (clone x temperature x light) were driven by fluctuations in DHA, or TSugars, or both. However, unlike the total nectar sugars, diurnal effects were absent for DHA. That is, DHA was relatively more stable than the larger sugars suggesting its independent regulation. Recently, Clearwater et al., (2018) observed that DHA production is not stoichiometrically linked to synthesis of the larger sugars and provided the more variable ratios of DHA: hexose than fructose: glucose as evidence of this. In addition, it was proposed that the selective secretion, reabsorption, or loss of DHA compared with the hexoses from aging flowers further reinforced the idea that DHA is produced and secreted separately from the hexoses.

To date, the mechanisms for floral nectar sugar and DHA secretion are unknown in Mānuka. It is suggested that the mechanisms driving sugar production in the two thermo-periods (day and night) may be different, as universally, nectar sugars may be derived directly from photosynthesis or from stored starches in nectaries or in some cases mobilised from photosynthates in storage organs (Pacini et al., 2003). It is

possible that an influx of sugars at night via phloem uploading could explain high nectar sugar concentrations in the early morning, since reduced respiration rates in cooler overnight temperatures typically increase sap sugars. Conversely, (short-term) increases in temperature result in increased respiration, in a near exponential relationship (Atkin & Tjoelker, 2003). Increases in TSugars across the day supported by rising temperatures i.e. with fluctuating glasshouse temperatures, could mean that photosynthesis by nectaries, as recently proposed by Clearwater et al., (2018), may also be contributing to nectar secretion. Future research to clarify whether Mānuka nectar sugars are derived from stored carbohydrate or direct from leaf photosynthesis or that of nectary tissue, could be carried out by simple defoliation tests (see Southwick, 1984).

The Sunlight Theory

Taken together, results suggest that light quality (sunlight factors and possible temperature x sunlight interactions) may be important in determining the nectar yield outcomes in Mānuka on any given day. Preliminary evidence for the strong influence of solar radiation on Mānuka secretion rates in the glasshouse was previously provided by Nickless (2015). In nature, solar irradiation influences the temperature of a sunlit flower (Corbet et al., 1993; Petanidou & Smets, 1996). Indeed, secretion in some species is reportedly reduced on cloudy or dull days. For example, 24 hour nectar production rates on cloudy days were 62% sunny day production in *Ipomopsis aggregata* (Pleasants & Chaplin, 1983). In the literature, the effects of sunlight (irradiance) on nectar characteristics are species-specific. For example, in milkweed, *Asclepius syriaca*, while sun/light was not a variable in that study, volumes were greatest under clear plastic compared with the more opaque fabrics when inflorescences were bagged to exclude insects (Wyatt, Broyles, & Derda, 1992). Similarly, when *Thymus capitatus*, a woody perennial species of Mediterranean regions adapted to consistently high temperatures, is grown at low temperatures and

irradiance, nectar secretion depends more on changes in light levels than on temperature (Petanidou & Smets, 1986).

Industry Relevance

The floral and nectar characteristics of the genotypes studied were well adapted to the cool-temperature conditions that might typically be encountered during the main flowering period of a NZ spring (NIWA climate data). That is, blossoming was initiated in widely divergent overnight temperatures of around 6°C and 15°C. In addition, secretion was not suppressed at the lower temperatures. The high degree of plasticity observed in the growth, flowering, and nectar traits in response to the different temperatures is not surprising given the wide distribution (environmental tolerance) of New Zealand Mānuka.

1.7-fold (70%) increases in shoot length, with a longer effective growing season (i.e. at temperatures favourable for vegetative growth) and faster growth rates at the warmer temperatures, resulted in around 25% more floral bud sites, on average, across the genotypes. Very broadly, based on these results and assuming a linear relationship between growth and temperature (Grace, 1988), incremental increases of 0.25 mm day^{-1} in shoot extension may be expected for a 1°C increase in $T_{MA.}$ This is equivalent to an additional 90 mm of primary shoot growth annually. Linearity between rates of development and accumulated temperatures have been found to apply within the temperature range of around 5 –20°C for temperate species (Grace, 1988). However as a cautionary note to extrapolating outside of the temperatures studied, non-linear thresholds (stepped) can sometimes apply (Porter & Gawith, 1999).

Using the equation of Nickless (2017)[16], NP under cooler conditions (lower daily temperature averages) would be expected to be greatest for the Orange cultivar (characterised in relative terms by high sugars, high floral density, and high normalised DHA) and under comparatively warmer conditions for the Black cultivar (high DHA, high floral density and a long bloom period). Relatively low brachyblast numbers and floral density in the Green cultivar, due to reduced outgrowth of laterals, could be problematic in achieving high nectar yields. It must be remembered that in a field situation there will be additional environmental factors together with pollinator activity and their interactions, the effects of which may be additive to those reported here.At this stage industry applications from the small number of clones studied remain elusive.

Limitations of This Study

In glasshouse experiments, there are practical difficulties of exposing plants to a single environmental (stress) factor, at the exclusion of all others. However combinations of (stress) factors are the more likely situation in nature. Glasshouse environments are perhaps better able to simulate real-life situations such as might be encountered in the field than are growth rooms, since temperature also rises and falls (fluctuates) in natural environments with changing day length and irradiation. Relative humidity in the glasshouse environment also fluctuates alongside any changes in temperature. Comparisons with growth room experiments, however, where it is sometimes possible to uncouple factors, can prove valuable. Because of limitations on Mānuka performance in growth rooms and cabinets that can be attributed to lower light levels, it was not possible in this study to conduct longer-term experiments in those environments.

[16] NP = FP * YD, where NP is nectar potential, FP is flowering period, and YD is (µg nectar/flower*DHA conc.)*FD where FD = a measure of floral density.

The temperature differential between the glasshouse growing-environments varied across the season with ambient conditions as is usual for glasshouse environments. That is, the temperature differences were smallest during the summer months (3 –4°C) and largest during the winter months (5 –8°C). However, the reported temperature minima of ≥ 5°C and ≥ 10°C in the two glasshouses (Cool and Warm resp.) were maintained throughout. Accordingly then, the T_{MA} range studied was fairly narrow i.e. ~17°C to 22°C, and fell well within the optimal range of growing temperatures for temperate species (reported to be around 20°C by Jones. 2014). Optimal temperatures for the operation of photosynthetic machinery are reportedly between 20 and 30°C (Jones, 2014), and as such it is unlikely then that the responses by the plants in this study represented stress responses (either cold or high temperature).

An additional significant experimental difficulty encountered in this study was the lack of flowering synchronicity across the clone-treatments. Because rates of flower development differed between clones and treatments, it was challenging to sample flowers at the same stage, on the same day (in all of the glasshouse-conducted experiments). Also, while nectar sampling was achieved within a nine-day window, ideally it would have been better (from a statistical perspective) to sample all of the clone-treatments on the same day thus removing any day effects and simplifying the statistical model. It was not logistically possible to achieve this because of the time taken for nectar sampling and the skills required for the task, precluding the use of untrained assistants. Alternatively, sampling blocks (experimental units) across the 9-day window would have been a more suitable approach to quantify any day effects. Remedying the phenological differences between the treatments in the long-term temperature experiment is a more difficult problem to address. Perhaps more regular nectar sampling at different points in each of the clone-temperature floral curves may have been a more useful approach. Greater efficiency of nectar sampling by multiple

trained nectar collectors would certainly extend nectar data collection to include more clones in future experiments.

Statistical Models in Use

In G x E studies, the statistical model in use has been one of the factors influencing the outcomes of phenotypic and genotypic comparisons across sites (Przystalski et al., 2008). The initial statistical approach taken in this study was to use mixed models (LMM and GLM) to answer the question: "Do plant responses in warm conditions differ from those in cool conditions in glasshouses or growth rooms?" Choosing the simplest model that provided the best fit to the observed data (West, Welch & Galecki, 2014) was a primary goal in model selection. The LMM included clone and temperature as fixed effects and position effects as random terms. LMM was an appropriate choice since the REML algorithm used to estimate the variance components has been shown to be numerically robust against missing or unbalanced data structures and non-orthogonality (Piepho et al., 2008), both of which were inherent in the data sets. However, in terms of the larger picture and to scale-up findings to a real-life *field* situation, MET-analyses employing Finlay-Wilkinson regression proved a useful approach in comparing the performances of the clones in the different environments. For example, the approach taken by GenStat® to assess the *relative performances* of the clones across the growing-environments uses *genotype* as a random effect and treatment (environment) as a fixed effect. The statistical question driving the analysis then becomes "Which of the three genotypes is more 'stable' (less sensitive to environment) across the temperature environments?" MET analyses are typically used in large-scale comparative studies of important annual or tree crops where the relative performance of *numerous* varieties growing at *multiple* field sites is evaluated. Therefore, its appropriateness as a model in this smaller study is debatable. However, it is argued that the 'intention' of the model still holds. That is, to characterise the

sensitivity of each genotype to environmental effects by fitting a regression of the environment means for each genotype on the average environment means.

Overall Conclusions

The observed growth and flowering responses in glasshouse-grown genetic clones, from three breeding lines varying in their expression levels of nectar DHA, to changes in ambient temperature were cultivar-specific and appeared to be independent of their nectar DHA concentrations. Rather, the different responses of the clonal lines appeared to correlate with their different growth habits (plant form). For example, the two upright cultivars responded similarly to the temperature treatments, while the semi-prostrate variety behaved very differently.

Flowers were mostly terminal on short shoots such that total floral capacity was shown to be highly correlated with lateral outgrowth and the production of brachyblasts (i.e. the *branching* process). It is possible that brachyblast numbers could set the limit for achievable nectar potential (NP) in Mānuka, since NP is a function of floral density (Nickless, 2015). However, further studies would be needed to establish this. Floral capacity (bud formation) in the clones was genotype-specific and directly influenced by the temperature conditions imposed in this study. It follows then, that in a plantation setting, any environmental constraints imposed during the main growing period will likely have important consequences for the subsequent floral and nectar traits. Outcomes may or may not be beneficial to nectar flow depending on cultivar specificity (GEI).

Nectar production was promoted by low overnight temperatures, and in high daytime temperatures together with sunlight. Nectar quality was highest under the warmer temperatures (and high light) especially in the warmer overnight conditions which acted to decrease total nectar sugars possibly due to higher respiration rates. While DHA and TSugars were plastic traits, the genotype rank orders for DHA/TSugar were reasonably

well correlated across the variable temperature and light conditions afforded by the growth rooms and glasshouses.

In conclusion, genetics (genotype, and the 2- and 3-way interactions between clone and temperature) were a strong determinant of the relative quality of floral nectar in the Mānuka clones. Temperature and light intensity appeared to be major environmental influences of nectar quality. The environmental plasticity of DHA content, independent of the larger nectar sugars in this study, adds support for its separate secretion and regulation in Mānuka floral nectar.

Future Directions

Further studies to uncouple light (photon flux density) and temperature influences on Mānuka nectar production would be highly beneficial.

Results reported here are for two-year-old plants in which the phenophases of growth and flowering were shown to be separated in time. There was, however, evidence in the third season of growth (i.e. at the end of the Experiment #2) for simultaneous vegetative outgrowth and blossoming occurring on shoots, in all three lines. No data was collected. While this may have been a physiological response to confined roots or nutrient deficiency, or water deficiency, it could be an example of meristems transitioning between determinate floral buds and indeterminate vegetative buds. Reversion of the inflorescence apex (determinate) to vegetative growth (indeterminate), or the phenomenon of *proliferation (*Figure 6.1 below) as described by Troll (1959) and cited in Weberling (1988), is characteristic of many members of the Myrtaceae family to which Mānuka belongs. The most common examples occur in the Australian genera of *Callistemon* and *Metrosideros* with recurring or successive zones of vegetative and reproductive growth on shoots (Webering, 1988). Troll suggested that this phenomenon might be controlled by climatic factors. The fact that the development of flower bearing systems and vegetative zones occurs successively (in a spatial sense) in the same shoot which continues growing over time, (i.e. meristems

alternating between vegetative and reproductive modes) under certain conditions could prove favourable in Mānuka with potential for a greater succession of waves of flowering, thus lengthening the flowering season and subsequently increasing flower numbers under inductive conditions. The way in which this phenomenon contributes new growth for future floral bud formation raises questions deserving of further study.

Figure 6.1 An example of 'proliferation' in Mānuka. That is, reversion of a previously floral meristem at the shoot apex (as indicated by the terminal fruit) to a vegetative meristem (as indicated by the shoot extension originating at the base of the bud). This phenomenon is commonly seen in other members of Myrtaceae.

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