An Investigation of Factors that Contribute to Dihydroxyacetone Variation Observed in New Zealand *Leptospermum scoparium*

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Abstract

Honey derived from *Leptospermum scoparium* (commonly known as mānuka) is known to have anti-bacterial activity that is not entirely accounted for by the presence of hydrogen peroxide.[1] This is known as non-peroxide activity (NPA). The discovery of this medical benefit has led to mānuka honey being a major export for New Zealand. In order to assure supply, mānuka trees are being investigated to determine why certain specimens yield honeys with a greater NPA than others. This might result in plantations of *L. scoparium* that would yield honey with a consistently high NPA.

The compound responsible for the NPA is a 1,2-dicarbonyl known as methylglyoxal (MGO).[2] The precursor to this molecule was found in nectar of the mānuka flowers and was identified as dihydroxyacetone (DHA).[3] An investigation of DHA in the nectar of *L. scoparium* across different regions of New Zealand was carried out by Williams (2012).[4] It was confirmed that trees vary within and between regions across New Zealand.

This thesis describes different investigations into why the variation of DHA observed in various mānuka flowers is so great. Flowers were collected during flowering periods from 2011-2013 and were frozen prior to processing. The extraction method used ten flowers (10F) and samples were analysed by gas chromatography with flame ionisation detection (GC-FID). The DHA quantity was expressed with respect to the total sugar (Tsugar) in the nectar (DHA/Tsugar) in order to allow for comparison between samples.

The study by Williams (2012) was extended to include the Northland region.[4] Wild *L. scoparium* var. *incanum* specimens were collected from this region and it was determined that these trees only produced a low to moderate amount of

DHA/Tsugar. Williams (2012) also investigated the DHA variability of trees in close proximity to each other as these are supposed to be genetically similar.[4] This study was repeated in a different region and the findings were the same; that is trees that were in close proximity to each other can have different DHA/Tsugar.

One possibility of why DHA is observed in mānuka flowers is that it is used to combat stress as a compatible osmolyte. This was tested using chemical additives and it was found that the DHA/Tsugar varied as a result of the Tsugar as opposed to the amount of DHA. This response was cultivar dependent.

Different flower physiologies were also investigated. This included the andromonoecious nature of mānuka and the specific colour change in the hypanthium of the flower. Male flowers were found to have a larger amount of DHA/Tsugar as a result of a higher level of DHA than hermaphrodite flowers. This could suggest that DHA is being used in the hermaphrodite flowers for processes such as seed production. The DHA/Tsugar of flowers with different hypanthium colour was shown to be dependent on the variety type. The source and reason for DHA still remains unclear and therefore further study is required.

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List of Abbreviations

σ	Standard Deviation
% RSD	Relative Standard Deviation Percentage
1F	Single Flower Test
10F	Ten Flower Test
20F	Twenty Flower Test
ANOVA	Analysis of Variance
AR	Analytical Reagent
ABG	Auckland Botanical Gardens
C_3	Three carbon chain
DCM	Dichloromethane
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DHA/Tsugar	Dihydroxyacetone: Total Sugar Ratio
DNPH	Dinitrophenylhydrazine
F-1,6-P	Fructose-1,6-Biphosphate
F/G	Fructose: Glucose Ratio
G3P	Glyceraldehyde-3-phosphate
GC	Gas Chromatograph

GC-FID	Gas Chromatograph- Flame Ionisation Detector
GC-MS	Gas Chromatograph- Mass Spectrometer
GD	Glycerol-3-phosphate Dehydrogenase
HA	Hydroxyacetone
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatograph- Mass Spectrometry
MGO	Methylglyoxal
m/z	Mass : Charge Ratio
NPA	Non-peroxide Activity
NSW	New South Wales, Australia
NZ	New Zealand
OPD	o-phenylenediamine
PFBHA	o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamide
QLD	Queensland, Australia
RuBP	Ribulose-1,5-biphosphate
SA	South Australia
spp	Species (plural)
subsp.	Subspecies
TAS	Tasmania, Australia

TMSI	1-(trimethylsilyl)imidazole
TPI	Triose Phosphate Isomerase
Tsugar	Total Sugar
var	Variety
UMF®	Unique Mānuka Factor
WA	Western Australia

1 Introduction

1.1 Non-peroxide Activity of Mānuka Honey

The treatment of human wounds and ailments with honey has been practiced for thousands of years. Honey's antibacterial activity originates from hydrogen peroxide which is produced as a result of the action of glucose oxidase and from honey's intrinsic hygroscopic nature, high sugar content and a low pH environment which all combine to prevent bacterial growth.[5] In 1988, a survey found honey originating from *Leptospermum scoparium* (commonly known as mānuka) had antimicrobial activity additional to that due to the presence of hydrogen peroxide.[1] This is known as non-peroxide activity (NPA).

In 2008 the compound responsible for the NPA was identified as methylglyoxal (MGO). This 1,2-dicarbonyl was identified by isolation and characterisation.[2] A correlation between the MGO content and NPA was successfully established.[2] Further work established the precursor to MGO as dihydroxyacetone (DHA) which is found in the nectar of the mānuka flowers.[3] In the same study, DHA was quantified in a range of honeys with varying ages. Initially, DHA existed at high concentrations in fresh honey but decreased as the period of storage increased; simultaneously the amount of MGO increased. There was a lack of mass balance between the degradation of DHA and formation of MGO in this study and this was hypothesised to be due to DHA undergoing side reactions in the honey matrix.[3]

As the compound responsible for the NPA character of manuka honey has been traced to the *L. scoparium* plant, it is possible to test individual trees and therefore make predictions of the MGO yield and NPA of the honeys to be derived from

these trees. This potentially allows for the trees to be selected and bred to yield mānuka honey with consistently high NPA.

1.2 *Leptospermum scoparium*

Leptospermum scoparium J.R Forst *et.* G. Forst is a New Zealand indigenous species with widespread distribution. It is a member of the large Myrtaceae family which comprises 133 genera and at least 3800 species.[6,7] This family has evolutionary centres in tropical and sub-tropical regions of the Southern Hemisphere which include Southeast Asia, Australasia, Central and South America.[6,8] A defining characteristic of this family is the placement of the ovary, being classified as either half-inferior or inferior*. This family is further identified by the nature of the plant's internal phloem, the presence of vestured pits† on the xylem structures, the presence of numerous stamens in the flower and oil glands in the leaves.[6]

The *Leptospermum* genus is known to contain 83 species including *L. scoparium*. This genus is known to be polyphyletic, meaning that it has characteristics that appear to be shared by a common ancestor but are rather the result of convergent evolution. One of these characteristics is the nature of the fruit valves. *L. scoparium* has a persistent woody fruit and therefore is one of the 13 *Leptospermum* species to be classified in the *L. myrtifolium* subgroup; also known as the group eight of the *Leptospermum* species.[9] This subgroup is also defined by its deciduous sepals.[6]

Due to its wide distribution across New Zealand, *L. scoparium* is known by a multitude of colloquialisms such as tea tree and kahikatoa but the Māori term

* Inferior refers to the placement of the ovary below the point of attachment of other flower parts.
† Vestured pits are bordered pits in the xylem that have minute outgrowths from the secondary cell wall of the pit chamber.

mānuka is the most commonly used name. The mānuka tree has an r-type plant strategy which has resulted in it having two essential ecological roles in the New Zealand environment.[10] The first role is permanent habitation in sites that are unsuitable for the establishment of climax forests. These areas are often nutrient poor, unstable or are exposed to extreme environmental conditions. Secondly, mānuka is a seral species. Its rapid growth rate, seed production and short life span makes it suitable to be a primary succession species.[6,10]

L. scoparium is a species that exhibits several polymorphisms due to its adaptation to differing environmental conditions across Australasia. The observed variation in the plants suggests that it is possible that the amount of DHA will vary amongst different mānuka plants. A few of the common polymorphisms are detailed below.

1.2.1 Stature

Mānuka often achieves two metres in height, although some trees have exceeded four metres. Because it is often found in harsh conditions, there are forms of *L. scoparium* that have adopted a unique stature. The prostrate forms differ from other plants of this species as its growth is ground-hugging as opposed to upright. An experiment, whereby seeds of prostrate plants were removed from their site of origin and grown in a uniform environment, revealed that there was retention of the tailing form. This suggests that this characteristic is genetically controlled which has most likely evolved as a result of strong pressures (such as high winds or infertile soil). Mānuka can also occasionally present as a dwarf form such as the Nanum variety. This form has smaller leaves than the other forms in addition to reduced internodes.[11]

1.2.2 Flowers

Many variations of mānuka have been observed but extreme "sports" exist in the wild. These wild varieties have flowers that are typically white in colour, approximately 12 mm in diameter and typically have a single row of five petals (known as single flowers). It is rare to see red-flowered plants in the wild although one outstanding variety was found. This specimen was named *L. scoparium* 'Nichollsii'[‡]. This variety was thought to be so exceptional that it was used as a parent plant for many cultivars and is the source of the gene(s) responsible for the vibrant red pigmentation seen in many garden cultivars. Another trait brought into cultivation is the double flower; this is the arrangement of smaller petals into two rows. This trait was initially observed in a wild variety known as Leonard Wilson. It is thought that this variety has been renamed Album Flore-pleno.

Both Nichollsii and Album Flore-pleno have now been superseded in the wild although they can be found in the United Kingdom. **Figure 1.1** illustrates common garden cultivars that have different flower morphologies.[11,12]

[‡] Cultivars will now only be referred to as their common name; the species name will be omitted.

Figure 1.1: Examples of garden cultivars that have different flower morphologies including: single pink flower (upper left), single red flower (lower left), double red flower (upper middle), dwarf white single flowers (lower middle) and double white flowers (right).

The average flower lifetime is 10 to 12 days (as measured by the degree of petal abscission). Flowering is observed in *L. scoparium* from October to January, for a period of 3 to 4 weeks. It has been observed that the flowering period of sympatric[§] species do not coincide. This strategy prevents interspecific hybridisation and reduces the competition for pollinating organisms.[7,13]

The flowers of the *Leptospermum* genus are andromonoecious; the plant bears both male and hermaphrodite flowers. The difference between these genders is illustrated in **Figure 1.2**. This reproductive strategy has several advantages: reduced nutrient and energy consumption for the production of male flowers, increased attraction of pollinators and reduction in the predation on the maternal flowers.[14,15,16] A study by Primack and Lloyd (1980) investigated the proportion of male to hermaphrodite flowers. It was found that flowers on the upper part of *L. scoparium* plants showed a higher percentage of hermaphrodite flowers and the

[§] Sympatric species are different species that are occupying the same geographical region.

proportion of these flowers increased as nutrients were provided to the plants. Additionally the proportion of the two flower types varied over two successive years, implying that the amount of male flowers produced is a result of environmentally induced physiological control.[14]

A study investigating the Australian species *L. myrsinoides* and *L. continentale* agreed with these conclusions.[16] It was also observed in the latter study that further into the flowering season, the number of male flowers increased. The male flowers only produced pollen for 6 days after anthesis but continually produced nectar over their lifespan thus attracting pollinators.

Figure 1.2: The andromonoecious nature of mānuka: hermphrodite (upper) and male flowers (lower). The hermaphrodite flower is easily identified by a prominent stigma.

This supports the theory that male flowers require less nutrients and energy to be produced and hence accumulate later in the season as nutrients begin to deplete. It was hypothesised that the floral buds near the leaves act as direct photosynthetic sinks and thereby are more likely to develop to bear both maternal and paternal characteristics. Those that are positioned inward have less access to nutrients and hence are more likely to develop to be male.[16]

Studies with these species and other Myrtaceae species also revealed that pollinators do not discriminate between the male and hermaphrodite flowers.[13,17]

1.2.3 Seeds and Fruit

Leptospermum scoparium has been classified as a member of the *L. myrtifolium* subgroup based on the observation that this species has a woody valved capsule that houses a great number of small seeds.[18,19] It was hypothesised that the woody nature of the fruit protected the seeds from fire thus making it suitable as a seral species. Due to the minute nature of the seeds, it seems most likely that their dispersal occurs via wind propagation.[7] Manuka has a low percentage of seed germination and it compensates by producing a vast amount of seeds. The age of the seeds does not seem to affect the chance of germination and hence they are able to remain on the tree for a few years.[19]

It had been observed by Harris (2002) that there was a difference in the time period of fruit retention in mānuka as not all trees retained their fruit for three years[**].[18] Some trees had retained their seed capsules, unsplit, for a long period of time whereas other trees separated their capsule valves and released the seeds within a year of formation. It was deduced that there was a correlation between the geographical origin of the plants and this valve separation with those located in the north of New Zealand having a lower degree of capsule splitting. Trees that have a greater proportion of capsule splitting and an early release of seeds were more likely to have broad leaf lamina.[18] This relationship between leaf shape and geographical origin are discussed in further detail below.

An investigation by Price and Morgan (2006) illustrated that the fitness (measured with respect to seed germination and seedling survival) of the mānuka trees is due

[**] This is in contrast to the kānuka plant that sheds its seeds annually.

to phenotypic plasticity rather than being determined genetically.[20] Different Australian *L. scoparium* plants were taken from designated regions and were grown in a uniform environment. There was a higher proportion of seedling survival in one site; there was no correlation between the survival rate and the site of origin. The same occurred for the seed germination.[20]

1.2.4 Leaves

The leaf shape and size of the mānuka plant is highly variable. The shape can range from being narrow lanceolate to ovate-coriac[††] depending on the geographical origin and the variety of the plant. Typically, the leaves are between 7 to 20 mm in length and 2 to 6 mm in width.[6,21] A study by Yin *et al*. (1984) investigated the variation in the leaf shape and size with respect to the latitude and altitude of the region of origin. It was initially observed that the leaf length shortened as the latitude and altitude increased. To confirm that this was due to genotypic variation, 17 seedlings from various sample sites were picked and grown in a uniform environment. The seedlings maintained their respective leaf shapes which confirmed that the variation observed is due to genetic differences. This is a clear example of the ecotypic differentiation that has occurred in different populations of *L. scoparium*.[22]

1.2.5 Leaf Oil

Mānuka leaf oil has been used for therapeutic purposes and is known to have an antimicrobial effect. It was found in various studies that this oil[‡‡] is dominated by its sesquiterpene content. The composition was found to vary with geographical origin; the oil derived from mānuka plants in the East Coast region of New

[††] Lanceolate means spear shaped whereas ovate-coriac leaves range from egg to heart shaped.
[‡‡] Mānuka oil has a low monoterpene content which is in contrast to kānuka oil; the latter is predominantly composed of α-pinene.

Zealand showed the highest antimicrobial activity. The antimicrobial activity was attributable to the high triketone content; more specifically to three β-triketones known as leptospermone, iso-leptospermone and flavanone.[21,23,24] Douglas *et al*. (2004) described ten oil chemotypes that classified mānuka oil from different regions of New Zealand.[25] These investigations were carried out by use of a steam distillation extraction technique. A study using an ethanol extraction found that there were triketones present in other regions of New Zealand although the East Cape oils have the greatest proportion of triketones present.[25] These other regions included the Coromandel, Waikato, Nelson, Auckland and Northland areas. In both studies the main constituent of the triketone content was leptospermone. With ethanol extraction, a significant amount of grandiflorone was detected in the samples collected from the East Cape and Nelson; due to the non-volatile nature of this triketone it was not extracted when steam distillations were utilised.[26]

1.2.6 Frost Hardiness

It has also been shown by various studies that frost hardiness of mānuka is another example of genetic variation. Seedlings of *L. scoparium* were transplanted from sites in Auckland and Christchurch to a uniform environment in order to eliminate environmental variation. It was found that in autumn the frost hardiness of the plant was dependent mainly on the altitude of origin and the temperature tolerance increased approximately 1 ^{0}C per 400m. However during winter, the frost hardiness seemed to be dependent on the latitude of origin. This research illustrated that the frost hardiness is a genetically linked characteristic that developed as a result of different selection pressures in that environment.[27,28] Earlier work by Warrington and Stanley (1987) investigated the frost hardiness of three different cultivars of *L. scoparium*. These cultivars included Martinii, Burgundy Queen and Nanum Huia.[29] Although these cultivars appear quite

different (single pink flowers, double red flowers, dwarfed form with single red flowers, respectively) they are all derived from a common ancestor, the Nichollsii cultivar.[12,30] Due to their genetic similarity, there was no observed difference in the frost hardiness of these cultivars.[29]

The variation seen amongst different mānuka populations may have arisen due to the different established ecotypes in New Zealand. Due to the difference in environmental pressures, the mānuka plants may have evolved to show physical and physiological differences from other members of their species; consequently different populations have developed into separate varieties. *L. scoparium* var. *incanum* is located predominantly in the North Island, particularly in Northland, the Coromandel and with some representation on the East Coast. The closely related variety, *linfolium*, is located exclusively in the Waikato region. In the more southern regions, the *myrtfolium* variety is most common. It was noted that there is an unidentified variety commonly seen on the East Coast and it this variety that has a high concentration of antimicrobial triketones in the leaf oils.[31] From all of the foregoing it seems likely that the DHA concentration will also vary between mānuka in different regions of New Zealand. This was confirmed in a study by Williams (2012).[4]

1.2.7 Other *Leptospermum* Species

As a part of this project, a limited selection of Australian Leptospermum species was investigated.

Brophy *et al.* (1998) has grouped the *Leptospermum* genus into nine groups based on the species' morphological and anatomical similarity.[9] *L. scoparium* belongs to group eight. This group also contains *L. continentale* and *L. juniperinum*. *L. continentale* is the closest relation to *L. scoparium*; differing only by the shape of

the leaves and the size of the fruit. *L. juniperinum* differs from the two aforementioned species by the arrangement of the leaves and the smaller size of their fruit.[32] *L. liversidgei* belongs to group six and differs from the foregoing species by their leaf appearance and the absence of stem flanges. *L. laevigatum* and *L. myrsinoides* do not have persistent fruit and are classified under group three. *L. laevigatum* differs from the all the other aforementioned species as it has more than five fruit loculi[§§]. A summary of all the morphological characteristics is provided in **Table 1.1**.

Research on the various *Leptospermum* species has predominantly focused on the leaf oil composition. An extensive analysis of the oil composition of these species was carried out by Brophy *et al*.[9, 33-36] *L. continentale* and *L. juniperinum* leaf oils contained considerable amounts of both mono and sesquiterpenes. The compounds that predominated were α-pinene,1,8-cineole and β-carophyllene. *L. liversidgei* oil was primarily composed of sesquiterpenes and β-carophyllene and (2*E*,6*E*)-farnesol were the main components.[33-34] There have been two chemotypes identified for *L. liversidgei*; one showing a high level of citronellal and the other a high level of neral or geranial. Citronellal is responsible for the lemon scent of the leaves and is known to be an effective mosquito repellent.[35, 37]

Windsor *et al*. (2012) investigated the MGO and DHA quantity in a variety of honeys derived from different *Leptospermum* species. Honey from *L. laevigatum* flowers manifested a low amount of both MGO and DHA. The *L. liversidgei* samples had a moderate amount of MGO but only a low amount of DHA. The quantity of DHA and MGO is indicative of the age of the honey. This is ultimately indicative of a tree with a high level of DHA.

[§§] Loculi are multiple compartments within the ovary. These chambers contain the seeds.

L. polygalfolium honey consistently had either a moderate or high amount of both MGO and DHA.[38] Although the amount of DHA in honey of different Leptospermum species has been investigated, the DHA/Tsugar in the nectar has not been studied.

Table 1.1: Various morphological features of various *Leptospermum* species.

Species	Group #	Height	Leaves*	Flowers	Ovary	Flowering period	Location†
L. scoparium	8	2m	Lanceolate to ovate-cordate 7-15 mm long 2-6 mm wide	Solitary 8-12 mm diam. White, pink and red	5-locular	Oct-Feb	NZ VIC TAS
L. continentale	8	1-2m	Lanceolate to narrow lanceolate 10 mm long 1-3 mm wide	Solitary 10 mm diam. White	5-locular	Oct-Jan	VIC SA
L. juniperinum	8	2-3 m	Narrow-elliptic or lanceolate 5-15 mm long 1-2 mm wide	Solitary 6-10 mm diam. White	5-locular	Nov-Dec	QLD
L. liversidgei	6	4 m	Narrow-obovate 5-7 mm long 1-2 mm wide Lemon scented	Solitary 10-12 mm diam. White or pink	5-locular	Jan	QLD
L. laevigatum	3	>4 m	Narrow-obovate 15-30 mm long 5-8 mm wide	2 flowers together 15-20 mm diam. White	6-11 locular	Aug- Oct	QLD,VIC,TAS SA WA
L. myrsinoides	3	1-2 m	Narrow-obovate to oblanceolate 5-10 mm long 1-3 mm wide	Solitary 10-15 mm diam. White or pink	4 or 5-locular	Oct- Nov	VIC SA

* Lanceolate = Long shape, wider in the middle with two pointed ends Ovate-cordate = Circular to heart-shaped Elliptic = Egg-shaped Obovate –Oblanceolate = Tear-drop range to a narrower obovate shape

† New Zealand (NZ), Victoria (VIC), Tasmania (TAS), South Australia (SA), Queensland (QLD), Western Australia (WA)

1.3 Nectar

The wide variation of phenotype observed between different populations of *L. scoparium* is a result of genetic factors. It therefore seems likely that the DHA variation observed to date may also be genetically determined.[4] As DHA is present in the nectar, a better understanding of the nectar is vital for this investigation.

1.3.1 Formation and Secretion

It is generally been accepted that nectar is predominantly composed of sucrose and the amount of hexose components is determined by the quantity of the invertase enzyme present in the plant's nectariferous tissue.[39] Often, the sugar ratio of the nectar has been established to attract a particular pollinator; thus, plants that are pollinated by bees exude a smaller volume of sucrose dominated nectar but it is more concentrated.[39,40] This would not have been the case for the evolution of the mānuka nectar in New Zealand as the European honey bee was not present for this to occur. Mānuka nectar is primarily composed of fructose and glucose.[4] This is typical of a generally pollinated plant. An extensive analysis of mānuka nectar showed an average fructose to glucose ratio (F/G) of approximately 1.65:1. This indicates that these hexose components may not be sourced from sucrose (which would have given a 1:1 ratio) but rather from a trisaccharide such as kestose. Alternatively, this ratio may be affected by a microbial presence in the nectar.[4] Investigations on bumble-bee pollinated plants showed that yeasts frequently reach high densities in the nectar. These yeasts drastically reduced the sugar concentration and altered the proportion of sucrose to its hexose components.[41]

Nectar is secreted through nectaries in the flowers which are vascularised by the phloem; the phloem network is responsible for transportation of solutes and water to the flowers.[39] The movement of the solutes in the phloem is dictated by the location of sources and sinks of these solutes hence the flow of these solutes will be under metabolic control as these sites will change over time.[42]

It has been observed in certain Myrtaceae species that secretion from the nectaries occurs through modified stomata; secretion has been known to also occur through trichome cells, secretory cell walls or by the rupture of the hypanthium cuticle. In the case of *L. myrsinoides* and *L. continentale* the cuticle only broke at the locations of the modified stomata. Due to the relation of these two species to *L. scoparium* it is likely that secretion occurs in a similar manner.[43]

A detailed pathway of nectar secretion for flowers has not yet been elucidated and as a result, the mechanism is fiercely debated amongst authors. It is commonly accepted that the vascular system provides the pre-nectar to the nectariferous tissue.[44] The movement of this pre-nectar can occur in two ways has been described by of two different secretion pathways: the symplasmic and apoplasmic models. The majority of the literature seems to support the symplasmic pathway. This model states that the pre-nectar moves from the sieve cells to the parenchyma cells, via a network of plasmodesmata[*]. The pre-nectar is then transported through the symplasm[†] in vesicles secreted by the endoplasmic reticulum or the Golgi body. It is in these vesicles in which carbohydrates are modified.[44,45] The nectar can leave the symplasm by either granulocrine or eccrine secretion; whereby nectar components are secreted by exocytosis or active

[*] Plasmodesmata are small channels which traverse the plant cell wall, forming inter-cellular bridges.
[†] The symplasm is the environment encased by the plasma membrane.

transport across membrane spanning pores, respectively.[42,46] The granulocrine mechanism is depicted in **Figure 1.3**.

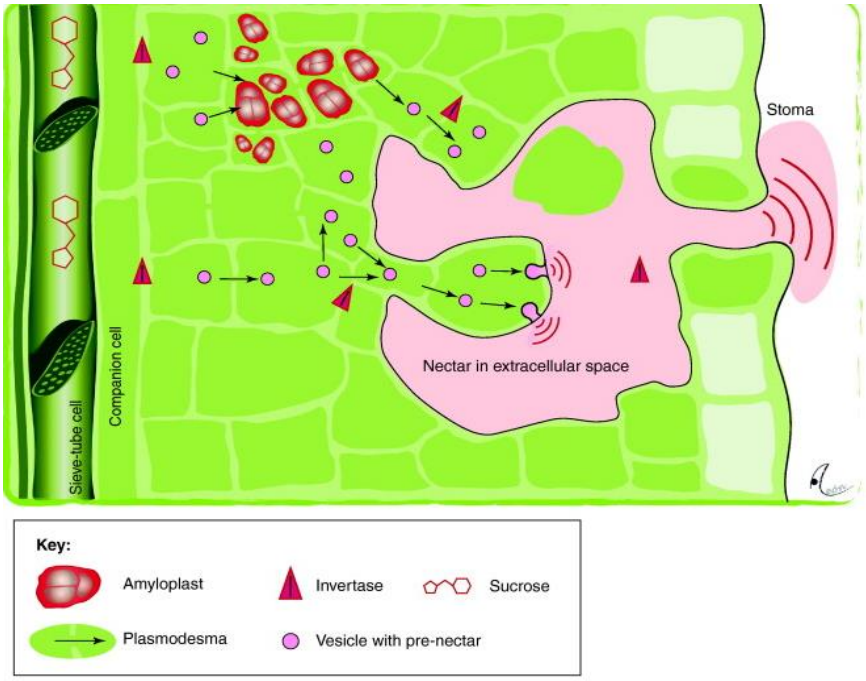

Figure 1.3: The symplasmic model of nectar secretion depicting granulocrine secretion (exocytosis) across the plasma membrane. After Heil (2011).[41]

Nectar secretion in Myrtaceae was hypothesised (by O'Brien (1996)) to occur by this model and Heil (2011) provides a collection of evidence that supports the symplasmic model with granulocrine secretion.[43,44] Kram and Carter (2009) support this model but further state that it does not discount the possibility of eccrine secretion and apoplasmic flow of nectar also occurring.[45]

The apoplasmic model involves the pre-nectar being released by the terminal phloem cells directly into the apoplasm[‡] where the transformation from sap to nectar occurs. This has been invalidated by both Heil and Vassilyev (2010).[44, 46] Vassilyev has also attempted to challenge granulocrine secretion as evidence indicated the endoplasmic reticulum does not produce secretory vesicles and the Golgi body only does so to accommodate polysaccharides.[46]

[‡] The apoplasm is the environment made up of the cell wall and intercellular spaces.

Recently a new secretion model has been proposed. It incorporates the symplasmic model but secretion into the apoplasm occurs by active transport across the plasma membrane (eccrine secretion). Once the nectar is in the apoplasm, it moves by a pressure driven mass flow. This flow is powered by water influx into the apoplasm. As a result, the nectar is moved into substomal cavities and secreted through modified stomata. This model can supposedly be applied to all nectaries irrespective of their makeup.[46] Although it might seem naïve to have a model account for nectar secretion in all plants (as the biodiversity of plants is vast), it is not unreasonable that this process is highly conserved.

In *L. myrsinoides* and *L. continentale* plants, the nectar was observed to be present as a thin film over the hypanthium[§].[13] This shallow nectar container is unique to the Myrtaceae family, providing pollinators more access to the exposed nectar. As the mānuka flowers are non-specialised (i.e. are suitable for all types of pollinators), the flower has evolved into a bowl shape which has allowed for a more accessible area. The arrangement of the stamina allows for a greater chance of the pollinator brushing against them and therefore collecting pollen.[47] This shape would lead to greater water evaporation resulting in an increase of the Tsugar.

As the phloem is responsible for providing the nectar constituents, it is possible that DHA is being produced in another area of the plant and is being transported to the flowers via the phloem network. Due to the aforementioned variability of solute movement in the phloem, it is not surprising therefore that a variation of DHA has been observed within the same mānuka plant.[4]

[§] O'Brien (1996) defines the hypanthium as the disc shaped structure positioned above the ovary.

1.3.2 Role of Environmental Effects on Nectar Production

The volume of nectar secretion and its components is a dynamic process. It is regulated by micro-environmental conditions. Two studies of *Asclepias syriaca* illustrated the effects that a change in the micro-environment has on the nectar production. A study of two clones of a *A. syriaca* plant grown in the same environment showed that the changes in this environment (such as the sunlight, temperature and humidity) had a greater effect than the age of the flower. It was also illustrated that the nectar production was not a uniform process and it peaked at about 50 hours after anthesis.[48] The second study confirmed these findings by use of the bagging technique[*]. Moreover, it was observed that the nectar volume increased when water was added to simulate humidity and rain and the sugar concentration was increased in proportion.[40] If this sugar increase did not occur, the concentration would be altered due to the hygroscopic nature of the nectar.

It was illustrated in a study on *Pyrostegia venusta* that when nectar is removed, the volume of the remaining nectar increased and the resulting sugar concentration is decreased. Conversely, if the nectar is not removed, it is often re-absorbed and re-expressed. This is a strategy for resource recovery and it is essential during ovule and ovary development. It is more acceptable to look at the secretion and reabsorption as a unified process which allows for a nectar homeostasis to be established.[43,49]

Canto *et al*. investigated the variation of the sugar concentration in the nectar between flowers on the same plant. This was investigated by using *Aquilegia vulgaris* subsp. *vulgaris* and *Aquilegia pyrenaica* subsp. *Cazorlensis*. Plants of these species were grown both outside and in a greenhouse to determine the role

[*] The bagging technique is the coverage of flowers with a material that prevents interference from pollinators. These materials included plastic bags, nylon mesh and mosquito nettings.

that pollinators played on the nectar composition. Flowers from the greenhouse-grown plants had very little variation whereas those sampled from plants outside the greenhouse had a significant variation. It was hypothesised that the variation observed could be due to the difference in sunlight exposure and therefore temperature of the flower's microenvironment; the removal of nectar by pollinators, addition of enzymes or microorganisms to the flower by the pollinator may have also had an effect.[50]

Other environmental effects such as stresses may have an impact on the nectar composition. When a plant is stressed, the plant will accumulate more solutes to combat the effect. For example, plants that are in a low water or in a high salt environment will produce highly soluble organic compounds that will decrease the water potential in the roots; resulting in an uptake of water from the soil. These compounds are termed compatible osmolytes and they do not interfere with cellular metabolism.[51] One of the most well-known of these compounds is proline. The production and accumulation of low molecular weight compounds has also been observed in the cold acclimation process when a plant is exposed to low temperatures. It is possible that DHA is being produced by mānuka in response to an environmental stress. Mānuka is known to inhabit harsh environments and this environment may have provided the necessary pressure for mānuka to have evolved to be constantly over-producing DHA. Conversely, if sugars are also being produced in response to an environmental stress, the overall DHA/Tsugar ratio observed in the nectar will decrease as a result.[51]

In previous sections it has been discussed that the variation of phenotype observed between mānuka plants was primarily a result of genetic control. The aforementioned investigations have illustrated that the nectar composition of all plants is also very much influenced by environmental factors.

1.3.3 Collection Methods

In order to quantify the amount of DHA present in the mānuka nectar, a method of nectar collection had to be established. The majority of the collection methods that have been used in the literature have developed for measuring the sugar content. Common methods of nectar collection involve removing nectar by the action of a micropipette or by use of a microcapillary tube, centrifuging individual flowers, absorption of the nectar by use of filter paper, washing the flowers in a solvent or rinsing the flowers with water.

The centrifugation technique was used by Swanson *et al*. (1950) to remove nectar from the small flowers of red clover. Although this technique was found to be suitable, it required specialised instrumentation.[52]

Morrant *et al*. (2009) investigated four of the collection methods outlined above using four different *Eucalyptus* species. To test the suitability of the utilisation of filter paper to soak up the nectar, the filter paper was cut into equilateral triangles. Each tip was dipped into the nectar and the filter paper was then soaked in distilled water for approximately 15 minutes. The filter paper was then agitated until it formed a pulp. Although this method is simple and convenient, it can only be used for qualitative analysis as it cannot remove all the nectar. For an estimate of the total sugar content, the filter paper can be soaked in an appropriate solvent, followed by either a calorimetric or chromatographic analysis.[53]

A micropipette or microcapillary tube can be used to measure the nectar volume. When used in conjunction with a refractometer the sugar content can be determined. In one study a micropipette (0.25 μL) was used to remove the nectar; the proportion of the nectar filling the tip was measured and the nectar was released into distilled water (2 mL). This method is appealing due to its simplicity

but there is risk of damage to the nectaries. As a result, amino acids and cellular sugars will be leaked into the nectar which will therefore affect the result. Moreover, it can be difficult to obtain samples that are less than 1 μL and that are relatively viscous. The study by Lanza *et al*. (1995) utilised this technique to determine the variation between flowers on the same plant, different plants located in the same population and plants located in different regions. It was found that there was no significant intra-plant variation which is in contrast to the findings of Canto *et al*.(2007).[50,54] Additionally, Stephens *et al*. (2010) utilised a micro-syringe to remove the nectar from *L. scoparium*. The nectar from 7 mānuka samples were analysed and MGO was supposedly found in the nectar in trace amounts.[55]

It was determined by Morrant *et al*. (2009) that the most effective methods for nectar collection involves either rinsing or washing the flowers. The rinsing method is the most efficient as it removes the most amount of floral sugars. The flowers were inverted over a vial and rinsed multiple times with distilled water (0.5 mL). Although there is a risk of cellular sugars being released into the nectar due to osmotic pressure, it is advantageous as it does not require removal of the flowers from the plant. It can also be utilised in experiments that involve repeated nectar extractions. Stephens *et al*. (2010) used this method to remove nectar from kānuka flowers. Nectar from 50 flowers was collected in a bulk container containing water (100 μL). An aliquot (10 μL) of the bulk water was pipetted to the surface of the flower and then re-collected into the bulk amount.[55]

Although rinsing is the most efficient method (as it yields the greatest amount of sugars) the washing method is the most convenient. Morrant *et al*. (2009) investigated the optimal time that five flowers should be soaked in distilled water (2mL). It was determined that soaking for 1 min was suitable; although only one

round of soaking extracts 60 to 90% of the total sugar content, it was determined to be sufficient. Prolonged soaking periods (from 20 min to 60 min) increased the risk of the extrafloral sugars being leached into the water.[53]

Many of the investigations involved nectar analysis of individual flowers. Several recent publications support pooling multiple flowers from the plants to get a more representative idea of the nectar composition of the whole plant. This method has been used by Adams *et al*.(2009), Morrant *et al*.(2009) and Stephens *et al*.(2010).[3, 53, 55] The pooling approach was used by Williams (2012) to extract the nectar from mānuka flowers of the same tree.[4]

1.4 Dihydroxyacetone (DHA)

DHA is the smallest ketose compound and is commonly known as triose[†]. It is seen as an intermediate in the general metabolism of many organisms (including plants and animals) but it is generated for commercial sale by the conversion of glycerol as a part of the metabolism of acetic acid bacteria. The bacteria capable of this reaction include the *Acetobacter* and *Gluconobacter* genera.[56-57, 58] It can crystalize in five forms with only one being monomeric. The DHA crystals provided by Sigma-Aldrich are in one of the dimeric forms but it rapidly undergoes dissociation when heated or dissolved in water. Dimerization of DHA in the aqueous phase will occur after approximately 30 days of storage at room temperature. Hence it is recommended that this compound is stored with refrigeration. DHA has been observed to be sensitive to the pH of its environment. With an optimal pH of 5, alkaline conditions promote DHA degradation to form hydroxyacetone (HA) and MGO whereas acidic conditions promote the formation of MGO and dimerization.[59]

[†] Triose is also the generic term to describe three carbon sugars

DHA was once used as an agent for the treatment of diabetes as it was found to have a more rapid uptake and greater rate of carbohydrate combustion than glucose.[60] A chance discovery found that DHA stains the skin as a result of DHA undergoing the Maillard reaction with the amines, amino acids and peptides located in the horny layer of the epidermis. This staining resembles a tan achieved by UV light exposure and consequently sunless tanning lotions have been developed containing DHA as the active ingredient. DHA is known to be innocuous and therefore it can be utilised in these lotions. Moreover, due to the high reactivity of this ketose, it does not penetrate the deeper layers of the skin. Therefore if an adverse reaction were to occur, it is easily removable.[59]

1.4.1 Dihydroxyacetone phosphate (DHAP)

The phosphorylated form of DHA (DHAP) is found in the majority of organisms as it is involved in several metabolic processes including photosynthesis. Photosynthesis is comprised of two essential processes; the light-dependent process whereby energy is stored in molecules such as ATP and NADPH and the light-independent process where this energy is utilised. These processes are depicted in **Figure 1.4**. The latter process is known as the Calvin-Benson cycle or the C_3 carbon fixation cycle.[61,62] It involves the fixation of carbon dioxide into 3-phosphoglycerate. This compound is then reduced in two steps to form glyceraldehyde-3-phosphate (G3P). DHAP is formed via an isomerisation reaction, of G3P, by triose phosphate isomerase (TPI). G3P and DHAP then combine to form fructose-1,6-biphosphate (F-1,6-P) which is involved in the regeneration of ribulose-1,5-biphosphate (RuBP), thereby allowing the cycle to continue.[62]

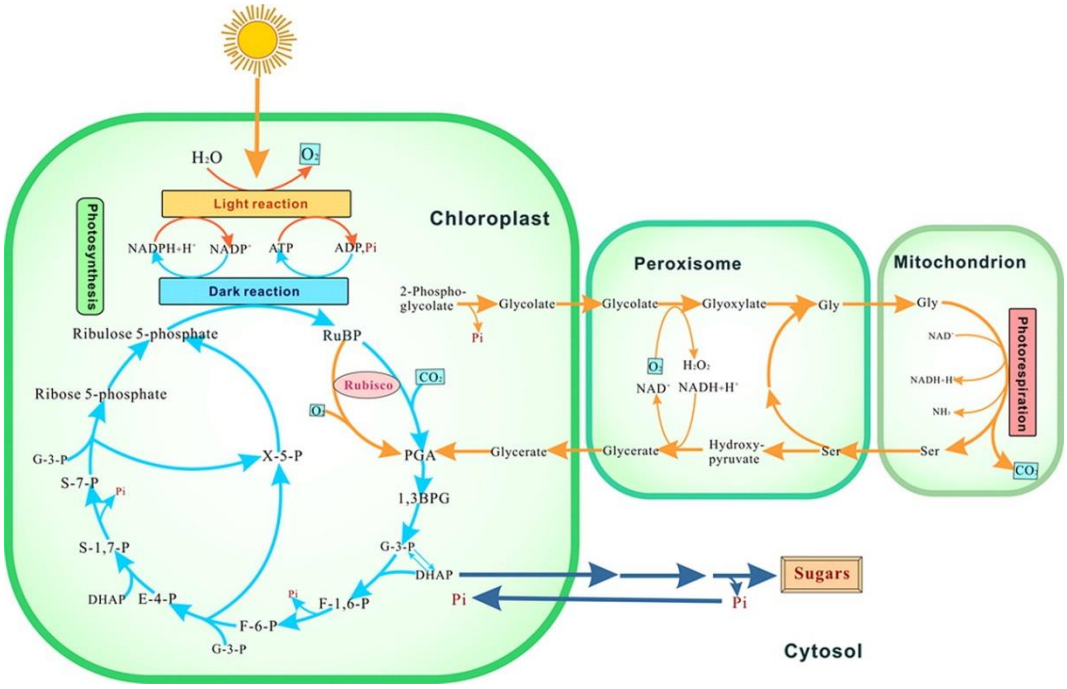

Figure 1.4: A summary of the light and dark photosynthetic reactions and the fates of intermediate compounds. DHAP is formed by an isomerisation reaction of G3P. This figure is reproduced from Luo *et al.* (2009).[58]

DHAP can also be formed by the pentose phosphate pathway in the chloroplast. This is the generation of primarily fructose-6-phosphate from glucose-6-phosphate. A by-product of this metabolic pathway is the DHAP isomer, G3P.[63]

The DHAP formed in the chloroplast has a multitude of fates. The majority of this triose phosphate is exported from the chloroplast to the cytoplasm via the triose phosphate translocator where it undergoes further enzyme catalysed reactions.[64] One of these reactions is gluconeogenesis which is the formation of hexose sugars from a non-carbohydrate source. Hexose sugars can be stored in the form of starch or used for sucrose synthesis. The reaction between DHAP and G3P forms F-1,6-P, a crucial molecule in the metabolic pathway due to its regulatory role.[65] However DHAP present in both the cytoplasm and chloroplast is also consumed to form pyruvate‡.

‡ Pyruvate is the precursor used in the Kelvin cycle during the respiration process

DHAP metabolism is closely linked to the plant's requirements for glycerol. Glycerol anabolism involves the reduction of DHAP (sourced from both the cytoplasm and chloroplast) to glycerol-3-phosphate by either a reductase or a dehydrogenase enzyme. The phosphate group is removed by a phosphatase enzyme to yield glycerol.[56] Glycerol is an essential component in the lipid synthesis.

It has been reported that glycerol regulation is used in response to variation in extracellular water concentration (or osmotic stress).[66,67] In *Dunaliella parva*, the intracellular glycerol concentration was found to vary in response to the external salt concentration. The glycerol variation was deduced to be a regulation of glycerol anabolism and catabolism. The catabolism of glycerol to DHA involves the action of two enzymes: a dehydrogenase (which is responsible for the dehydrogenation of glycerol to DHA) and a kinase (an enzyme responsible for the phosphorylation of specific substrates). This catabolism occurs in most organisms by the initial phosphorylation of glycerol by glycerol kinase and then the subsequent action of glycerol-3-phosphate dehydrogenase (GD) to form DHAP. It can also occur by the dehydrogenase enzyme oxidising glycerol to free DHA, followed by phosphorylation by the kinase to form DHAP.[68] Glycerol regulation has been found to occur only by intracellular enzymatic reactions.[66] This variation in glycerol concentration has also been observed in the yeast *Sacchromyces cerevisiae*.[67]

1.4.2 Sources of DHA

Although DHAP is utilised in many different process and is not unique to mānuka, the question then becomes why do mānuka flowers exhibit high quantities of the non-phosphorylated form of DHA? It is as yet unknown whether

the DHA is originating from the plant itself or from a microbial species present in the nectar.

As mentioned, glycerol catabolism occurs by the action of two enzymes: a dehydrogenase and a kinase.[68] One possible mechanism by which DHA might accumulate is if *L. scoparium* breaks down glycerol by the initial action of GD and the DHA accumulation could occur as a result of a faulty DHA kinase. If this process occurs in the flower, the resulting accumulation of DHA could be removed by secretion into the nectar. Another possible mechanism of DHA accumulation is if there is a mutation in the GD enzyme. This could have resulted in an enzyme with lower substrate specificity; subsequently oxidation of non-phosphorylated glycerol to DHA could be occurring. This would only be successful if DHA kinase was not present. DHA accumulation as a result of these possibilities could also occur in the microorganisms present in the flowers of *L. scoparium.*

The TPI enzyme is involved in many of the metabolic processes that occur not only in plants, but other organisms as well. The source of DHA may be a result of a malfunctioning TPI enzyme. Chen and Thelen (2010) investigated the TPI enzyme and its role in *Arabidopsis thaliana* seed development. Seeds contain large quantities of starch reserves (provided by the mother plant) to ensure survival until the plant is able to photosynthesise; starch is eventually converted to triacylglycerides which will yield glycerol in order to produce sucrose. The TPI enzyme has a large role in these metabolic processes and those that occur as the seed becomes autotrophic[§]. When the TPI gene was mutated in *A. thaliana*, an accumulation of glycerol, DHAP, G3P and MGO was observed.[69]

[§] Autotrophic means the ability of the plant to synthesise its own food from inorganic sources

It is possible that in mānuka, the TPI gene contains a mutation that reduces the expression and function of the resulting protein product; therefore resulting in the accumulation of the aforementioned compounds. Dephosphorylation of DHAP by DHAP phosphatase would prevent the formation of MGO which occurs by non-enzymatic phosphate elimination of DHAP. Accumulation of MGO and DHAP is toxic to plant development (seedling germination and plant growth respectively) and therefore production of DHA may be a viable alternative pathway to prevent such damage.[69]

Another possible but unlikely source of DHA is the Lobry de Bruyn-van Ekenstein rearrangement of D-glyceraldehyde occurs in basic conditions.[70] Cleavage of glucose in basic conditions could also result in DHA accumulation. This reaction would be supported in the stromal space of the chloroplast as it has been illustrated to be slightly basic. The Benson-Calvin cycle previously described occurs in this region and has an optimal pH of 8.1.[71] Additionally, the phloem exuded in the secretory cells is slightly alkaline; thus creating a supportive environment for the aforementioned rearrangement.[42] The DHA would be rapidly phosphorylated to be incorporated in the essential metabolic processes occurring in the chloroplast. The accumulation would only occur if the expression and action of DHA kinase (which phosphorylates DHA) was impaired.

A third possible, but unlikely, source of DHA might from a microorganism by the reaction between xylulose-5-phosphate and formaldehyde (derived from methanol). This reaction is a part of a novel pentose phosphate cycle that has been observed in methanol utilising yeasts; known as the DHA pathway. It is thought that DHA is formed as a result of a transketolase enzyme. This enzyme catalyses the transfer of an aldehyde component from a pentose sugar to formaldehyde which results in G3P and DHA. This was shown to occur in the yeast *Candida*

boidinii.[72] Formaldehyde originates from methanol which has been shown to be produced in substantial amounts by all C_3 plants.[73] For the DHA to accumulate, the action and/or expression of DHA kinase and TPI would have to be hindered.

Therefore the source of DHA found in the mānuka flowers remains uncertain and required investigation in future studies.

1.4.3 DHA Quantification

Various HPLC methods have been used to quantify DHA. Initially, attempts were made by Adams *et al*. (2009) to detect DHA using the same method as was used for MGO and HA detection.[3] This method involved separation by a size exclusion and ligand exchange HPLC system and detection of the various components was achieved by a refractive index detector.[3] The introduction of a derivatising agent, o-phenylenediamine (OPD) allowed detection of the two analytes of interest: MGO and DHA. An optimal result of the derivatization of DHA is achieved in an acidic acetate buffer. Simultaneous MGO derivatization and quantification in this buffer cannot be achieved as MGO qunioxalines accumulate from carbohydrates present in the sample; this results in an over estimation of the MGO content. MGO is separately assayed using an alkaline buffer.[74]

A more suitable derivatising agent for the carbonyl-containing analytes is O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA). This compound reacts via its terminal amine to form a compound that has reduced polarity and greater volatility. Derivatization with PFBHA produces stable α-β unsaturated carbonyl and 1,2-dicarbonyl derivatives. This derivatization allows for dicarbonyls to be distinguished from hydroxycarbonyls and allows for the identification of unambiguous molecular weights of the derivatives in mass

spectral detection.[75] Simultaneous determination of DHA and MGO is possible by this method.

PFBHA derivatization is not always suitable for highly polar molecules and thus an alternative derivatising agent was investigated by Richardson *et al*. (2000). Use of 2,4-dinitrophenylhydrazine (DNPH) attaches a dinitrophenyl group to the polar analyte; it allowed for an increase in the molecular weight and for the separation of the peaks in the mass spectra from the early-eluting interfering peaks. The reaction of DNPH with polar compounds proceeds in the same manner as PFBHA. Despite its advantage over PFBHA, it was illustrated that it did not achieve superior sensitivity. Rather it was the authors suggestion that derivatization with DNPH and detection by LC-MS should be used to supplement the GC-MS analysed PFBHA derivatives.[76]

An investigation into PFBHA derivatization studied the most suitable extraction solvents for a variety of oxygenated organic analytes and the stability of these resulting derivatives. $^{13}C_3$ -labelled compounds with varying degrees of oxygenation were analysed. Acetone, MGO, HA and pyruvic acid were the chosen model compounds for a carbonyl, dicarbonyl, hydroxycarbonyl and keto-acid system. The extraction solvents studied included hexane, methyl-*tert*-butyl ether and dichloromethane. As expected, the multi-functional carbonyl compounds had a greater solubility in the more polar solvents and a greater extraction of the analyte was achieved. Hence dichloromethane was the most suitable solvent for derivatised MGO and HA. The stability of these derivatives was studied in 4 ^{0}C refrigeration. It was advised that the time between sampling and analysis should be minimised as the concentrations of the derivatives varied significantly throughout storage. The compounds were found to be stable for up to 38 days of refrigeration if storage was required.[77]

If necessary, improvement of the sensitivity and volatility, for analysis by GC, can be achieved by silyation. The addition of *bis*(trimethylsilyl)trifluoroacetamide replaces any hydroxyl groups present with trimethylsilyl groups, therefore achieving greater volatility. This silyation method was utilised by Charles (1999) to detect MGO and HA at limits at the pptv (part per trillion by volume) level.[78] This method was attempted by Williams to improve the sensitivity of the desired analytes by GC-FID detection.[4] It was found that silylation by Tri-Sil™ caused a peak overlapping with HA to form as a result of the unreacted amine group of PFBHA being silylated.[4] Consequently, Williams (2012) used 1-(trimethylsilyl)imidazole (TMSI) as the silylating agent in the nectar analyses.[4] Occasionally, there was still a small interference from the silylating agent but it was overcome by use of the peak splitting function in the integration software.

1.5 Aim of this Investigation

The study by Williams (2012) refined a method that allowed for the detection of DHA in the nectar of the mānuka tree and used this method to investigate the DHA being produced by varieties of mānuka that were localised to certain regions around New Zealand.[4] This study illustrated that there is a large inter-regional DHA/Tsugar difference (approximately 0.00240 mg/mg) in the DHA being produced but there may also be a difference within the region of interest.[4] The East Cape region was the most promising region and one of the aims of the current study is to further investigate this region. The investigation of mānuka in the Northland region will be expanded upon as Williams (2012) did not have a significant sample size from which to draw conclusions.[4] This region predominantly contains the *L. scoparium* var. *incanum* variety and will be compared to mānuka found in the East Cape region.

It has been suggested that the DHA/Tsugar of a mānuka plant is possibly the result of genetic influence.[4] In order to test this hypothesis and the inheritability of DHA production, garden varieties of different phenotypes will be tested. Furthermore, the current study aims to investigate the different characteristics of the mānuka plant that might be giving rise to the DHA/Tsugar variation observed between similar trees. This will hope to improve the understanding of nectar production in the mānuka plants. Finally, specimens from Australian *Leptospermum* species will be investigated to determine whether the high levels of DHA observed in the nectar of *L. scoparium* is shared across the genus.

2 Method

2.1 Materials

Sucrose (99+%), D(-)-fructose (99+%), D-mannitol (98+%), citric acid (99.5%), DHA (dimer, 97%) and HA (technical grade, 90%) were all sourced from Sigma-Aldrich; D-(+)-glucose was obtained from BDH Laboratory.

O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamide hydrochloride, PFBHA, (derivatization grade) and MGO (43.2%) were sourced from Fluka Analytical. 1-(Trimethylsilyl)imidazole (TMSI) was purchased from Thermoscientific. Dichloromethane (DCM) (AR) and *n*-Heptane (HPLC grade) were obtained from Ajax Finechem. Liquid nitrogen was supplied by BOC and Milli Q deionised water was obtained from a Barnstead E-pure water system at 17 MΩ. All future references to water mean Milli Q unless otherwise stated.

Analysis of *Leptospermum* species at the University of the Sunshine Coast used the following materials:

HA (technical grade, 90%), DHA (dimer, 97%), MGO (40%), Sucrose (99+%), D-mannitol (98+%), D(-)-fructose (99+%), citric acid (99.5%), D(+)-glucose and TMSI were sourced from Sigma-Aldrich. PFBHA was sourced from Alfa Aesar. DCM (AR) and *n*-hexane (AR) were obtained from Merck Australia. Liquid nitrogen was supplied by BOC Gases.

2.2 General Methods

L. scoparium samples were freeze-dried using a Labonoco Freeze Dry System and the freeze drier utilised at the University of the Sunshine Coast was a ThermoSavant MODULYOD-230 system.

2.3 Sample Collection Information

Mānuka samples collected in this study were predominantly from the Northland, East Cape and Waikato regions. The sites investigated in this survey range from latitudes 34 ^{0}S and 37 ^{0}S and longitudes 172 ^{0}E and 179 ^{0}E. The Northland samples were collected over the 2010/2011 flowering season. The sites of these trees were not associated with known hive sites; rather they were randomly chosen. Sites located in the East Cape region had previously been investigated by Williams (2012) in 2009 and 2010.[4] These sites are located near hive sites and due to commercial sensitivity will be discussed using generic labels. Particular trees from Williams' survey were chosen and samples were collected within a 50m radius of the chosen specimen. These samples were collected in November 2011. Additionally, mānuka trees cultivated in the gardens of the University of Waikato (UoW) and Auckland Botanical Gardens (ABG) were utilised in this survey. These flowers were collected over the course of 2 years in 2012 and 2013. For commercial sensitivity, cultivar names will be replaced using a numbered code.

Australian *Leptospermum* spp samples were predominantly collected from the North Coast of New South Wales (NSW) although one sample was collected from Beerwah in South East Queensland (QLD). The NSW sites ranged from latitudes 28.56 °S and 28.76 °S and from longitudes 153.56 °E and 153.58 °E. These samples were collected from December 2012- February 2013.

To ensure that a sufficient amount of flowers were collected for analysis, trees that bore a substantial amount of flowers were chosen as were trees flowering during the aforementioned flowering periods. This creates an unavoidable bias. Unfortunately, due to the limited amount of information on the source of an excess amount of DHA in mānuka flowers this bias cannot be avoided.

2.4 Flower Collection Methodology

When possible, the mānuka trees were sampled on a fine day. It would have been ideal to avoid sampling during a period of rain although in some circumstances, this could not be avoided. During collection, the mānuka tree was tagged with a unique identification number and particular details were recorded. These details included information on the location of the tree (GPS coordinates, elevation, natural and man-made features). Additionally, certain characteristics of the mānuka plant were noted such as the height and whether it was infested with noticeable black sooty mould. See **Appendix 6.1** and **6.2** for the advice given to collectors.

Flowers were collected from all over the tree and discrimination between hermaphrodite and male flowers was avoided. Flowers were either individually picked or they were scraped off between two fingers which moved from the base of the twig toward the tip. Samples were stored in air-tight plastic locking bags and chilled until they could be transferred to a freezer (-20 ^{0}C). Additionally, a herbarium sample was collected for the Northland and East Cape samples. This involved the removal of a branch that bore leaves, flowers and the seed capsules. The samples were stored in the herbarium located at the UoW and later moved and deposited in the Auckland Herbarium.

2.5 Nectar Extraction and Analysis

Methodology previously established by Williams (2012) included a twenty flower test (20F) and a single flower test (1F).[4] These involved using twenty flowers or a single flower per replicate, respectively. Due to a limited amount of sample collected per tree, the 20F test was altered to ten flowers per replicate as opposed

to twenty (10F). This change has been verified and is discussed in **Section 3.1**. Both methods utilise ten replicates per sample.

Flowers (10) were counted for each replicate and left to soak (20 min) in water (2 mL). Ideally, whole flowers were chosen and both male and hermaphrodite flowers were included. For the 1F test, one flower was used per replicate and left to soak in water (1 mL) for the same amount of time. The flowers were removed upon the completion of the extraction and aliquots were used for the sugar and DHA analysis.

2.5.1 Sugar Analysis

Prior to the addition of nectar, the mannitol internal standard was added (5 μL, 5mg/mL) to the Gas Chromatograph (GC) vial. A nectar aliquot was then added (20 μL for the 10F and 50 μL for 1F) and the solution was freeze dried preferably overnight. TMSI was added (50 μL) to the dry sample and left to stand (1 hour) for derivatization to complete. Upon completion, heptane (1 mL) was added prior to analysis by Gas Chromatograph-Flame Ionisation Detector (GC-FID).

2.5.2 DHA Analysis

A nectar aliquot was added (200 μL for 10F or 400 μL for 1F) to a vial containing the HA internal standard (5 μL, 0.5 mg/mL). The derivatising agent PFBHA (50 μL, 20 mg/mL in a pH 4 0.1M citric acid buffer) was added and the samples were left to stand (1 hour). DCM (2mL for 10F or 1 mL for 1F) was added and the samples were frozen. After the aqueous layer had frozen, the DCM was extracted into a GC vial. Derivatization was completed by adding TMSI (50 μL) and standing (1 hour) prior to analysis by GC-FID.

In addition to sample preparation, six standard samples were prepared. The volumes of the DHA and MGO components are shown in **Table 2.1.** The volume was made up by addition of water and these standards underwent the same treatment outlined above.

Table 2.1: Volumes of MGO (1.5 mg/mL) and DHA (1.5 mg/mL) in standard solutions.

Standard #	Volume of DHA (µL)	Volume of MGO (µL)
1	10	0
2	8	2
3	6	4
4	4	6
5	2	8
6	0	10

2.6 Gas Chromatography-Flame Ionisation Detector (GC-FID)

The sugar and DHA analyses were carried with an Agilent Technologies 7890A GC system comprised of a split/splitless inlet, a flame ionisation detector (FID) and with an Agilent Technologies 7683B autosampler and a 10 µL syringe. Separation was achieved with a Zebron ZB-5 30m x 0.32 mm x 0.25 µL GC capillary column. Agilent Technologies GC Chemstation Rev.B.04.03[54] software was used to run the instrument. The syringe was cleaned with five washes of DCM prior to injection and five washes of acetone post-injection to avoid sample contamination and the accumulation of suspected silylation by-products that have solidified.

For both analyses, the injection size was 2 µL. The FID detector was maintained at 300 ^{0}C with a constant gas flow of 30 mL/min of hydrogen, 400 mL/min of air and 10 mL/min of make-up gas.

For the sugar analysis, the inlet was configured to be in the splitless mode, at 280 ^{0}C with a 2 mL/min septum purge flow. The carrier gas (hydrogen) flow was set

to 4 mL/min. The oven program began at 100 ^{0}C (1 minute hold) and was ramped to 200 ^{0}C at a rate of 30 ^{0}C/min. A second ramp increased the temperature to 250 ^{0}C at a rate of 10 ^{0}C/min and a final ramp increased the temperature to 300 ^{0}C at a rate of 30 ^{0}C/min.

For the DHA and MGO analysis, the inlet was set at 250 ^{0}C in the splitless mode with a 2 mL/min septum purge flow. The carrier gas (hydrogen) was configured to 2 mL/min. The oven program had an initial temperature of 130 ^{0}C with a five minute hold period. The temperature was increased by 10 ^{0}C/min until the final temperature of 250 ^{0}C was achieved. This was held for 6 minutes.

2.7 Gas Chromatography-Mass Spectrometer (GC-MS)

The Australian *Leptospermum* spp. samples were analysed on a Perkin Elmer Clarus 580 Gas Chromatograph coupled to a Perkin Elmer Clarus SQ85 Mass Spectrometer. This system is comprised of a split inlet and a Perkin Elmer Elite-5MS 30m x 0.25 mm x 0.25 µm column. The autosampler was equipped with a 5 µL syringe; it was rinsed twice both prior and post injection with DCM. The carrier gas (helium) flow was set to 1 mL/min for both the sugar and DHA analyses.

For the sugar analysis the inlet was configured to shut the split from 0.20-1.00 minute and open with a 50:1 ratio; it was maintained at 280 °C. The oven program was the same as what was outlined in the aforementioned section. Compound ionisation was at 70eV electron impact, analysing *m/z* +70-440 over 4.50-11.00 minutes.

For the DHA analysis, the inlet was configured as for the sugar analysis except the temperature was maintained at 250 °C. The oven program was the same as

that outlined in **Section 2.6**. Compound ionisation was at 70eV electron impact, analysing *m/z* +45-440 over 4.50-23.00 minutes.

2.8 Data Processing

The DHA and sugar GC chromatogram peaks were manually integrated using the GC Chemstation program for the *L. scoparium* samples. TurboMass Ver 6.0.0 was used to analyse the GC-MS results for the *Leptospermum* spp samples. An example of the gas chromatographs from this work is shown in **Appendix 6.3**.

The following sections show how these peak areas were utilised to determine the total amount of DHA in the nectar. The quantity of DHA present is expressed as a ratio to the total amount of sugar present in the nectar (*i.e*. DHA/Tsugar). Data processing was carried out using Microsoft Excel Ver: 14.0.6129.5000 (32-bit) and Minitab Ver: 16.2.3. Statistical analyses included the use of one-way ANOVA tests at 95% confidence.

2.8.1 Sugar Analysis

The total amount of sugar (Tsugar) present in the nectar aliquot is determined from the amount of fructose and glucose present in the nectar with respect to the mannitol internal standard (as shown in **Figure 2.1**). The following peaks are integrated: three fructose peaks (4.82, 4.85 and 4.88 min), two glucose peaks (5.20 and 5.60 min) and the mannitol peak (5.40 min). Sucrose is not included as it is not detectable in the mānuka nectar and therefore contributes an insignificant amount to the total amount of sugar.

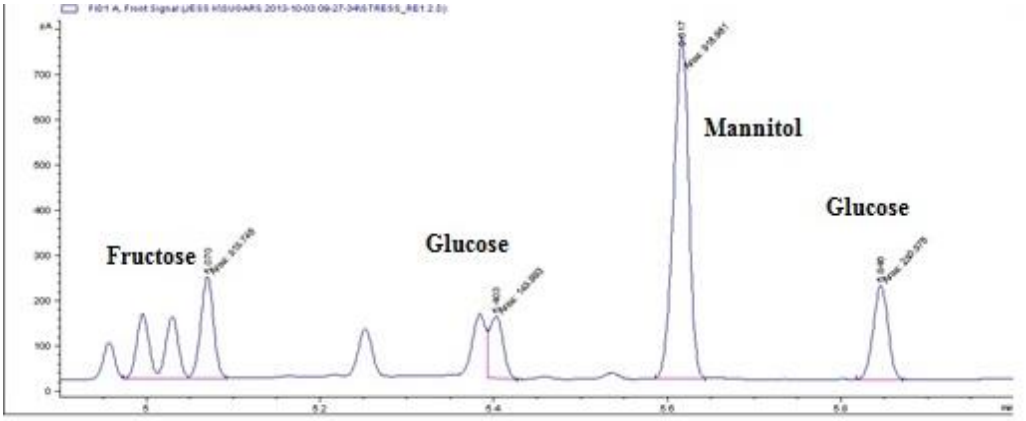

Figure 2.1: An example of a sugar chromatograph from a mānuka nectar sample. From left to right three fructose peaks, the first glucose peak, the mannitol internal standard peak and the second glucose peak are integrated**.**

Equations 2.1 - 2.3 are used to determine the Tsugar value. The sugar term in these equations is representative of either glucose or fructose. The resulting value is the total amount of sugar in the 200 µL aliquot of nectar (or 400 µL for 1F). For the response factor curves, see **Appendix 6.4**.

$$\frac{\text{Sugar}}{\text{Mannitol}} = \frac{\text{Sugar Peak Area}}{\text{Mannitol Peak Area}} \quad \textbf{(2.1)}$$

$$\text{Sugar (mg)} = \frac{\left(\frac{\text{Sugar}}{\text{Mannitol}}\right) \times \text{Mannitol Volume(mL)} \times \text{Mannitol conc.} \left(\frac{\text{mg}}{\text{mL}}\right)}{\text{Sugar/Mannitol Response Factor}} \quad \textbf{(2.2)}$$

$$\text{Tsugar (mg)} = [\text{Fructose (mg)} + \text{Glucose (mg)}] \times 10 \text{ (8 for 1F)} \quad \textbf{(2.3)}$$

- Glucose/ Mannitol Response Factor = 0.5335
- Fructose/ Mannitol Response Factor =0.8217

2.8.2 DHA Analysis

The amount of DHA in the 200 µL of nectar (or 400 µL for SFT) is determined by use of a standard curve and Equations 2.4 and 2.5. The ratio of DHA and HA peak areas is plotted against DHA (mg) which gives a linear calibration curve shown in **Figure 2.3**. The corresponding peaks are shown in **Figure 2.2**. Each standard point is representative of an independent preparation.

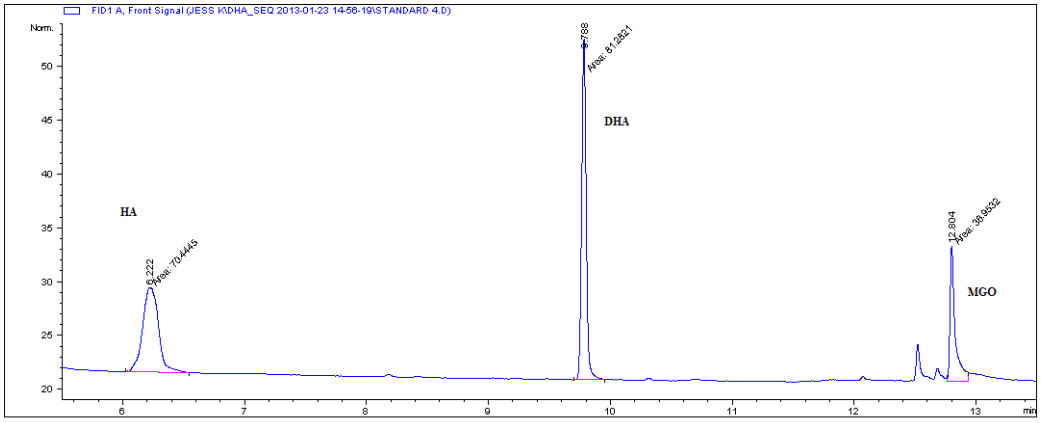

Figure 2.2: An example of a DHA chromatogram of a mānuka nectar sample. Peaks left to right represent HA (6.32 min), DHA (9.84 min) and MGO (12.85 min). The largest MGO peak is integrated as this peak will be the easiest to integrate if MGO is observed in the nectar sample (although it almost never is detected in the nectar).

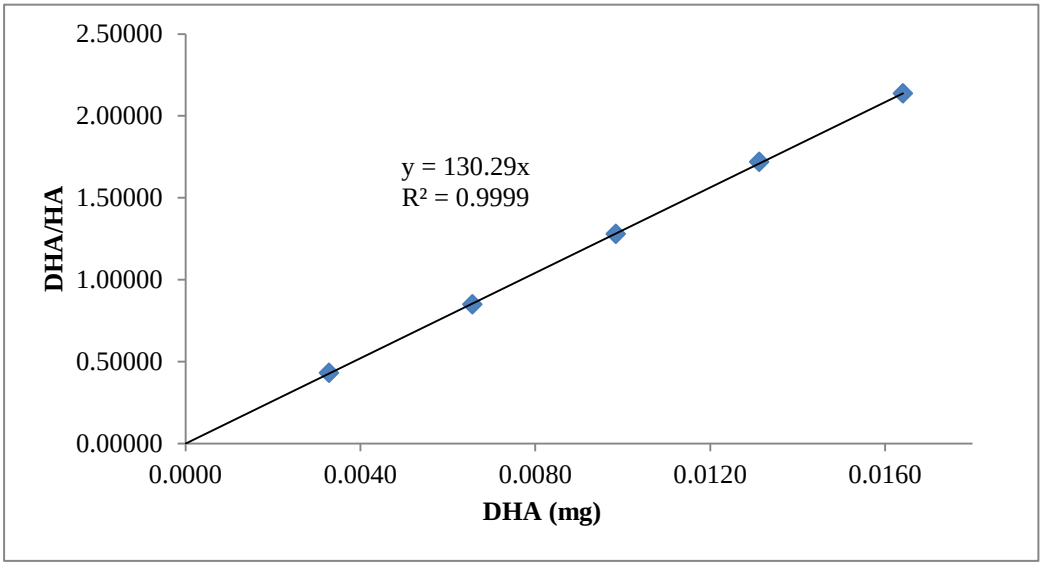

Figure 2.3: An example DHA standard curve (DHA/HA vs DHA (mg)).Each point represents an independent preparation.

$$\frac{\text{DHA}}{\text{HA}} = \frac{\text{DHA Peak Area}}{\text{HA Peak Area}} \quad \textbf{(2.4)}$$

$$\text{DHA (mg)} = \frac{\text{DHA}/\text{HA}}{\text{Gradient of Standard Curve } (\text{mg}^{-1})} \quad \textbf{(2.5)}$$

The DHA/Tsugar value is then determined from **Equation 2.6**.

$$\frac{\text{DHA (mg)}}{\text{Tsugar (mg)}} = \text{DHA (mg)} \div \text{Tsugar (mg)} \quad \textbf{(2.6)}$$

The DHA/Tsugar is determined for each of the ten replicates for the sample. These values are then averaged to give a mean DHA/Tsugar value for the tree.

2.9 Outliers

Due to the relatively small sample size used in this analysis, an abnormal DHA/Tsugar value occasionally resulted due to the combination of experimental error and variation within the tree itself. If the relative standard deviation (% RSD) exceeded 25 % for the ten replicates, the Grubbs' outlier test was utilised to ascertain whether an outlier was present. This was kept consistent with outlier analysis by Williams (2012).[4]

Initially, the single outlier test was applied to data to determine the presence of an outlier. This is given by **Equation 2.7.**

$$G = \frac{|\bar{x}\text{-}x|}{\sigma} \quad \textbf{(2.7)}$$

(where $\bar{x}$ is the mean of all the replicates, x is the mean of the replicate of interest and σ is the standard deviation of all the replicates).

If the G value exceeded 2.176, it was said with 95% confidence that the individual value was an outlier.[79]

If, after the removal of the single outlier, the % RSD was still greater than 25 % it was assumed that the outlier is a part of a pair of outliers. The outlier pairing involved either the largest and smallest values, the two largest or the two smallest values. These mean pairings could have skewed the DHA/Tsugar value and hence the presence of outlier values was not immediately noticeable. In order to determine whether this is the case, two tests were utilised. These tests are shown in **Equations 2.8 and 2.9**.

Equation 2.8 represents the test for the outlier pairing involving the largest and smallest value.

$$G=\frac{x_n-x_1}{\sigma} \quad \textbf{(2.8)}$$

(where x_n represents the replicate with the largest value and x_1 represents the replicate with the smallest value).

It was said with 95% confidence, that this pairing were outliers when the G value exceeded 3.68.[79]

Equation 2.9 represents the test for the two largest/smallest values.

$$G=1-\left(\frac{(n-3)\sigma^2_{n-2}}{(n-1)\sigma^2}\right) \quad \textbf{(2.9)}$$

(where n is the number of replicates and σ_{n-2} is the standard deviation of the replicates excluding the two possible outlier values).

It was said with 95% confidence that when G exceeded 0.7695 this value pairing were outliers.[79]

If after the removal of a pair of outliers the % RSD remained greater than the 25% threshold, the variation observed was attributed to be a result of the whole set of replicates as opposed to one or two individual replicates.

3 Results

The study by Williams (2012) left some unanswered questions and therefore certain investigations were undertaken to add to this work.[4] This included a survey of Northland and the investigation into the effect of tree proximity on the DHA/Tsugar. Before either could be done, the soaking method had to be altered because a poor season resulted in flower collections that were limited in size.

3.1 Ten Flower Test (10F)

Due to the limited amount of sample collected at the beginning of this project, the number of flowers per replicate had to be reduced. The number of flowers was halved as was the extraction volume (10F) and this was tested alongside the original 20F test. A summary of the results is provided in **Table 3.1** and the DHA/Tsugar values are illustrated in **Figure 3.1**.

Table 3.1: A comparison of the DHA/Tsugar and F/G values obtained for samples analysed by the 20F and 10F tests.

Tree	Flowers per Replicate	DHA/Tsugar (mg/mg)	F/G Ratio
Niwa	20	0.00305	1.57
	10	0.00299	1.57
OP	20	0.00207	1.87
	10	0.00211	1.84
VB003	20	0.00097	1.65
	10	0.00101	1.69

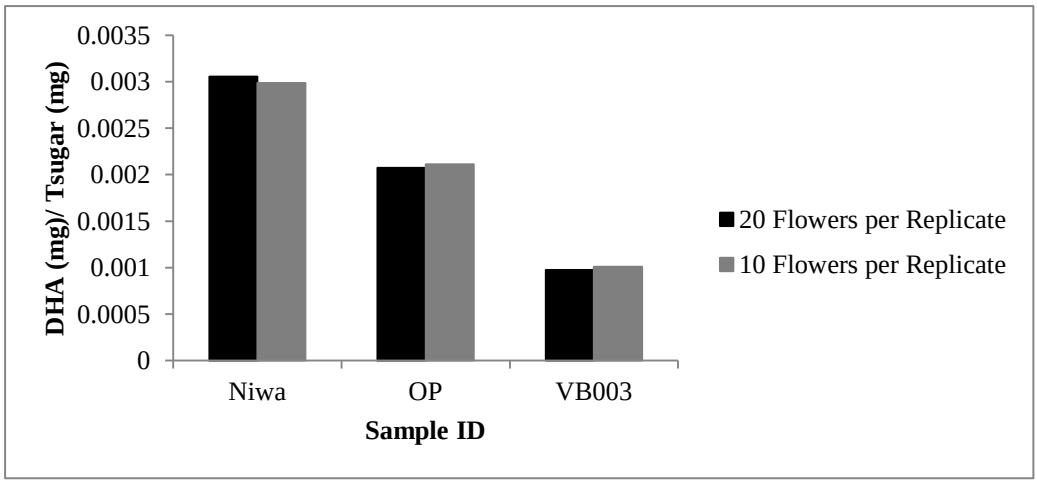

Figure 3.1: The DHA/Tsugar (mg/mg) of samples analysed by both the 20F and 10F tests. These two tests involved changing the number of flowers per sample (20 and 10 flowers, respectively) and altering the extraction volume (4mL and 2mL, respectively).

Statistical analysis showed with 95% confidence that the difference between the results obtained in the 20F and 10F tests was insignificant (p=0.9). As expected, the percentage relative standard deviation (% RSD) increased in the 10F test. However, this increase did not exceed the 25% limit and therefore the 10F test was utilised for the majority of analyses.

3.2 *L. scoparium* var. *Incanum* Survey

A study by Stephens investigated the factors responsible for the Unique Mānuka Factor® (UMF) variation of mānuka honey collected from different sites across New Zealand; it was found that the variation was not completely accounted for by these factors.[31] Hence it was hypothesised that this variability was a result of different ecotypes present across different regions. Williams (2012) sampled many trees across various New Zealand regions but did not include Northland in this investigation.[4]

The *L. scoparium* var. *incanum* variety is generally thought to be localised to the North Island; more specifically to Northland and Coromandel. The *incanum* variety is identified by the lanceolate shaped leaves, pink colouring of the stamina

and the white-pink single flowers.[31] In the current study, the *incanum* variety found in the Northland region was investigated to determine whether high DHA production is present in the *incanum* variety. The samples were identified by observation in the field; unfortunately formal confirmation at the Auckland Herbarium has not yet been completed*. The trees investigated in this survey were collected from the Northland, Auckland and Coromandel regions. It has been reported that *incanum* is also found in the East Cape growing alongside an unidentified variety.[31] Williams reported that some trees located in the East Cape produce large amounts of DHA; however the variety of these trees remains unknown.[4]

The results of this survey are expressed in **Figure 3.2**. For simplicity of discussion, the DHA/Tsugar values will be defined as either low (<0.001 mg/mg), moderate (0.001- 0.002 mg/mg) or high (>0.002 mg/mg). Absolute DHA values will be defined as low (<0.0005 mg), moderate (0.0010- 0.0015 mg) and high (>0.0015 mg). Absolute Tsugar values will be defined as low (<0.200 mg), moderate (0.200-0.500 mg) and high (>0.500 mg).

* Formal confirmation is to be carried out by P. de Lange.

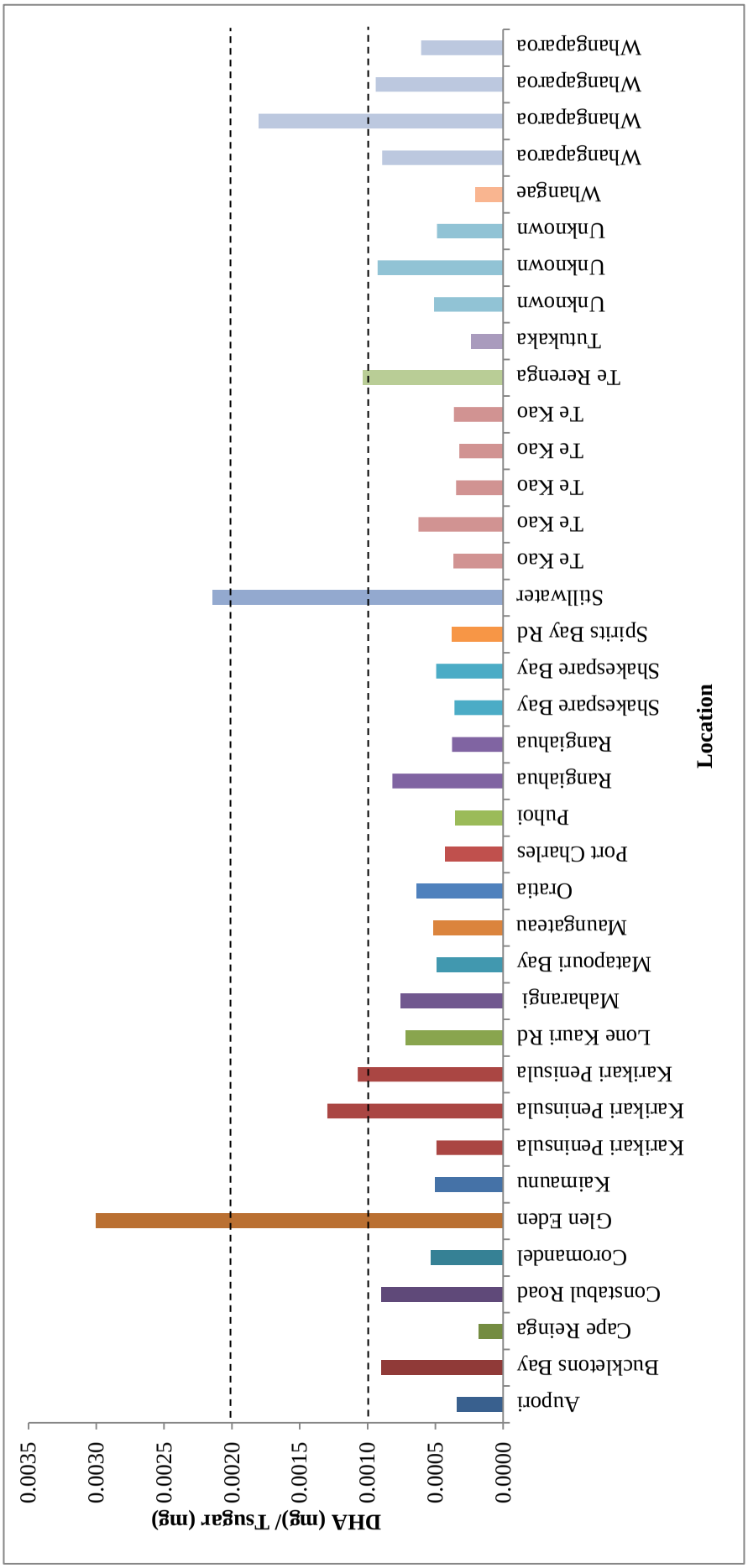

Figure 3.2: The DHA/Tsugar values of the *L. scoparium* var. *incanum* samples collected as a part of the Northland Survey. These samples were collected across the Northland, Auckland and Coromandel regions. The dotted lines represent the low, moderate and high DHA/Tsugar levels (<0.001, 0.001- 0.002 mg/mg and >0.002 mg/mg, respectively).

As illustrated in **Figure 3.2** there are two high producers amongst this sample set. The sample with the largest amount of DHA/Tsugar detected (0.00300 mg/mg) was sampled from a municipal site in the Auckland area. Therefore it may be possible that this sample is a garden cultivar; thus explaining the significant difference in DHA production. A Grubbs outlier test was applied to this sample and the sample from Stillwater; both of these samples were deemed as outliers and thus they were removed from further analyses.

As this survey encompassed multiple regions, the sample set was further separated into two zones. Zone 1 included samples from Northland whereas Zone 2 included samples from Auckland and the Coromandel. The classification is shown in **Table 3.2**. The distribution of the average DHA/Tsugar for each sample within these zones were analysed and the results are expressed in **Figure 3.3**.

Table 3.2: Samples were broadly classified into two zones; Zone 1 encompassed Northland whereas Zone 2 was comprised of samples from the Auckland and Coromandel regions.

Region	Number of Samples	Zone*
Spirits Bay Rd	1	1
Te Kao	5	1
Aupori	1	1
Cape Reinga	1	1
Karikari Peninsula	3	1
Kaimaunu	1	1
Rangiahua	2	1
Whangae	1	1
Matapouri Bay	1	1
Tutukaka	1	1
Lone Kauri Rd	1	1
Maungateau	1	2
Buckletons Bay	1	2
Maharangi	1	2
Port Charles	1	2
Puhoi	1	2
Whangaparoa	4	2
Shakespeare Bay	2	2
Te Rerenga	1	2
Coromandel	1	2
Constabul Rd	1	2
Oratia	1	2

* Zone 1 contains the Northland specimens and Zone 2 contains those from the Auckland and Coromandel regions. The latter zone included samples with a latitude greater than 36.3 °S. The sites are ordered from North to South.

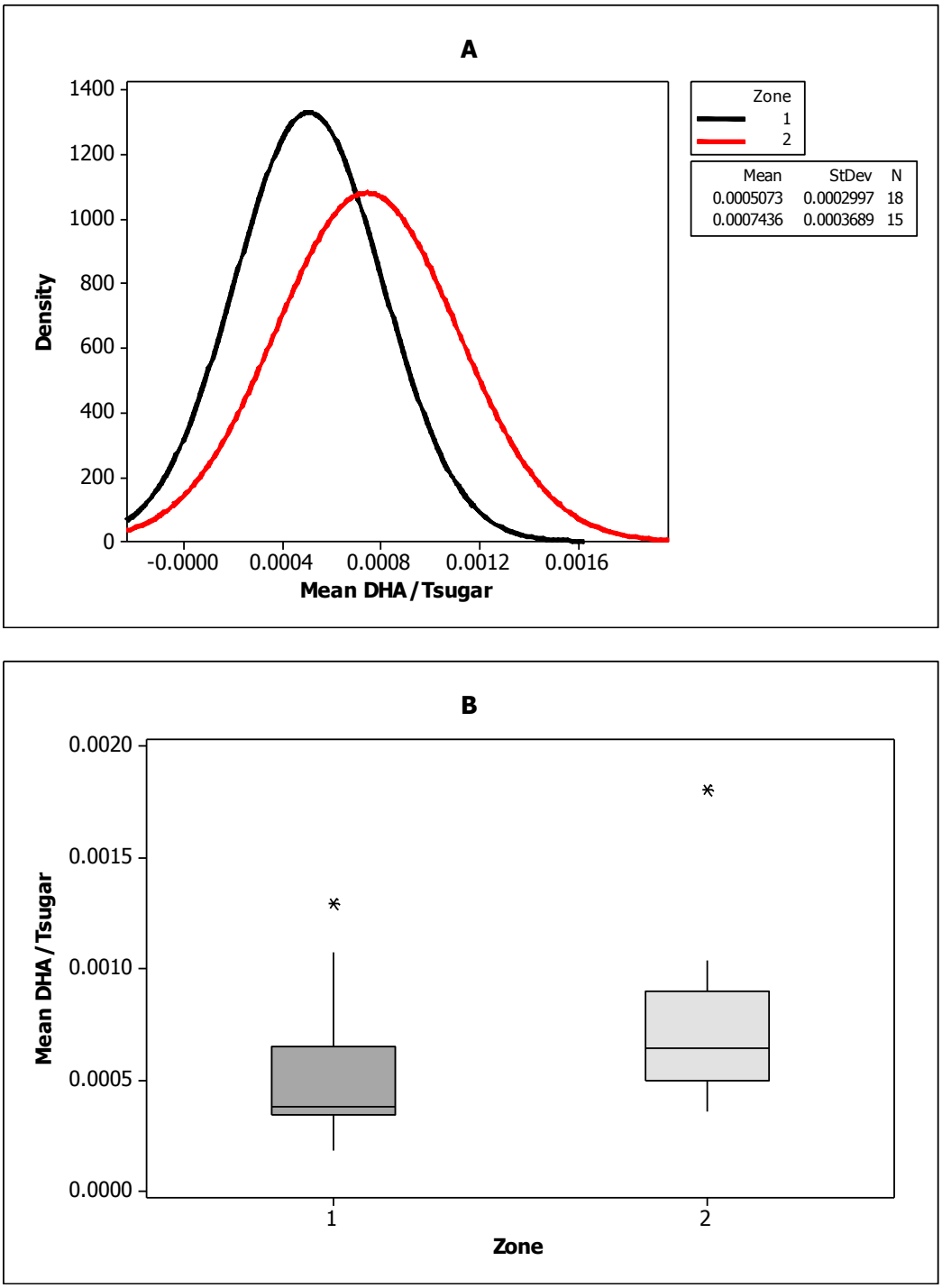

Figure 3.3: The distribution of the average DHA/Tsugar values for each sample encompassed in Zone 1 (Northland) and Zone 2 (Auckland and Coromandel). This is shown as a curve (A) and a boxplot (B).

Figure 3.3A illustrates that the Northland samples (Zone 1) are clustered on one side of the mean for the zone therefore resulting in an asymmetrical distribution. Thus it can be concluded that the majority of these samples produce low amounts of DHA. In contrast, samples classified under Zone 2 have a distribution similar to that of a normal distribution.

Figure 3.3B illustrates that there is one outlier in each of the zones additional to the two previously rejected from the bulk sample. This was confirmed with the Grubbs Outlier Test. The outlier in Zone 1 is one sample located in the Karikari Peninsula and outlier in Zone 2 is one of the samples located in Whangaparoa.

The foregoing suggests that the Northland region has a wide variation of DHA which ranged from low to moderate. For comparison, the samples tested on the East Cape by Williams (2012) produced a moderate to high amount of DHA; the 2010 mean was 0.00141 mg/mg.[4] This does not rule out the possibility that specimens of the incanum variety are responsible for the high DHA production in the East Cape as opposed to trees of the unidentified variety. A DNA analysis would need to be carried out to fully discern which variety is responsible for the East Cape production pattern.

3.3 Tree Proximity

In order to determine whether intra-regional DHA variation was able to be attributed to environmental differences, trees in close proximity to each other were sampled in the Coromandel region.[4] There was a significant amount of variation between trees which suggested that DHA variation was more likely to be a result of a genetic factor as these trees occupied the same environment. However, this study was only done with a limited amount of trees and only in one site. In order to further investigate this phenomenon a larger sample set and other sites were investigated. Known high and low DHA producing trees in the East Cape region were chosen and trees within a 50 metre radius were sampled. This was done in four locations and each location included multiple sampling sites. The sites are depicted in **Figure 3.4**. For larger images with an appropriate scale, see **Appendix 6.5**.

Due to the poor weather conditions that prevailed in 2011, many trees were not flowering during the collection period and therefore samples were limited in size. As a result, these analyses were carried out using the 1F test; where possible, it was repeated three times to ensure a representative DHA/Tsugar value being obtained[*]. Due to the unfortunate weather conditions, the original trees in this survey were not re-sampled in 2011 either because the trees were not flowering during the collection period or because it was deceased or had been removed from the site.

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[*] With the exception of this section, the 10F test was used for all other analyses.

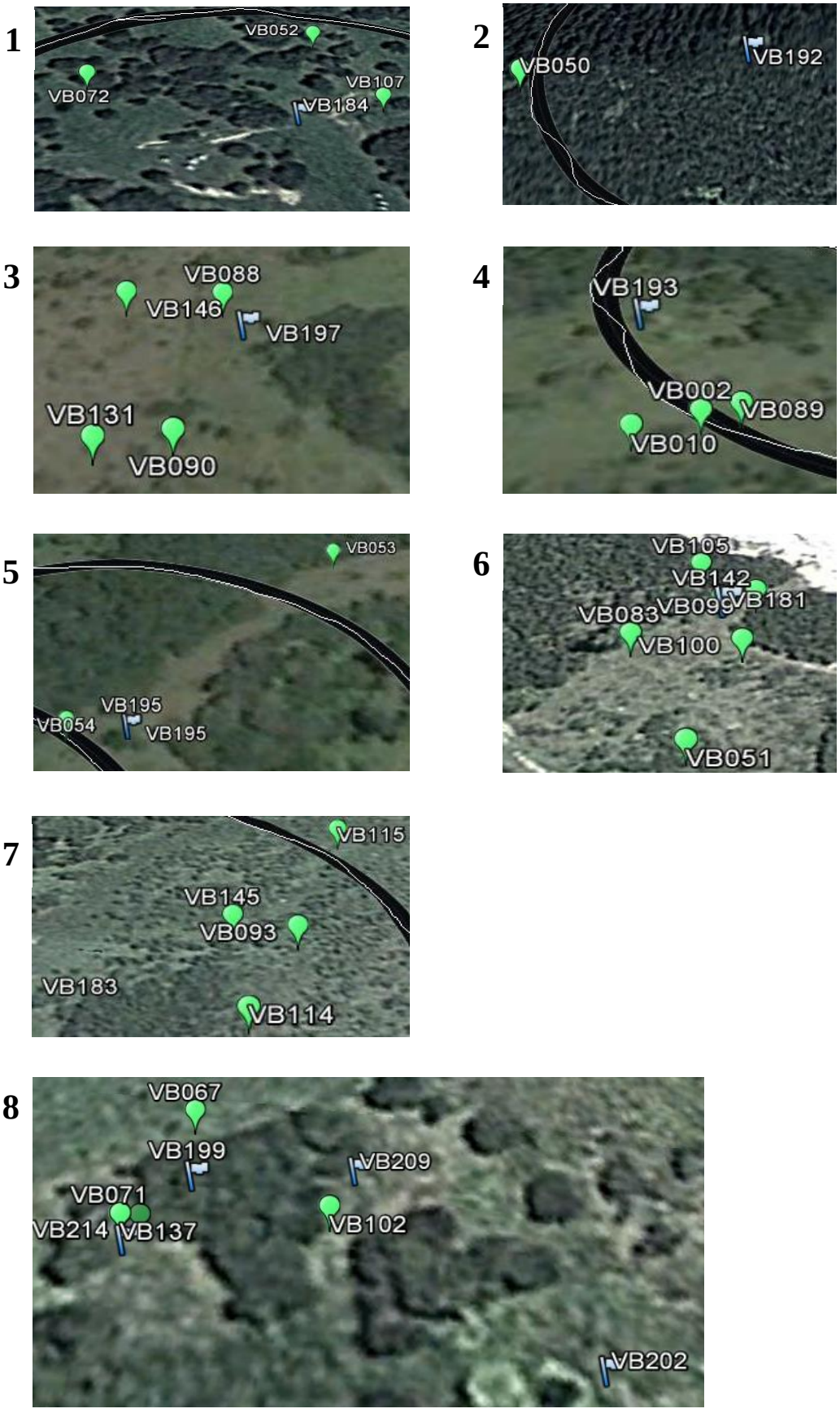


Figure 3.4: The sites that were sampled in the East Cape region depicting the relative positioning of 2011 sampled trees (shown as a green icon) with respect to the chosen 2010 tree(shown as a flag). Locations one to four are encompasses sites 1-2,3-5,6-7 and 8 respectively. The 2011 samples were within a 50m radius of the original tree.

3.3.1 Location One

From this isolated rural location, two sites were sampled. In each site, the original tree was a low DHA producer. The DHA/Tsugar values for both sites are shown in **Table 3.3**.

Table 3.3: A summary of the DHA/Tsugar and F/G values for the trees in Site 1 and Site 2. The trees highlighted in red are the chosen 2010 trees (the DHA/Tsugar result is sourced from Williams' study (2012)).[4] The trees highlighted in green are specimens analysed by the 1F test. One tree did not produce DHA that was able to be detected (NQ).

Site	Specimen ID	DHA/Tsugar (mg/mg)
1	**VB184**	0.00032[a]
	VB052	0.00049
	VB074	NQ[b,c]
	VB107	0.00082
2	**VB192**	0.00086[a]
	VB050	0.00039

[a] Values were obtained from Williams (2012).[4]
[b] NQ = non-quantifiable
[c] The Tsugar for this specimen was 0.24622 mg (a moderate amount).

For both sites, the 2011 samples showed different DHA/Tsugar values than the chosen 2010 tree. One-way ANOVA tests showed with 95% confidence that the 2011 trees in Site 1 and 2 are significantly different to their respective 2010 tree ($p<0.05$). This test did not include VB074. This specimen did not produce a quantifiable amount of DHA but sugar was detected and quantified. In both of the sites, none of the specimens' DHA/Tsugar fell within error of the 2010 original tree (see **Appendix 6.6**). Although the DHA/Tsugar results are shown to be significantly different, the trees are all low producers. If DHA overproduction is predominantly determined by genetic factors, this result is to be expected.

3.3.2 Location Two

From this rural location, three sites were sampled. The first site was chosen due to the presence of a low to moderate DHA producing tree whereas the other two sites

were chosen based on two low producers. Site 4 and 5 are positioned adjacent to one another. The DHA/Tsugar values for this location are depicted in **Table 3.4**.

Table 3.4: A summary of the DHA/Tsugar and F/G values for the trees in Site 3,4 and 5. The trees highlighted in red are the chosen 2010 trees (the DHA/Tsugar result is sourced from Williams' study (2012)).[4] The trees highlighted in green are specimens analysed by the 1F test.

Site	Specimen ID	DHA/Tsugar (mg/mg)
3	**VB197**	0.00095
	VB088	0.00141
	VB090	0.00071
	VB131	0.00261
	VB146	0.00271
4	**VB193**	0.00088
	VB002	0.00079
	VB010	0.00104
	VB089	0.00066
5	**VB195**	0.00081
	VB053	0.00080
	VB054	0.00032
	VB140	0.00063

For Site 3, there is a broad range of DHA/Tsugar values varying from low to high. This is consistent with observations made by Williams (2012) in the Coromandel test.[4] The 2011 specimens have significantly different DHA/Tsugar values to that of the original tree, VB197 ($p<0.05$). However, the DHA/Tsugar of VB088 was within the error limits for the 2010 original tree (see **Appendix 6.6**).

In Site 4 only one tree (VB010) was considered to be significantly different from VB193 with 95% confidence ($p<0.05$). All of the 2011 trees fell within the error of VB193. Site 5 is positioned right next to the aforementioned site and only contains one significantly different tree (VB054) from the original tree. This tree did not fall within the error of VB195 (see **Appendix 6.6**).

3.3.3 Location Three

This location is positioned alongside a river and from here, two sites were sampled. The location is prone to flooding and therefore there may be an additional stress on these specimens compared to the previously discussed samples. Site 6 was chosen around a moderate DHA producer, VB181, whereas Site 7 in this location was chosen around a low producing tree, VB183. The DHA/Tsugar values of these sites are depicted in **Table 3.5**.

Table 3.5: A summary of the DHA/Tsugar and F/G values for the trees in Site 6 and Site 7. The trees highlighted in red are the chosen 2010 trees (the DHA/Tsugar result is sourced from Williams' study (2012)).[4] The trees highlighted in green are specimens analysed by the 1F test.

Site	Specimen ID	DHA/Tsugar (mg/mg)
6	**VB181**	0.00133
	VB051	0.00053
	VB083	0.00064
	VB099	0.00186
	VB100	0.00109
	VB105	0.00088
	VB142	0.00107
7	**VB183**	0.00034
	VB093	0.00242
	VB114	0.00248
	VB115	0.00091
	VB145	0.00095

Site 6 contained three low producers and three moderate producers; only VB100 was proven to be similar to the original tree, VB181 by a one-way ANOVA test ($p<0.05$). However, the DHA/Tsugar of trees VB099, VB100, VB105 and VB142 all fell within error of the original 2010 tree as given by Williams' (2012) study (see **Appendix 6.6**).[4]

It was expected that the trees in Site 7 would give a low to moderate amount of DHA/Tsugar as observed in the previous site. However, two trees gave high DHA/Tsugar values and the other two gave borderline moderate DHA/Tsugar

values; all the 2011 specimens differed significantly in their DHA production from the 2010 tree and none overlapped within error of this tree (see **Appendix 6.6**). These specimens were located in a swamp. It is known that *L. scoparium* does not thrive in swampy conditions and hence VB093 and VB114 may be producing DHA in response to this stress accentuated in the wet conditions in 2011. These results show that although a common environment or stress may be experienced by all specimens, the responses of individual trees seem to be different as a result of their genetic makeup.

3.3.4 Location Four

Only one site was chosen from this well maintained farmland. Three specimens were chosen from this site in 2010; two moderate producers and one high producer. These trees had been selected for their phenotype and were deliberately planted in rows. The DHA/Tsugar values are summarised in **Table 3.6**.

Table 3.6: A summary of the DHA/Tsugar and F/G values for the trees in Site 8. The trees highlighted in red are the chosen 2010 trees (the DHA/Tsugar result is sourced from Williams' study (2012)).[4]

Site	Specimen ID	DHA/Tsugar (mg/mg)
8	**VB199**	0.00312
	VB202	0.00130
	VB214	0.00145
	VB137	0.00421
	VB070	0.00200
	VB067	0.00195
	VB071	0.00168
	VB102	0.00044

Unlike in previous sites, the DHA/Tsugar values are consistent with the 2010 trees with the exception of VB102. This tree did not fall within the DHA/Tsugar error of the original three trees. This sample had not been deliberately planted but was growing naturally in close proximity to the other specimens. The trees are

either moderate or high DHA producers. This location was the only location that had significant annual variation, as found by Williams (2012).[4] The 2011 samples are similar to the 2010 DHA/Tsugar values. This suggests that the DHA overproduction may be controlled by more than one factor (both genetic and environmental). This site also contained a tree that is extremely high producing (VB137) which represents a possible parent tree for breeding.

As previously discussed, one possibility for the overproduction of DHA is stress. These samples appear to be under no drought or excess water stress. However, the trees may not have enough exposure to sunlight and are in very close proximity to each other such that their branches interlock. This may be a source of stress hence resulting in the overproduction of DHA.

3.3.5 Conclusion

Samples across different sites were tested to determine whether the DHA production was similar across all trees in the site. These trees should theoretically be genetically similar (as they are likely to be derived from one parent) and they are in a similar environment. It was expected that neighbouring trees would yield a similar DHA/Tsugar value to that of the 2010 original tree. This was not the case.

The DHA/Tsugar values of every tree in each location were investigated (see **Appendix 6.6**). Each location was more similar to the 2010 DHA/Tsugar measured by Williams (2012) with the exception of Location One.[4] This location was similar to the 2009 DHA/Tsugar values.[4] This investigation showed that there is substantial variation of trees within one site and definitely within one location. The evidence also suggests that the NPA of the honey produced from these sites will not be consistent from year to year.

3.4 *L. scoparium* Cultivars

Numerous garden cultivars have been bred from exceptional individual specimens of naturally occurring varieties grown in rural New Zealand. Williams (2012) investigated one tree grown in a Hamilton suburban garden that was termed Waikato Gold.[4] This tree produced a high amount of DHA and as a result, garden cultivars have been investigated more thoroughly. The result of this investigation is shown in **Figure 3.5**.

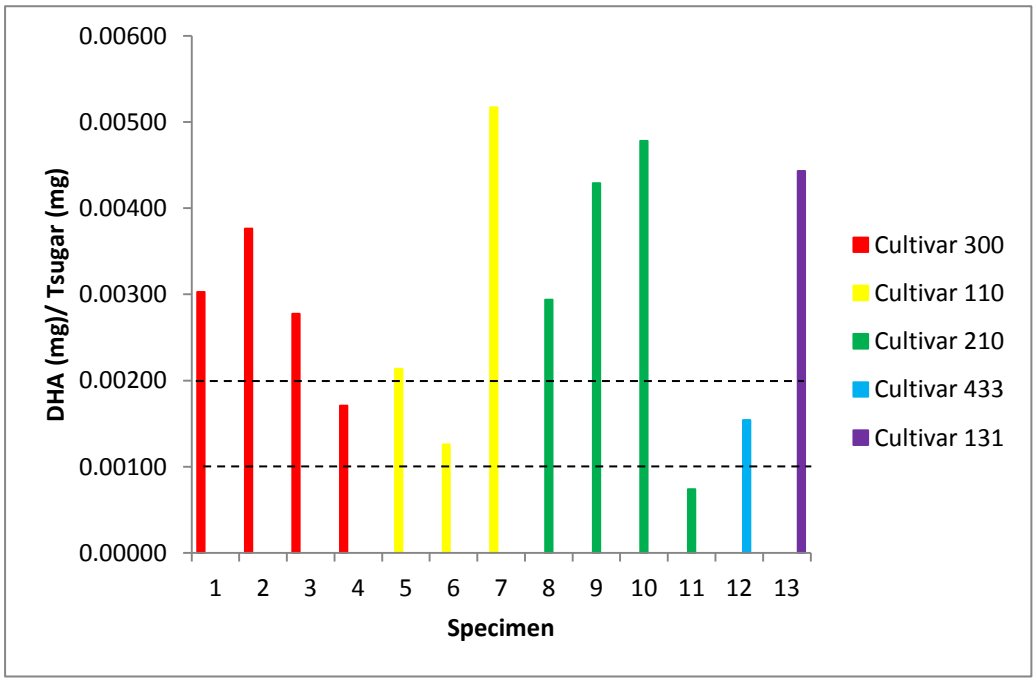


Figure 3.5: The DHA/Tsugar of *Leptospermum scoparium* garden cultivars that have coloured single flowers. The dotted lines represent the low, moderate and high DHA/Tsugar levels (<0.001, 0.001-0.002 mg/mg and >0.002 mg/mg, respectively).

The parentage of Waikato Gold was investigated to determine the inheritability of the overproduction of DHA. Waikato Gold will be referred to as cultivar 300. Three specimens of one parent (cultivar 110) were investigated. Williams (2012) observed that there was a lot of DHA variability between trees and therefore, as expected, there was significant variation between the trees of this cultivar.[4] However, the DHA/Tsugar range is moderate to high (greater than 0.001 mg/mg) exceeding the amount produced by most wild varieties.

The second parent, cultivar 200, is reported to be extinct in New Zealand but its closest relative was tested. This is cultivar 210.[11,12] Although there was significant variability between specimens of cultivar 210, three trees produced very high levels of DHA/Tsugar.

In preliminary trials, single specimens of other cultivars were tested. Four specimens were analysed but only two yielded quantifiable DHA. Cultivar 433 produced a moderate amount of DHA/Tsugar whereas the cultivar 131 specimen test was a high producer. These two cultivars bear single flowers whereas cultivars 431 and 432 bear double flowers. These double flowered cultivars did not produce a detectable amount of DHA although they did produce a quantifiable amount of Tsugar. These Tsugar values and those for other cultivars with no apparent absolute DHA are shown in **Appendix 6.7**. For the difference in the flower morphology of single and double flowers, refer to **Figure 1.1**.

Another double flowered specimen was tested which will be referred to as cultivar 425. This cultivar has white/pink double flowers. Like many of its double flowered counterparts it is related to cultivar 400, the first known double flowered cultivar.[12] Similar to other double flowered cultivars, DHA in cultivar 425 could not be quantified. From the foregoing, it appears that cultivars with the double

flower allele may not have a genetic predisposition for the overproduction of DHA.

With the exception of one specimen, all the cultivars that have been investigated have had pink or red flowers. A one-way ANOVA analysis showed with 95% confidence that all the varieties were significantly different to each other ($p<0.05$) with the exception of the 300 and 210 ($p=0.205$). This could be explained by the parentage of these two cultivars as they are both bred from 200. This cultivar was originally found in the Canterbury region of New Zealand.[11] As this region can be exposed to harsh weather conditions, it could be possible that an overproduction of DHA evolved as a result of a selection pressure.

In order to investigate further whether the overproduction of DHA is related to a particular trait, nineteen different varieties were sampled in one day from the ABG. As they were all sourced from the same site, environmental variation could be eliminated. Some of the aforementioned varieties were sourced from this site in previous years but never in the same year. Cultivars are selected for breeding based on the colour and arrangement of the petals, and the plant form. Therefore these nineteen varieties included all the possible combinations of plant stature, colour and nature of the flower. Unfortunately, prostrate plants were not included in this analysis.

Figure 3.6 illustrates the relationships between cultivars belonging to the series bred from cultivar 500 and the levels of DHA that was produced.

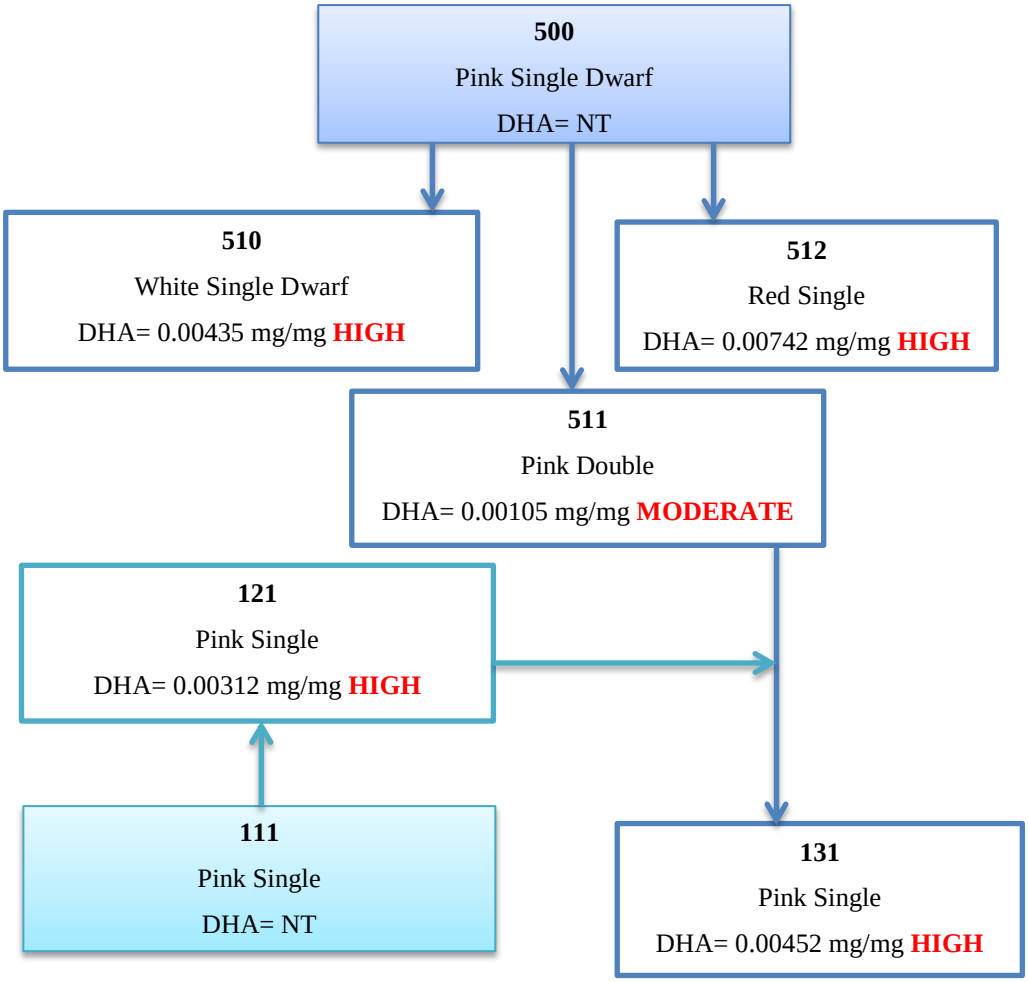


Figure 3.6: A flow diagram depicting the cultivars in the family tree bred from cultivar 500. This type of mānuka has a reduced stature. The parents shown represent the seed parents; information on the pollen parent is not available. Some cultivars were not available to be tested (NT) and are shaded as a result. The DHA range is expressed in red.

The single flowered cultivars in this group, especially cultivar 512, produce high levels of DHA. Cultivar 131 is a hybrid between 511 and 121; which of these is the seed parent remains unknown. Judging from the singled flower nature of cultivar 131, it appears as if the double flowered characteristic may be recessive.[80] This would explain why 131 is a high producer despite its double flowered parentage.

As shown above, the double flowered characteristic appears to suppress DHA production. Interestingly cultivar 511, a double flowered cultivar is a moderate DHA producer. It may be that the overproduction of DHA is stress related, attributable to the dwarf nature of the plant. A smaller stature would result in the reduction of the plants exposure to sunlight and thus result in a stressed state. DHA could be being produced to compensate for this stress.

The presence of the double flower gene may be responsible for a reduction in the expression of the gene responsible for DHA overproduction. Therefore other double flowered cultivars were investigated to determine whether they too were low or moderate producers. The relationships between the cultivars and their DHA productions are summarised in **Figure 3.7**.

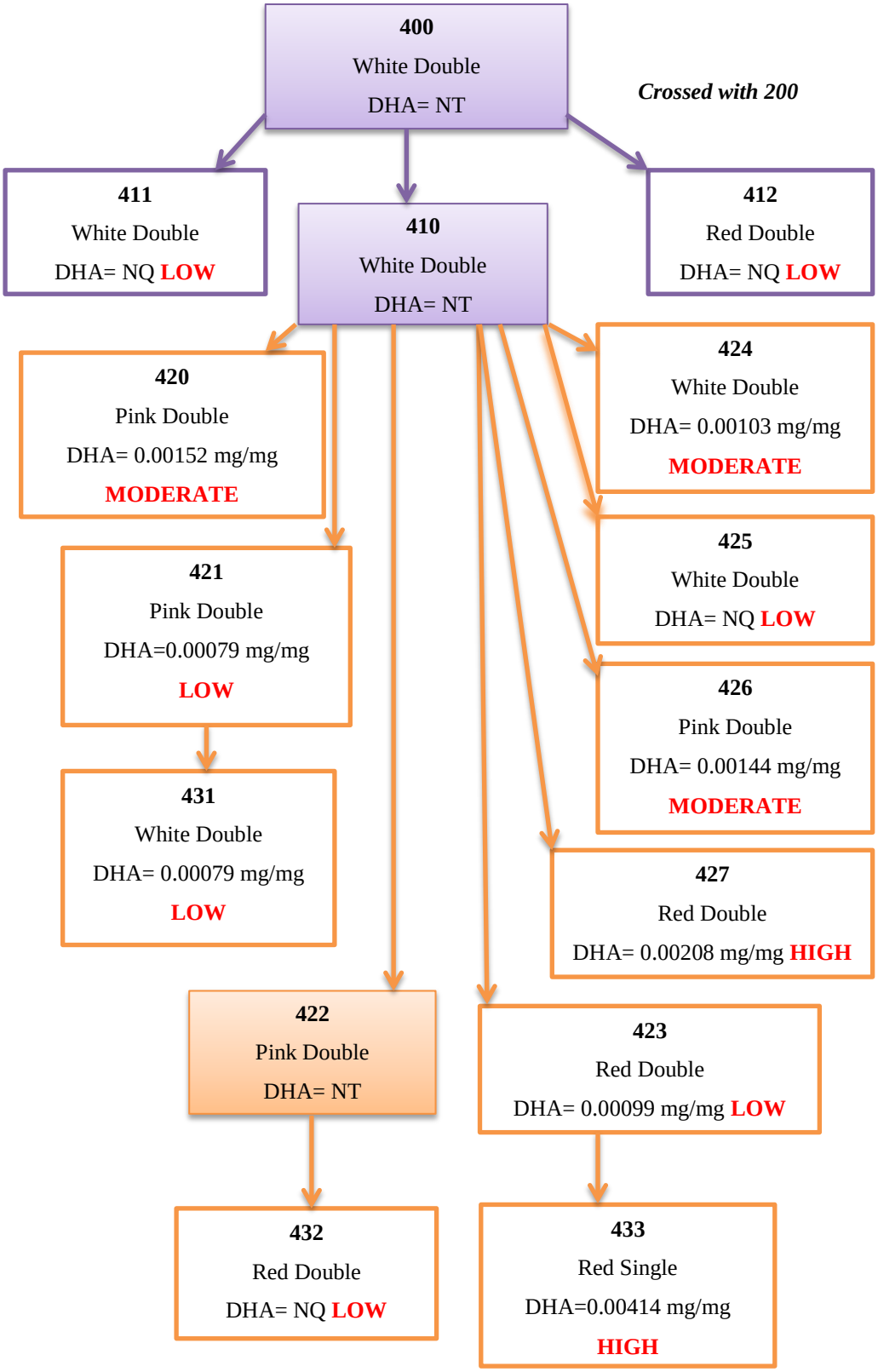


Figure 3.7: A flow diagram depicting the cultivars in the double flowered series. The parents shown represent the seed parents; information on the pollen parent is not available except for cultivar 412. Some cultivars could not be tested (NT); these have been shaded. Other cultivars did not produce a quantifiable amount of DHA (NQ) although sugar was detected (see **Appendix 6.7**). The DHA range is highlighted in red.

The double flowered series showed that, with the exception of 427, all of these cultivars are low and moderate DHA producers. Cultivar 427 is a red double cultivar. The high production of DHA could be explained by the red pigment of the petals; and thus its relation to 200. However, cultivar 412 was bred from cultivars 200 and 400 and it did not produce a quantifiable amount of DHA

It has been reported in the literature that 433's seed parent is 423.[12] As 433 has red single flowers and 423 has red double flowers, it seems unlikely that 423 is in fact the seed parent. There was a mānuka plant at the ABG labelled as 423; however this plant had single red flowers. This tree was collected in 2009 and tested.[81] With an average DHA/Tsugar of 0.00383 mg/mg it is possible that 433 was bred from this plant as opposed to an actual 423 tree. This would explain the single flower characteristic and the high DHA/Tsugar value of 433. The possibly misattributed plant has similar characteristics to 210.

The single flower series is illustrated in **Figure 3.8**. Cultivar 210 was not able to be collected from this site; however it was already known to be a high DHA producer. Unfortunately, there is a lack of information on the parentage of the 600 specimen.

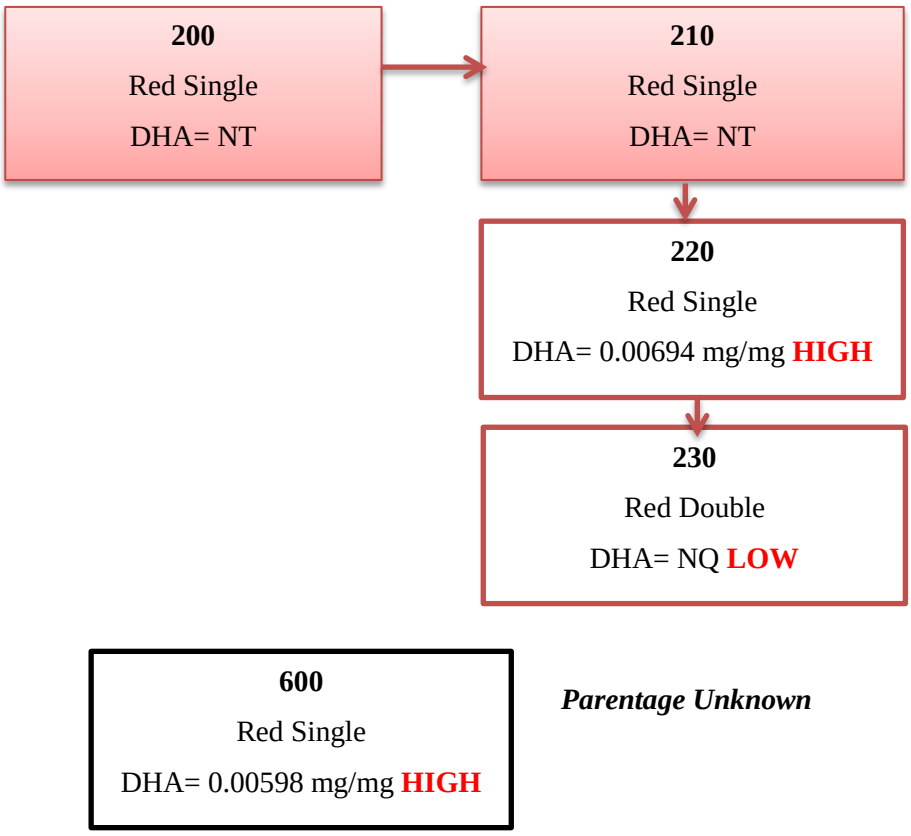


Figure 3.8: A flow diagram showing the single flowered series. The parents shown are the seed parents; no information is available for the pollen parent. The entire parentage of 600 is unknown. Some of the parent cultivars could not be tested (NT); these are shaded as a result. One cultivar did not produce a quantifiable amount of DHA (NQ) but did produce a quantifiable amount of Tsugar (see **Appendix 6.7**).

This series further confirms that single flowered cultivars express greater amounts of DHA than their double flowered counterparts. Further analysis of the different cultivars is required to determine whether this finding has a genetic basis.

As there are obvious differences between the different types of cultivars, it seems that the DHA production is a result of genetic predisposition. Therefore if mānuka cultivars were to be chosen for large-scale plantations, coloured single flowered cultivars should be considered for breeding. As these cultivars produce such a high level of DHA, a more consistently high level of NPA could be achieved.

3.5 Flower Gender

The *Leptospermum* species are known to bear both hermaphrodite and male flowers. Although there have been observations of the proportion of hermaphrodite to male flowers and whether pollinator discrimination occurs, there has not been an investigation into the chemical nature of these two flowers types.[16]

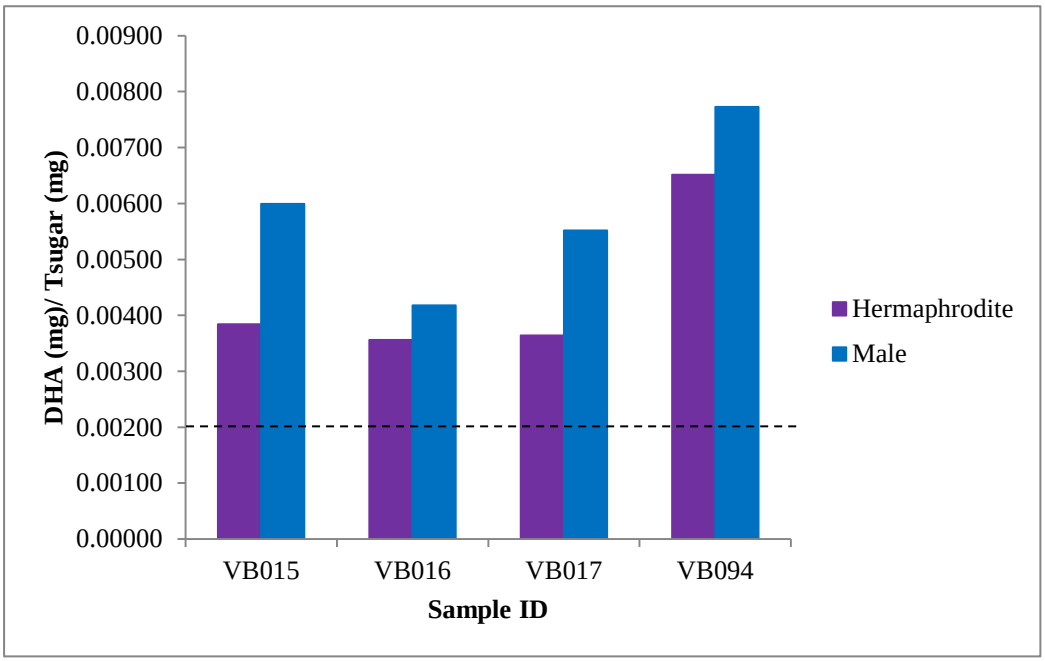


Figure 3.9: The DHA/Tsugar of hermaphrodite and male flowers of mānuka trees. The dotted lines represent the high DHA/Tsugar level (0.00200 mg/mg).

Table 3.7: The nectar composition of hermaphrodite and male flowers collected from various mānuka trees.

Tree	Gender	DHA (mg)	Tsugar (mg)	DHA/Tsugar (mg/mg)	F/G Ratio
VB015	Hermaphrodite	0.00048	0.12356	0.00384	2.00
	Male	0.00072	0.11805	0.00599	1.87
VB016	Hermaphrodite	0.00060	0.16666	0.00356	2.01
	Male	0.00080	0.19074	0.00418	2.07
VB017	Hermaphrodite	0.00079	0.21256	0.00364	1.84
	Male	0.00118	0.21237	0.00552	1.99
VB094	Hermaphrodite	0.00088	0.13248	0.00651	1.83
	Male	0.00098	0.12487	0.00773	1.70

As reflected in both **Table 3.7** and **Figure 3.9** there is a distinct variation in the nectar of the two genders. A one-way ANOVA test found with 95% ($p=0.000$) confidence that the DHA/Tsugar of the hermaphrodite and male flowers were significantly different. This difference seems to be attributable to the variation of DHA in the nectar as the total amount of sugar did not vary significantly between the male and hermaphrodite flowers (as indicated by further one-way ANOVA analyses). Nor was the F/G ratio consistently different; suggesting that the DHA is not sourced from the breakdown of the hexose components of the nectar. However, this DHA/Tsugar difference does not change the high classification of the trees.

The foregoing results could suggest that the DHA, whilst it is expressed in both flowers, is being utilised in the hermaphrodite flowers. This could be explained by the formation of triacylglycerides in the hermaphrodite flowers. As previously mentioned, these lipids are stored in the seeds as a source of chemical energy and carbon. Triacylglycerides are synthesised from glycerol and fatty acids; the precursor to glycerol is DHAP.[69] It is possible that DHA is exported to both types

of flowers but it is phosphorylated by a kinase enzyme and utilised by the hermaphrodite flowers. Consequently the amount of DHA being expressed would vary across the flowering season.

Williams (2012) investigated the DHA/Tsugar of one mānuka plant over a three year period (2009-2011).[4] The DHA/Tsugar varied significantly from 2009 to 2010 but remained relatively the same from 2010 to 2011 whereas the F/G ratio remained relatively constant across this three year period.[4] This tree was mature over the sampling period and hence the difference cannot be attributed to age; however the tree subsequently died in 2012. According to Williams, the Waikato region was in a drought at the conclusion of 2009.[4] Due to the environmental stress on the plant or impending senescence, the majority of the DHA being sent to the flowers may have been utilised in triacylglyceride synthesis. The rate of this synthesis could have increased to ensure that the chance of survival of seeds was maximised. Consequently, less DHA would have appeared in the nectar.

3.6 Hourly Nectar Composition Variation

The reason for the overproduction of DHA has been hypothesised but it still remains unknown. It also remains unknown whether DHA is produced in the flowers or it is produced in other parts of plant and is transported to the flowers by the vascular system. The hourly variation of the DHA/Tsugar was investigated to determine whether DHA and Tsugar production was uniform. While it is recognised that bees do not sample at a certain hour during the day, the results of this study could provide information on the optimum time to collect samples for analysis.

Three trees were tested; one of which is the same as cultivar 300. This specimen is labelled Tree A. The other two trees were the same as cultivar 210 and are labelled as Trees B and C. In order to take into account the different in nectar between male and hermaphrodite flowers, these two flower genders were tested separately. These samples were tested in triplicate. The DHA (mg), Tsugar (mg) and DHA/Tsugar (mg/mg) results are summarised in **Figure 3.10**, **Figure 3.11** and **Figure 3.12**, respectively.

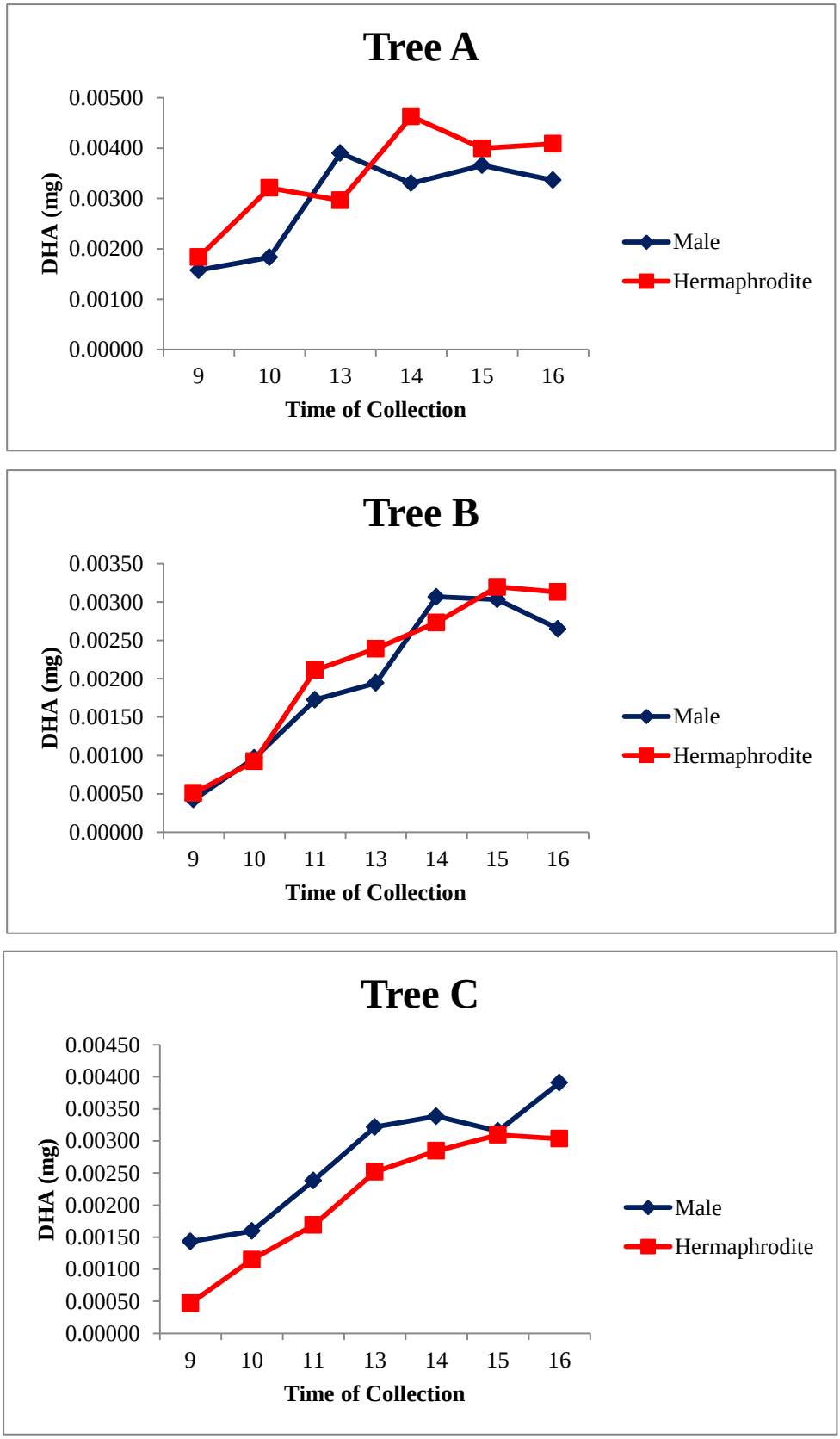


Figure 3.10: The hourly variation (from 9am to 4pm) of the absolute amount of DHA (mg) detected in the nectar of Tree A, B and C. Nectar was collected from both male and hermaphrodite flowers separately.

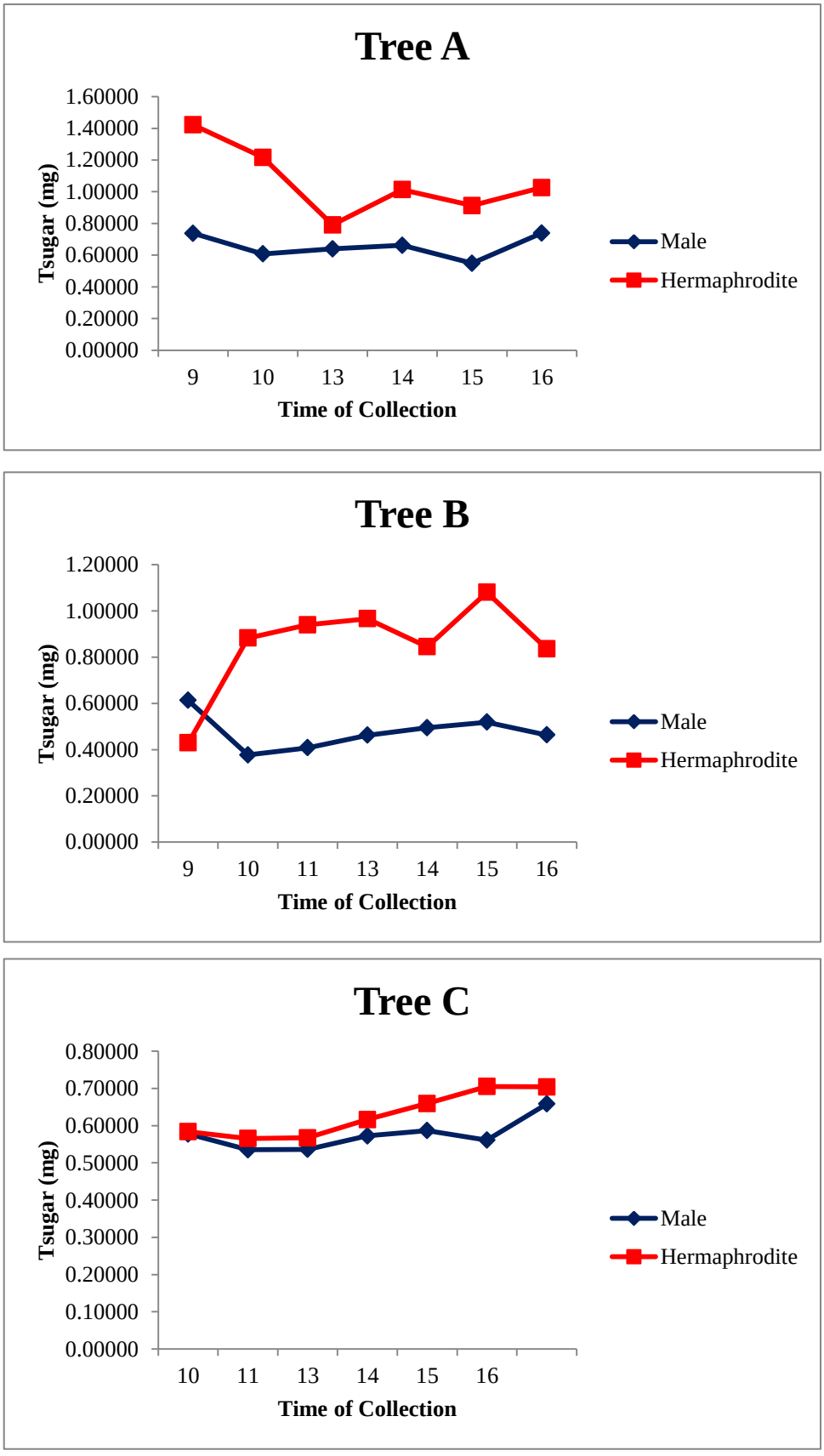
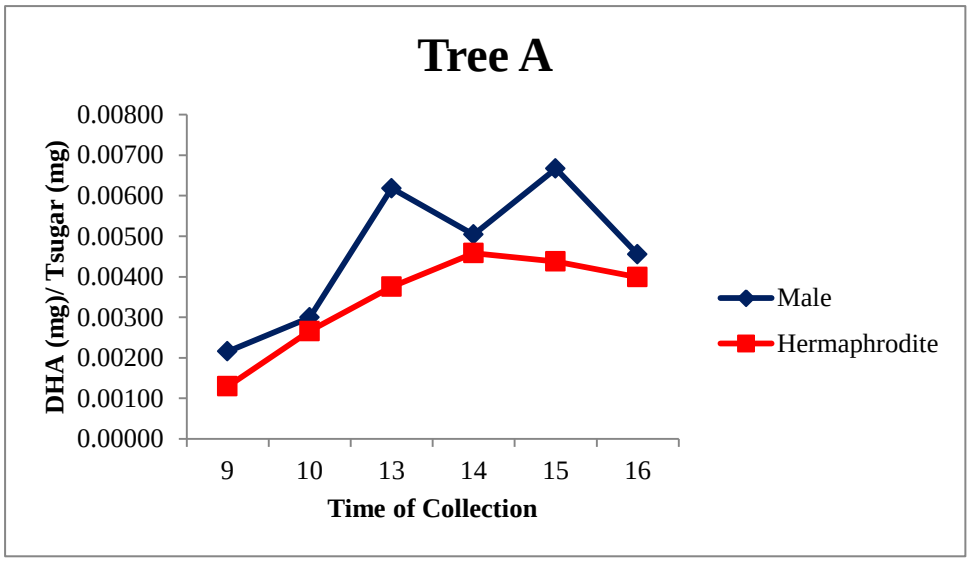
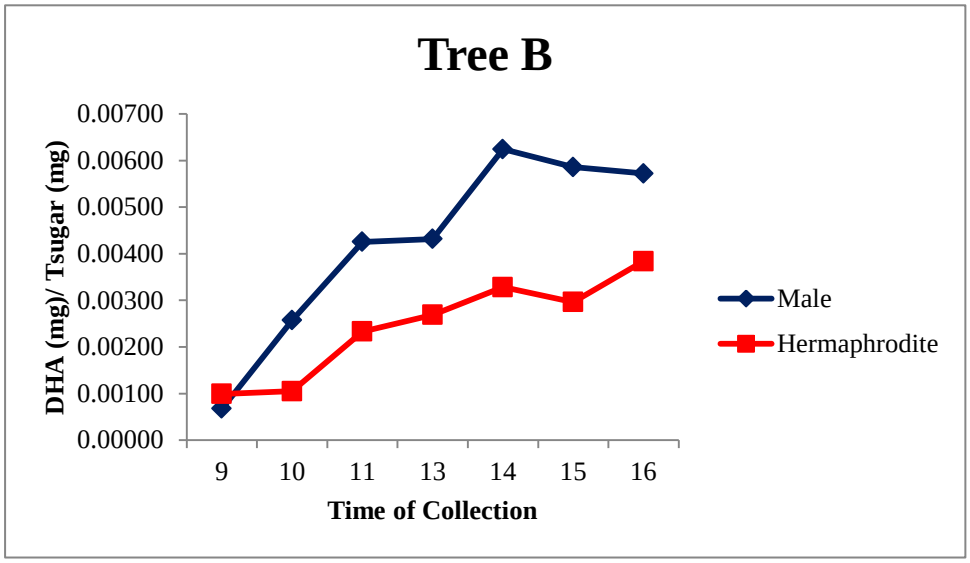


Figure 3.11: The hourly variation (from 9am to 4pm) of the absolute amount of Tsugar (mg) detected in the nectar of Tree A, B and C. Nectar was collected from both male and hermaphrodite flowers separately.





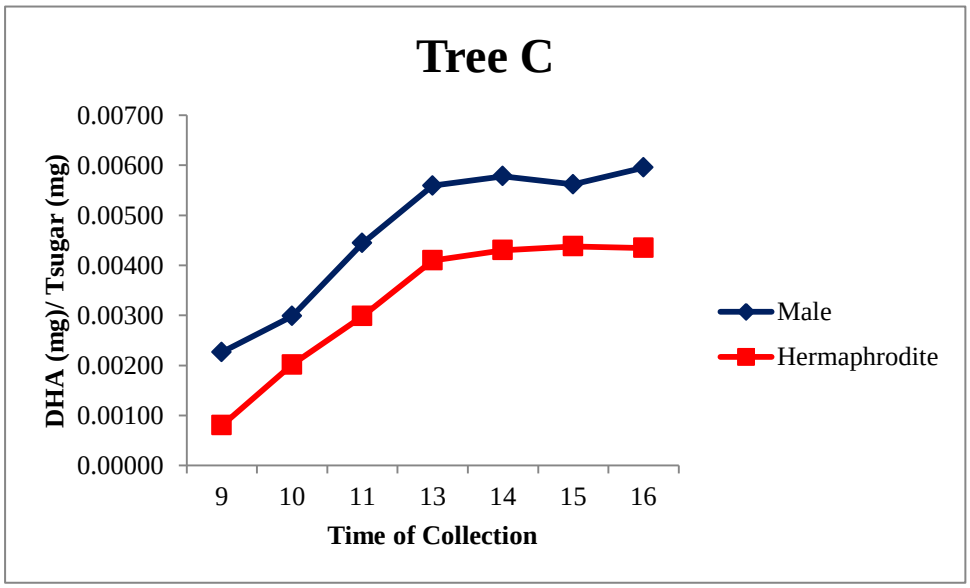


Figure 3.12: The hourly variation (from 9am to 4pm) of the DHA (mg)/Tsugar (mg) detected in the nectar of Tree A, B and C. Nectar was collected from both male and hermaphrodite flowers separately.

There are a few general trends that are obvious in the foregoing figures. **Figure 3.10** illustrates the change in the amount of DHA (mg) in the nectar as the day progresses. In all trees, it is evident that there is an increase in the DHA over the course of the day. This was clear for both the male and hermaphrodite flowers. The difference between the amounts of DHA at 9am compared to 4pm was found to be statistically significant by a one-way ANOVA test with 95 % confidence. The majority of the differences between the absolute amounts of DHA of the hermaphrodite and male flowers were found to be statistically insignificant ($p>0.05$).

The change in the Tsugar amount over the day is shown in **Figure 3.11**. Unlike the DHA results, it was found that the Tsugar did not vary a lot in both hermaphrodite and male flowers. The difference between 9am and 4pm was found to be significant in the hermaphrodite flowers of Tree A and C and in the male flowers of Tree B ($P<0.05$). The difference between the male and hermaphrodite flowers was found to be significant with 95% confidence in both Tree A and B but not in Tree C.

As with the trend observed in the amount of DHA, the DHA/Tsugar of the three trees increased over the course of the day. The difference between the start and end of the day was found with 95% confidence to be significant in all flower types and across all trees. As a result of the hermaphrodite flowers having a greater proportion of Tsugar present in the nectar overall, they have a smaller DHA/Tsugar compared to their male counterparts. The majority of this difference was found to be significant by one-way ANOVA tests ($p<0.05$).

In conclusion, the time of collection of sample does impact the DHA/Tsugar result. This should be taken into consideration in future collections. In order to

further investigate the reason for this variation, a more detailed study should be conducted on one tree. The number of replicates would need to be increased and it should be conducted over a longer period. Additionally, it is recommended that the experiment is carried out in a greenhouse to avoid interference by pollinators as it has been shown that pollinators can remove up to half of the available nectar.[43]

3.7 Flower Hypanthium Colour

Colour changes are often observed in flowers. These colour changes can be either generalised or specific and are thought to be beneficial to both the plant and the pollinator; the pollinators are encouraged to visit flowers with the greatest reward and those that are at the optimal reproductive stage.[82] The specific change of the mānuka hypanthium surface colour from green to red, as the flower ages, is of interest. Both male and hermaphrodite flowers exhibit this change. Examples of flowers with green and red hypanthium colouring are shown in **Figure 3.13**.

In *Chamelaucium uncinatum* (a member of the Myrtaceae family), it was found that pollinators did not visit flowers with a red nectary surface. This colour change has been hypothesised to signal insect pollinators that there is a lack of nectar reward provided by the flower.[43] It is unknown whether pollinator influence affects the DHA ratio.

Figure 3.13: The difference of hypanthium colour in manuka flowers. The flower on the left is a cultivar 210 plant and has a green centre. The stigma is reduced and the stamina are inward facing. The flower on the left is a cultivar 300 flower with a red centre. It's stigma is fully formed and the stamina are no longer inward facing. A green hypanthium is indicative of a young flower and it changes to red as it ages.

Williams (2012) has previously investigated the DHA/Tsugar of flowers with different hypanthium colour using the 1F test; no significant difference was found.[4] Due to the limited sample size of the aforementioned study, it was repeated in the current study using a greater number of flowers to confirm whether the DHA/Tsugar varied with hypanthium colour and therefore as the flower aged. **Table 3.8** summarises the nectar composition of three specimens with differing hypanthium colour and **Figure 3.14** illustrates the DHA/Tsugar difference.

Table 3.8: A summary of the nectar composition of mānuka flowers with either a green or red hypanthium.

Sample ID	Type of Cultivar	Hypanthium Colour	DHA (mg)	Tsugar (mg)	DHA/Tsugar (mg/mg)
VB001	300	Green	0.00037	0.15600	0.00222
		Red	0.00094	0.26274	0.00358
VB002	510	Green	0.00014	0.06162	0.00233
		Red	0.00062	0.13053	0.00472
VB015	510	Green	0.00021	0.08530	0.00250
		Red	0.00064	0.18877	0.00332

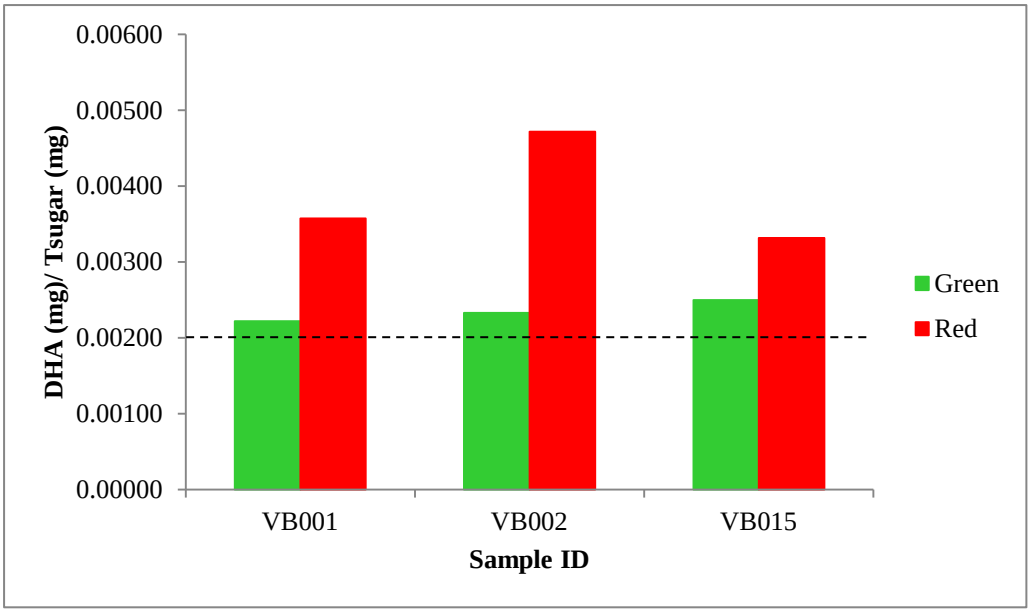


Figure 3.14: The DHA/Tsugar of manuka flowers with either a green or red hypanthium surface. VB001 is a different cultivar than the other two samples (cultivar 300 and 210, respectively). The dotted lines represent the high DHA/Tsugar level (0.00200 mg/mg).

There is a distinct difference in the nectar composition between the two flower types. In all three samples, the flowers with red hypanthium colouring have a greater amount of both DHA and Tsugar than flowers with green hypanthia; these results were found to be significant ($p<0.05$). This therefore resulted in a significant difference in DHA/Tsugar ($p=0.000$). However, the difference between these two flower types does not change the high DHA/Tsugar classification of the trees.

According to O'Brien's (1996) investigation of *C. uncinatum* the hypanthium is green during nectar secretion and the transition of the hypanthium colour to red signals the end of nectar secretion.[43] *L. scoparium* flowers seem to exude nectar continuously throughout the flower's lifetime as was also observed with *L. myrsinoides* and *L. continentale*.[13] As mānuka did not evolve with bees, bee behaviour cannot be related specifically to the hypanthium colour change. At this stage, it is unknown whether bees prefer one flower type over the other. It seems likely that this colour change is associated with senescence but it is interesting

that the solid content of the nectar increases with senescence. One would expect that the nectar would become less concentrated with age. It could be that the red colouring acts as an invitation to all pollinators, signalling a greater amount of nectar being produced.

The variation in DHA/Tsugar of flowers with different hypanthium colouring may be partly attributable to the variation observed between male and hermaphrodite flowers (as illustrated in **Section 3.5**). In order to investigate whether the variation is actually due to the hypanthium colouring, only hermaphrodite flowers with different hypanthium colouration were investigated. Two of the original trees were sampled again for this analysis however the third did not have a sufficient amount of flowers for sampling. A similar tree was chosen as a replacement. The VB001 tree is a 300 cultivar whereas the other two samples are 510 cultivars. A summary of the nectar composition is provided in **Table 3.9**.

Table 3.9: A summary of the nectar composition of hermaphrodite flowers with either a green or red hypanthium.

Sample	Cultivar	Hypanthium Colour	DHA (mg)	Tsugar (mg)	DHA/Tsugar (mg/mg)
VB001	300	Green	0.00097	0.46725	0.00203
		Red	0.00288	0.49005	0.00585
VB015	510	Green	0.00085	0.17716	0.00483
		Red	0.00042	0.10330	0.00425
VB016	510	Green	0.00027	0.07559	0.00349
		Red	0.00024	0.07342	0.00325

Unlike in the original investigation, these results were not all in agreement The VB001 flowers with red hypanthia had a greater proportion of DHA, Tsugar and a greater DHA/Tsugar. This result agreed with the original study. Conversely VB015 and VB016 had a reduced amount of both DHA and Tsugar in flowers with red hypanthia. The DHA/Tsugar of the different flower types did not change

the high DHA/Tsugar classification of the tree. This could suggest that, in VB015 and VB016, the nectar production is being reduced. It could also indicate that nectar has been sampled by pollinators.

This study did not analyse the change in hypanthium colour in the male flowers as by the time the hermaphrodite results were achieved, the flowering period had passed. This will need to be addressed in a future study as the variation observed in the original study could be attributable to the male flowers.

As the red hypanthium colour signals a flower approaching senescence, the flower may be experiencing an age-related stress that could be altering the nectar composition. Unfortunately the source of DHA is still unknown and this does not explain the lack of change exhibited by the cultivar 510. This data could suggest that the two cultivars deal with stress differently (as expanded on in **Section 3.9.1**). Alternatively, the differing results may indicate that these cultivars are actually different species of *Leptospermum* as they do not share a common ancestor.[83]

Hence in order to fully know whether DHA production alters with age in all cultivars, this investigation needs to be repeated to include varieties of different genotypes. It should also include the identification of different flower stages of *L. scoparium* as this will help identify when nectar is being exuded and when DHA is being maximised in the flower cycle. Male and hermaphrodite flowers should be sampled and analysed separately and the time of sampling should be taken into consideration.

3.8 Cultivar 300 Clones

It still remains unclear whether genetics have a greater effect on DHA than the environment. From the aforementioned studies it appears that there is a genetic predisposition to DHA overproduction. This was further investigated with a pot study. Several cuttings were taken from a cultivar 300 sample (specimen 2 in **Figure 3.5**) and nurtured in pots. These samples are genetically identical and therefore if the environment does not have an effect on DHA production, it was expected that the DHA/Tsugar would be uniform across all the cuttings. The DHA/Tsugar values of the parent and the clones are illustrated in **Figure 3.15**.

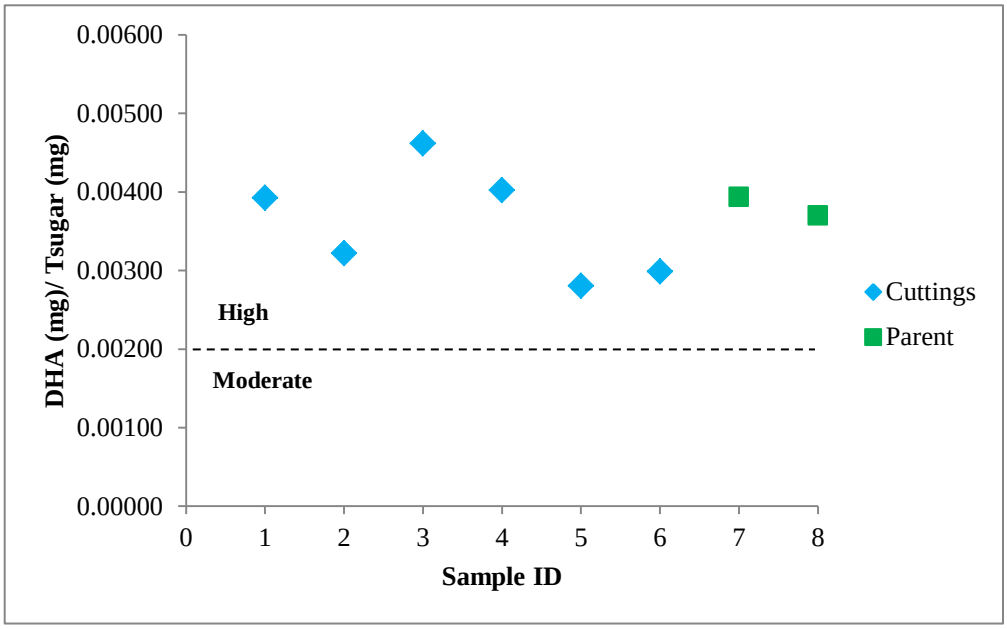


Figure 3.15: A comparison of the DHA/Tsugar of a parent specimen of cultivar 300 and it's genetically identical cuttings. The dotted lines represent the high DHA/Tsugar level (0.00200 mg/mg).

There was no significant difference between the parent samples (p=0.377). However, it can be said with 95% confidence that only cuttings 1 and 4 were the same as the parent samples (p=0.808 and p=0.455, respectively). If the DHA production was entirely genetically determined, it would be expected that the DHA/Tsugar value was the same in every sample. Although these samples were

thought to be under identical environmental conditions, the plant would be more sensitive to environmental fluctuations while grown in a pot. In order to ensure environmental conditions would be uniform for all plants, propagation should occur in a greenhouse. Despite the significant difference, all of the cuttings are still high DHA producers. This indicates that genetics are having a dominant effect but the environment modifies the DHA production slightly.

The environmental fluctuations occurring could affect the ratio of male to hermaphrodite flowers on the plant as illustrated by Primack and Lloyd (1980).[14] As there is no differentiation between these two flower types in the current methodology, some of the variation observed may be due to the proportion of male to hermaphrodite flowers included in the analysis. It is recommended that future sampling should only include hermaphrodite flowers which will give the minimum amount of DHA/Tsugar produced by the plant. While it is understood that bees do not differentiate between these flowers types, it would give a more representative indication of the variation that is occurring between plants.

3.9 Stresses

One possibility for the overproduction of DHA observed in mānuka is that it is produced as a compatible osmolyte. This is a low molecular weight organic molecule produced in times of stress; particularly when it is necessary to lower the water potential in the plant to ensure water uptake still occurs.[51] This was briefly tested in two ways.

3.9.1 Chemical Stress

The variation observed with the cuttings experiment suggests that DHA production is, to a certain extent, affected by the microenvironment of the plant. This theory was investigated by adding certain fertilisers and chemicals to cultivar

300 and 210 cuttings in pots; these additives are summarised in **Table 3.10**. When possible, only hermaphrodite flowers were sampled to limit the variation observed due to the difference in flower gender (see **Section 3.5**). This experiment was conducted off site by a horticulturist and consequently, flowers from multiple cuttings were pooled.

Table 3.10: Summary of the materials added to pots of multiple 210 and 300 cuttings.

Stress Number	Additive
1	Triabon*
2	Potassium chloride
3	Sodium chloride
4	Calcium carbonate
5	Magnesium carbonate
6	Asolon*
7	Potassium sulphate
* Triabon is a slow releasing balanced fertiliser and Asolon is a blend of nitrate compounds.	

The results of this investigation are summarised in **Table 3.11**. One-way ANOVA tests were utilised to determine the significance of these results (at a 95% confidence level).

Table 3.11: A summary of the nectar composition of the pooled 210 and 300 cuttings when under stress. The stressor number refers to **Table 3.10**.

Cultivar	Stressor #	DHA (mg)	Tsugar (mg)	DHA/Tsugar (mg/mg)	F/G Ratio
210	**Control**	**0.00114**	**0.56471**	**0.00209**	**1.89**
	1	0.00080	0.35528	0.00233	1.92
	2	0.00117	0.44841	0.00266	1.95
	3	0.00103	0.35537	0.00288	1.83
	4	0.00107	0.38385	0.00279	1.92
300	**Control**	**0.00152**	**0.40850**	**0.00359**	**1.68**
	2	0.00157	0.75966	0.00208	1.82
	3	0.00170	0.63681	0.00277	1.76
	4	0.00158	0.78480	0.00203	1.89
	5	0.00141	0.65616	0.00215	1.74
	6	0.00141	0.61808	0.00227	1.74
	7	0.00169	0.67797	0.00252	1.82

In all the stress tests, the DHA/Tsugar were significantly different from the control ($p<0.05$). Addition of a slow releasing fertiliser to the cuttings was carried out for the 210 cultivar. This was the only test to yield a significantly different absolute amount of DHA (mg) value ($p=0.003$). The addition of this fertiliser reduced the absolute amount of DHA.

In all the other stress tests, it was the Tsugar (mg) value that differed significantly; therefore resulting in the significantly different DHA/Tsugar values. The Tsugar values of the stressed 210 cultivar decreased whereas that of the 300 stressed cultivar increased. The reason for this is unknown. The F/G ratios for 210 were statistically indistinguishable whereas five out of six stress tests resulted in significant different F/G ratios for the 300 cuttings.

From the foregoing, it appears that there is a compositional change in the nectar when the plant is exposed to some form of chemical stress. The stress seems to affect the amount of sugar being secreted in the nectar. However, the change in response to stress seems dependent on the cultivar. The decrease in sugar exudation in the nectar would result in a lower water potential in the plant (as the sugars are retained) and therefore water uptake through the roots would still occur. The cultivar 300 plants seem to respond to the stress by reducing water exudation in the nectar thereby resulting in a more concentrated nectar.

The DHA content did not change in either cultivar as a result of these stresses except upon the addition of the slow releasing fertiliser. It appears that when the tree is experiencing favourable growth conditions, less DHA is produced. The Tsugar response to these tests was dependent on the cultivar possibly suggesting that these cultivars could be different species of *Leptospermum*.[83] In order to

determine whether every cultivar has a unique response to chemical stress, a more extensive study would be required.

3.9.2 Effect of Black Sooty Mould

It is also possible that the overproduction of DHA may be the result of a physiological process or a localised stress. A localised stress common to mānuka is the infestation of black sooty mould.

Black sooty mould is a fungus that feeds on the honeydew produced by the scale insects that infest mānuka.[6] This fungus is *Capnodium walteri* sacc.[84] It was originally thought that the resulting death of mānuka was due to a reduction in photosynthesis. Although not disproven, this theory has been replaced with the idea that the scale insect is removing essential nutrients from the mānuka plant, hence resulting in death.[84]

During sampling of the 2011 East Cape samples, the degree of sooty mould coverage was recorded. This coverage was used in this trial as an indicator of infestation by the scale insect. The scale ranged from 1 to 5 where 1 indicated no black mould and 5 represented a thick layer of the fungus. The relationship between the DHA/Tsugar production of the specimen and the degree of sooty mould was investigated. This is illustrated in **Figure 3.16**.

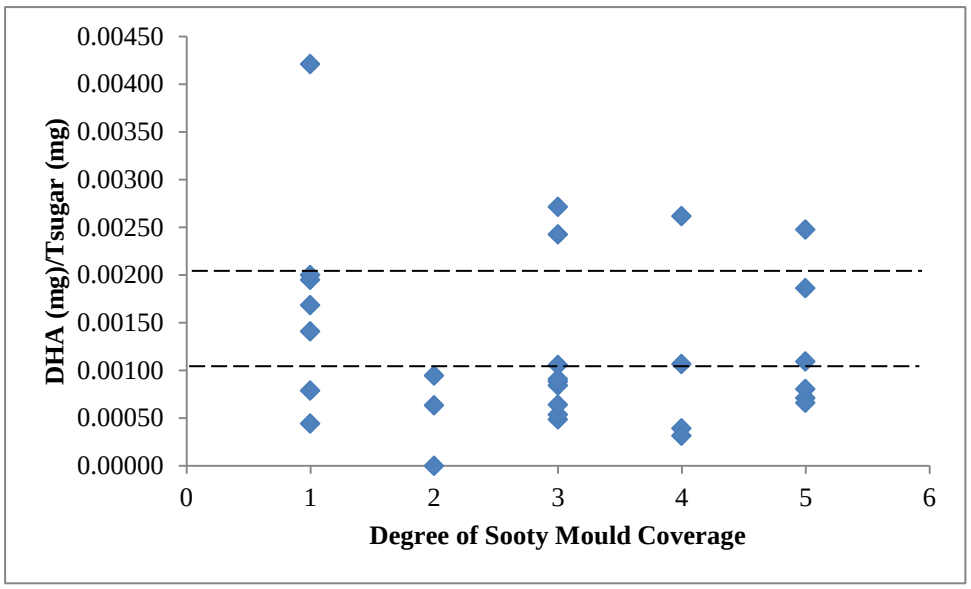


Figure 3.16: The relationship between the DHA/Tsugar production of 2011 specimen and the coverage of black sooty mould. The degree of coverage was ranked from 1 to 5 (no coverage to heavy infestation). The dotted lines represent the moderate and high DHA range (0.001 and 0.002 mg/mg, respectively).

It appears that there is no relationship between the DHA/Tsugar and the degree of coverage by the fungus. Additionally, there was also no relationship with the absolute amount of DHA (mg). Thus infestation by scale insect does not affect the mechanism that produces DHA.

3.10 Other *Leptospermum* Species

To establish whether DHA overproduction is a feature of the species or the genus a limited investigation of Australian *Leptospermum* species was undertaken. It is already known that unifloral honeys from *L. polygalfolium* have been shown to contain moderate to high amounts of MGO.[38] However the nectar composition of other *Leptospermum* species has not been investigated. An investigation was carried out on *L. liversidgei*, *L. juniperinum* and *L. laevigatum* collected from the NSW region of Australia. Unfortunately, Australian *L. scoparium* could not be analysed as this investigation took place at the University of the Sunshine Coast,

QLD which is outside the range of this plant. *L*. Merinda, a cultivar of Australian origin and grown in New Zealand, was also tested.

Six samples were analysed and their composition is summarised in **Table 3.12**.

Table 3.12: A summary of the DHA/Tsugar and F/G values for different Australian *Leptospermum* species.

Species	Sample #	DHA/Tsugar (mg/mg)	F/G Ratio
L. liversidgei	1	0.00273	1.88
	2	0.00084	2.05
L. juniperinum	1	NQ [a]	1.91
	2	0.00386	1.77
	3	0.00159	1.76
L. laevigatum	1	NQ [a]	1.61
L. 'Merinda'	1	0.00069	1.96
[a] NQ = non-quantifiable			

As with *L. scoparium*, there is substantial intraspecial DHA/Tsugar variation. A one-way ANOVA analysis was applied to members of the same species and members of different species. It showed with 95% confidence that the DHA/Tsugar of samples within the same species were significantly different as were the DHA/Tsugar of samples *L. liversidgei* and *L. juniperinum*. The significant difference between the *L. liversidgei* samples might be accounted for by difference in environment; the first sample is from Beerwah , QLD whereas the second was collected from the NSW region. A one-way ANOVA analysis showed with 95 % confidence that the F/G ratios of the different species are statistically indistinguishable. The F/G ratios of these Leptospermum species are consistent with what is observed for *L. scoparium*.

The DHA/Tsugar values of one *L. liversidgei* and one *L. juniperinum* sample can be deemed as high. This indicates that *L. scoparium* is not the only *Leptospermum*

species capable of producing honey with a high NPA value. All the observed values are consistent with the range of values observed in the East Cape region of New Zealand.

L. 'Merinda' is a cultivar of Australian parentage and is often inaccurately labelled as a *L. scoparium* variety. It is a hybrid cross between *L.* 'Pink Cascade and *L.* 'Aphrodite'; both of these cultivars were also assumed to be varieties of *L. scoparium*.[85] In actual fact, Pink Cascade is a cross between *L. polygalfolium* and *L. continentale* (a species closely related to *L. scoparium*) and Aphrodite was formed from *L. spectabile* seeds.[86-87] This low DHA producing sample was collected from a Hamilton, N.Z suburban garden. However, since some Australian species have tested with high levels of DHA, other garden cultivars of Australian origin should also be investigated.

4 Conclusion and Future Recommendations

This research investigated which features of *L. scoparium* might determine the variability of the DHA/Tsugar. These aspects included considerations of plant physiology and genetic inheritability. A conclusion of these results are summarised below, followed by recommendations on what should be further investigated.

4.1 Conclusion

This study investigated various factors that could be responsible for the variability of DHA/Tsugar that Williams (2012) observed.[4] Williams did not investigate the Northland region as a part of a regional survey and hence this was investigated to complete the survey of the North Island honey producing locations.[4] It was found that the majority of samples from this region produced low to moderate amounts of DHA further confirming that different ecotypes produce differing amounts of DHA. Another extension of Williams' study was the investigation of mānuka trees positioned in close proximity to each other.[4] This investigation confirmed Williams' preliminary observations; despite presumably genetic similarity, trees in close proximity have variable DHA/Tsugar.

It is possible that the amount of DHA in mānuka nectar is determined genetically. Different cultivars were investigated to determine the validity of this theory. It was found that certain cultivars have more DHA/Tsugar present in their nectar; in particular those with single flowers and coloured petals. This finding supports the theory that certain mānuka have more of a genetic predisposition toward DHA overproduction. However an investigation into cuttings from a cultivar showed that despite being genetically identical to the parent, the DHA/Tsugar of the cuttings could vary (whilst still remaining in the same range). This result does not

disprove this theory but does indicate that there are other factors involved in determining the amount of DHA in the nectar with respect to the Tsugar.

Another hypothesis of the presence of DHA in the nectar of *Leptospermum* species is that it is a compatible osmolyte; a low molecular weight organic molecule that is produced by the plant in times of stress. DHA could be responsible for lowering the water potential in the plant as to allow continual uptake of water. This theory was briefly tested by use of chemical additives. Interestingly, it was the Tsugar that varied in the plant and the direction of this variation was dependent on the cultivar type. A more specific stress to mānuka was also investigated; the infestation by scale insects as evinced by the coverage with black sooty mould but no relationship between the degree of infestation and the DHA/Tsugar was found.

Certain aspects of the flower physiology were investigated. As mānuka is known to be andromonoecious, the nectar composition of the male and hermaphrodite flowers was investigated. The male flowers had a greater amount of DHA/Tsugar; this was confirmed with an hourly nectar analysis. It was hypothesised that the DHA is being utilised in the hermaphrodite flower for seed production. However, the hourly variation study showed that it might be the variation of Tsugar that causes the difference in DHA/Tsugar observed between the male and hermaphrodite flowers. This study also showed that the DHA/Tsugar increased over the course of the day and that in future collections, time of sampling needs to be considered.

Flower age (as judged by the hypanthium colour) was also investigated. Again, the results were dependent on the cultivar type. Cultivar 300 had a greater amount of DHA/Tsugar in older hermaphrodite flowers whereas specimens of cultivar 210

showed no significant difference. As the cultivars do not share a common ancestor, it is not unreasonable for the nectar production to be different.

These results raise more numerous questions that still need to be addressed.

4.2 Recommendations for Future Research

Suggestions for future research have been made throughout the results chapter. They are expanded upon in Sections **4.2.1**, **4.2.2** and summarised in **4.2.3**.

4.2.1 Aspects relating to *L. scoparium* plant physiology

So far, the research conducted supports the theory that DHA overproduction is a result of a genetic predisposition. It has been assumed that there are different ecotypes of mānuka around New Zealand and there is evidence to suggest that certain varieties of mānuka produce more DHA than others.[4] In order to truly know whether this is the case, genetic testing is required. It is possible that there may be different species of *Leptospermum* in New Zealand that have been grouped as *L. scoparium*. This too would have to be confirmed by genetic testing. With genetic information on mānuka trees, it would be easier to make more sound conclusions about why DHA is overproduced to a greater extent in some trees.

A part of this study was the investigation into the inheritability of DHA in garden cultivars. Information on the family trees of the cultivars tested was primarily sourced from one source. In order to further investigate the inheritability, more information would need to be gathered on the parentage of these cultivars. A more thorough understanding of the genetics of the seed and pollen parent would be required.

The information gathered about these cultivars suggested that single flowered cultivars produce a greater amount of DHA than double flowered cultivars (some

of the latter did not produce any at all). However, this study did not take into account the flower stage and flower age. A study on *L. continentale* and *L. myrsinoides* samples showed that nectar is produced throughout both hermaphrodite and male flowers lifetime.[16] Despite the fact that these species are closely related to *L. scoparium*, particularly *L. continentale*, an independent investigation of mānuka flowers will be required. An investigation into the change of nectar composition at certain identified flower stages may show a point of where DHA production is increased. This would help answer questions regarding why DHA is produced. This should also be done across multiple varieties and cultivars to determine whether the trends observed occur across all members of the species.

In addition to mapping the DHA change as the flower ages, the Tsugar change should also be investigated. It would be interesting to test the Tsugar change in response to many different factors. It might be that DHA is continually produced after a certain point but it is the Tsugar that is responsible for the DHA/Tsugar variability.

An attempt was made to monitor how DHA changes with age by testing flowers with different coloured hypanthia. This investigation suggested the change in nectar composition is dependent on the cultivar. However, only hermaphrodite flowers were used in this study. It is highly recommended that an investigation into the change in nectar composition as the male flowers age should be done in the future. This investigation also did not take into account distinct flower stages, nor did it take into account the effect pollinators had on the flowers collected. As pollinators would probably have an effect on the nectar composition, this study should be repeated in a greenhouse, should include multiple cultivars and take into account different flower stages.

The hourly variation of DHA/Tsugar was investigated. This indicated that the DHA/Tsugar increases over the course of the day. In order to confirm this result, the study should be repeated using a greater number of replicates. This should be done on one tree over a longer period of time. Again, this should be done in a greenhouse to limit variability in the DHA/Tsugar result. It would also be interesting to determine how the DHA/Tsugar changes over the course of the flowering period.

According to O'Brien (1994), pollinators do not discriminate between male and hermaphrodite flowers.[16] It would be interesting to determine whether this is the case for *L. scoparium*. It would also be interesting to investigate a bee's behaviour toward flowers with different hypanthium colouring. If pollinators do show a preference to a certain flower type, the sampling method should be adjusted accordingly.

This study only investigated the DHA present in the flower's nectar. In order to understand where DHA originates and why it is produced, other parts of the plant should be investigated. Williams briefly investigated other regions of the plant and found traces of DHA in the leaves and twigs.[4] This should be expanded upon. As the nectaries are vascularised by the phloem, an investigation into the phloem and the xylem should be conducted to determine whether DHA or a possible DHA precursor is present.

One theory that accounts for the presence of DHA in mānuka nectar is that is acts as a compatible osmolyte. This was briefly investigated through a chemical stress study. While the cultivars did not show an increase of DHA, both cultivars 210 and 300 altered their sugar concentration differently in response to this stress. This should be further investigated with certain samples being exposed to

differing degrees of stress. The plants were left for a period of 2 weeks before the flowers were sampled. It would be interesting to monitor the long-term effect that the stress would have on the plant. Other stresses such as drought, reduced exposure to sunlight and ageing should also be thoroughly investigated.

A limited investigation of Australian *Leptospermum* species was undertaken to determine whether this was a feature shared across the genus. While some specimens showed a high amount of DHA, more species and a larger number of individuals should be investigated before any conclusions can be drawn. It would be interesting to investigate the DHA concentration of species that are closely related to *L. scoparium* (such as *L. continentale*) and compare it to that of species more distantly related.

4.2.2 Collection and Methodology

Currently, there is no discrimination between different flower types in the collection protocol. It was shown that male flowers have a greater proportion of DHA in their nectar with respect to hermaphrodite flowers. In order to get a minimum average DHA/Tsugar value for a sample, it is recommended that only hermaphrodite flowers are sampled. These are abundant on *L. scoparium* plants and are easier to remove from the plant. Secondly, flowers of a certain stage should only be picked. This will lower the variation seen for each sample. Lastly, it seems that the DHA/Tsugar does vary throughout the day and the time of sampling should be taken into consideration in order to limit the variability of the result.

It is recommended that flowers are removed from the tree by popping them off the base of their branch as opposed to scraping them between two fingers. While it does increase the collection time, it preserves the flower structure which would

minimise the release of extra-floral sugars. Currently, flowers are stored in air-tight bags and frozen as soon as possible. Williams (2012) recommended that an investigation be carried out to determine whether freezing is actually detrimental to the measurement of DHA/Tsugar.[4] This could not be investigated as a part of this project and therefore should be considered in future projects.

The current methodology involves the preparation of 10 replicates per sample. It is recommended that this number is reduced to five or six although it is recognised that this will increase the % RSD. This change will also decrease the overall run time on the GC and more samples could be processed. As this methodology could be used for commercial analysis, it is recommended that ten flowers per replicate are maintained. More flowers would require a longer collection time which could be inconvenient. Additionally, if the number of flowers per replicate needs to be reduced, the DCM extraction volume could be reduced to ensure a more concentrated solution is obtained. The current limitation to this project is the GC run time for the DHA analysis. It has a total run time of 23 minutes; DHA elutes at roughly 10 minutes. The run is extended to elute slower moving compounds. Hence it is recommended that the temperature program of this method is revisited to see if a shorter run time is possible. This would allow for more samples to be processed.

4.2.3 Summary

Plant Physiology	• Genetic Testing of *Leptospermum* cultivars and varieties. • Investigate nectar composition at distinct flower stages of different flower phenotypes. • Investigate how the nectar composition changes with age in male flowers. • Investigate the hourly variation of DHA/Tsugar more with more replicates and over a longer period of time. • Investigate pollinator behaviour towards flower of different gender and age. • Analyse other regions of the plant for presence of DHA • Investigate microbial communities in the flower for possible source of DHA. • Investigate different the effect of different stresses over a long period of time. • Further investigate other species of the *Leptospermum* genus.
Collection and Methodology	• Sample only hermaphrodite flowers at a particular flower stage. • Reduce the number of replicates per sample and improve the GC temperature program. • Continue appropriate experiments in a greenhouse to avoid interference from pollinators.

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6 Appendices

Appendix	Title
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6.7	Tsugar values for Cultivars with NQ DHA

6.1 Collection Information

Sampling of Mānuka Trees:

- 2 small bags of flowers should be collected
- A DNA specimen
- Take a Herbarium sample (do not do if tree has been previously sampled)
- Take photo for future reference
- Accurately fill out specimen information form

Flower samples

- Pick the flowers from the tree and place in bag. Scrape flowers off the branch between 2 fingers or preferably remove the flower by squeezing the base of the flower. Make sure to gently press all air out of the bags.
- Mark the bag with the tree code with vivid. Place inside an appropriately labelled larger bag.

DNA sample

- Collect 2-3 branch tips (with lots of fresh growth) the size of your index finger
- Place in coffee filter supplied and place this in the small Ziploc bag containing the blue granules of drying agent
- Press the bag flat to exclude air and seal immediately to keep the sample dry
- Mark the bag with the tree code and place inside a larger labelled bag.

Herbarium sample

- 3 branches of foliage about 20cm long
- Foliage should include leaves, flowers and capsules
- Place inside the larger bag and label appropriately.

Record of the Tree

- Specimen information form: Fill out accurately, satisfying as many sections as possible. Note any additional important information on the collection form.
- Photo: Take the photo prior to sampling and ensure the entire tree is in captured. Also take a picture of the flowers to identify the variety.

6.2 Specimen Collection Form

Location Information

1. Tag number:_____
2. GPS coordinates: S_____

   E_____
3. Elevation:_____
4. Location:_____
5. Time of collection: _____
6. Date:_____
7. Weather:

Pouring Down		Light Rain		50/50		Fine but overcast			Clear, Sunny
1	2	3	4	5	6	7	8	9	10

Tree Information

8. Tree Details

A	Single Mature Remnant Tree
B	Single Young Regenerating Tree
C	Mature Natural Stand
D	Young Regenerating Natural Sequence (e.g farm land going wild)
E	Mature Tree located within mixed Natural Bush Sequence (e.g forest)
F	Plantation Planting [Mature\Young\Mixed] (select one)
G	Garden planting [Mature\Young\Mixed] (select one)

9. Position in Stand

Inside Stand	Edge of Stand	Slightly Apart from Stand	Spread out Stand	Not in a Stand
1	2	3	4	5

10. Flower cycle:

Flower Buds only		50/50 Buds/Flowers		Fully in Flower			50/50 Flowers/Old Flower		Dead/Old Flowers
1	2	3	4	5	6	7	8	9	10

11. ~Height:_____

12. Flower colour

White	Strong Pink	Pale Pink	Mix Pink/White	Other(state)
A	B	C	D	E

13. Scent:

No Scent				Very Strong
1	2	3	4	5

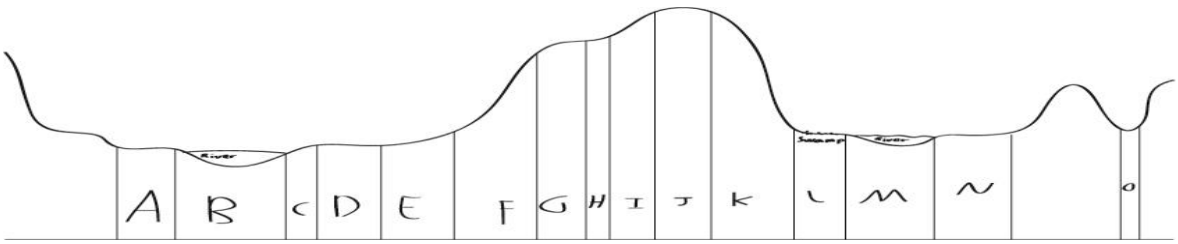
14. Black Fungus covered

none	little			Thick layer of fungus
1	2	3	4	5

Natural Geophysical Features

15. Land Feature

	Examples		
A	river terrace	I	saddle between two tops
B	in river	J	hill top largest point in immediate area
C	on river bank	K	very steep slope
D	plain like flat land	L	swamp like land
E	transit from flat to hill country	M	
F	side of ridge, hill, valley	N	floodplain next to river
G	top of a ridge line going towards a hill top	O	bottom of valley/gully
H	flat area on top of a ridge		



16. Direction Slope facing

N	NE	E	SE	S	SW	W	NW

17. Fresh Water Source :

Pond	Swamp	Creek	Stream	River	None
A	B	C	D	E	F

18. Distance to Coast:_____
19. Exposed to Wind: y / n

Manmade Influences

20. Land treatment

A	Trees growing on rich fully managed farmland
B	Trees growing on semi managed farmland
C	Trees growing on impoverished unmanaged farmland
D	Trees growing on land left to grow wild
E	Trees growing on managed residential land
F	Trees growing on unmanaged residential land
G	Trees growing as garden cultivars
H	None of the above

21. Stock camping under Trees:

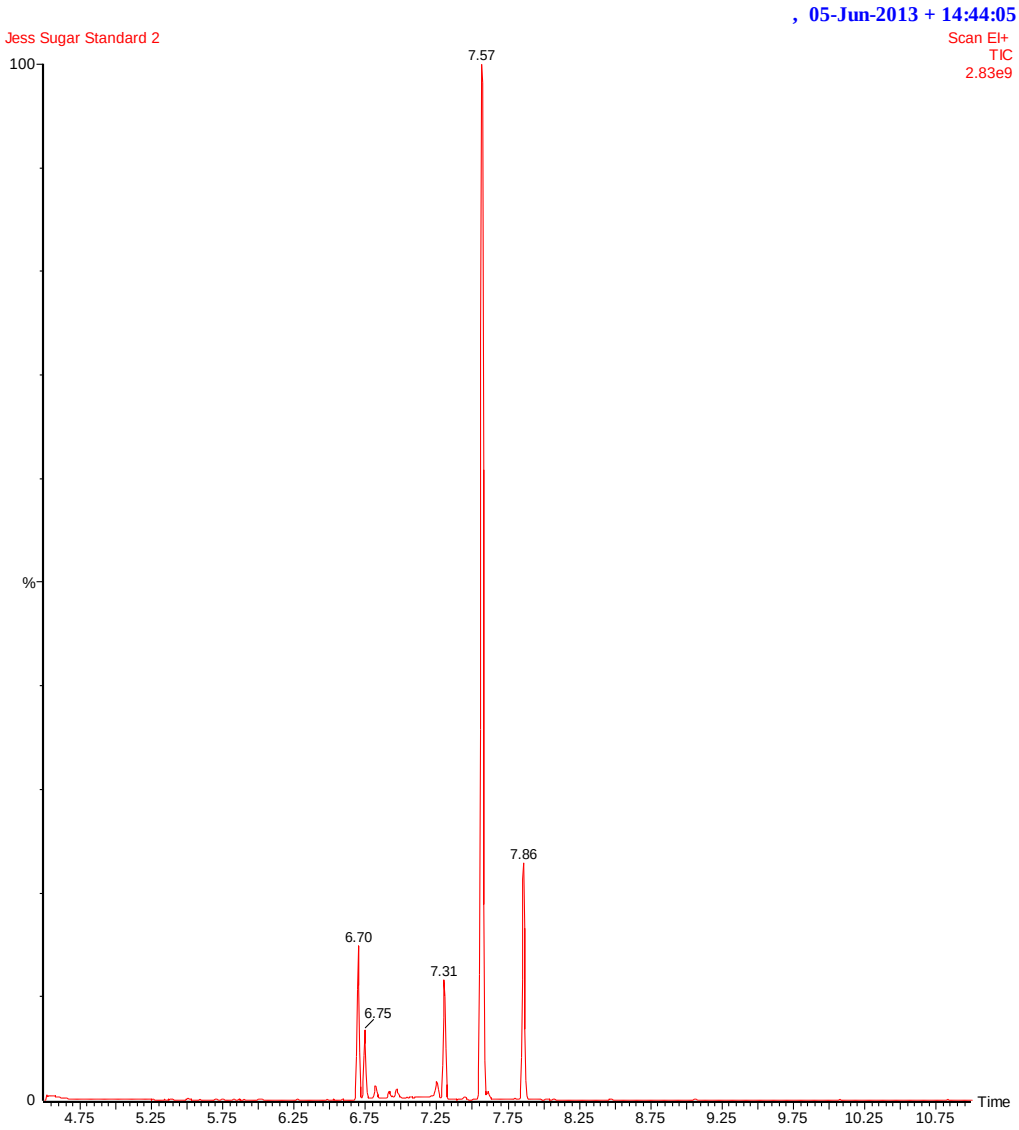
Cow	Sheep	Goat	All	None
A	B	C	D	E

22. Next to road(road defined as a public road): y / n

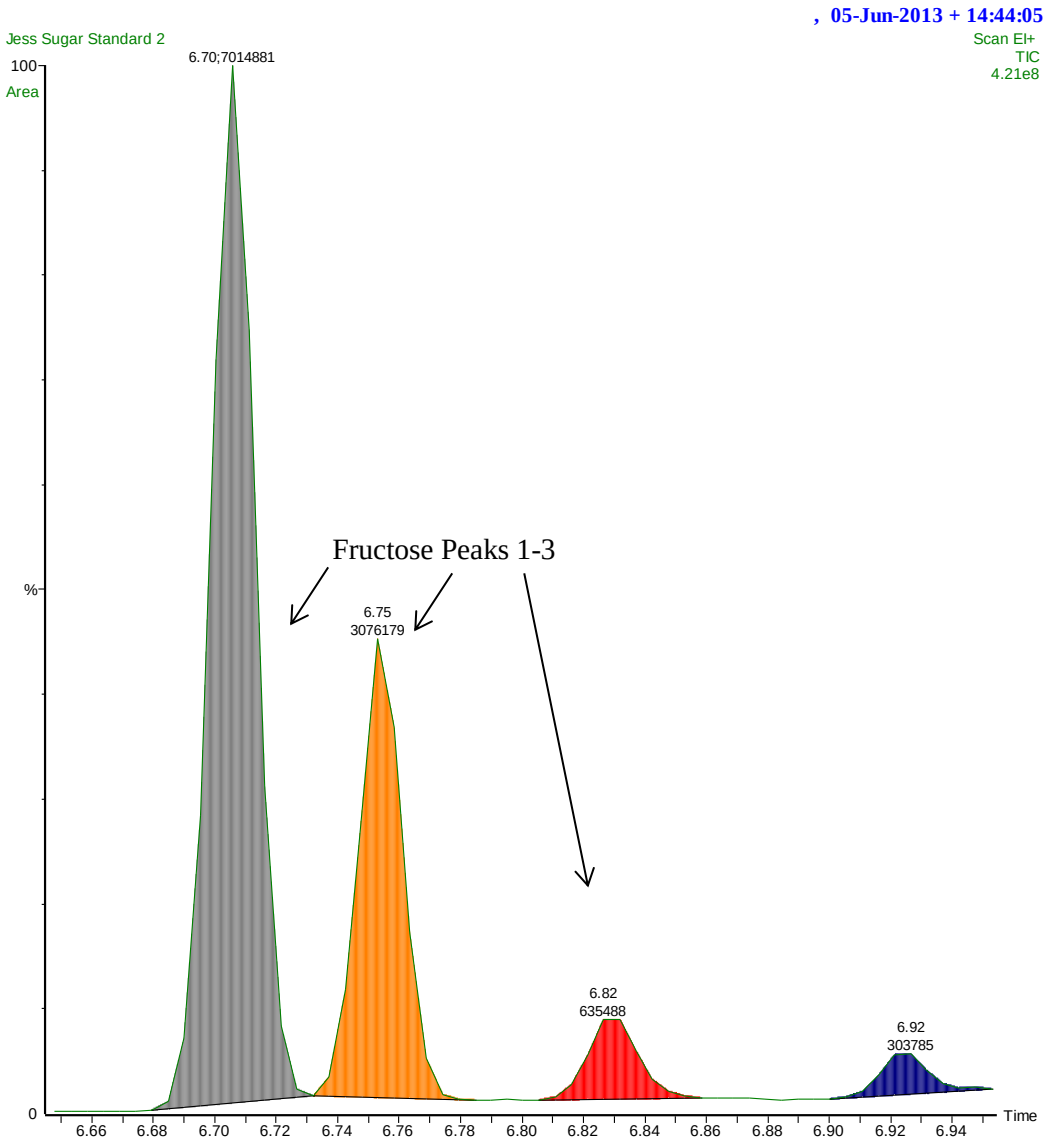
6.3 Example of Gas Chromatogram for *Leptospermum* spp.

These were obtained by GC-MS analysis at the University of the Sunshine Coast.

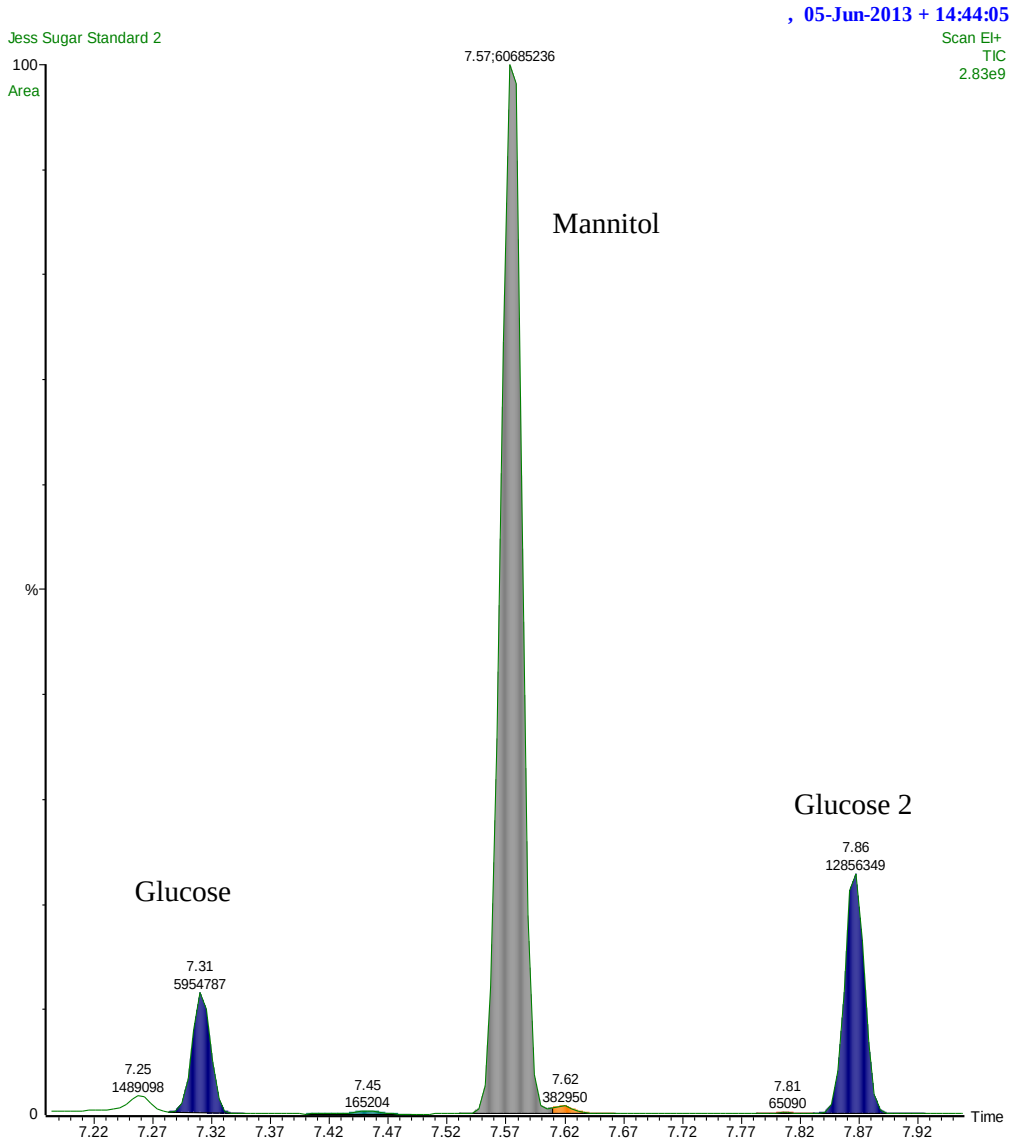
6.3.1 Full Sugar Chromatogram



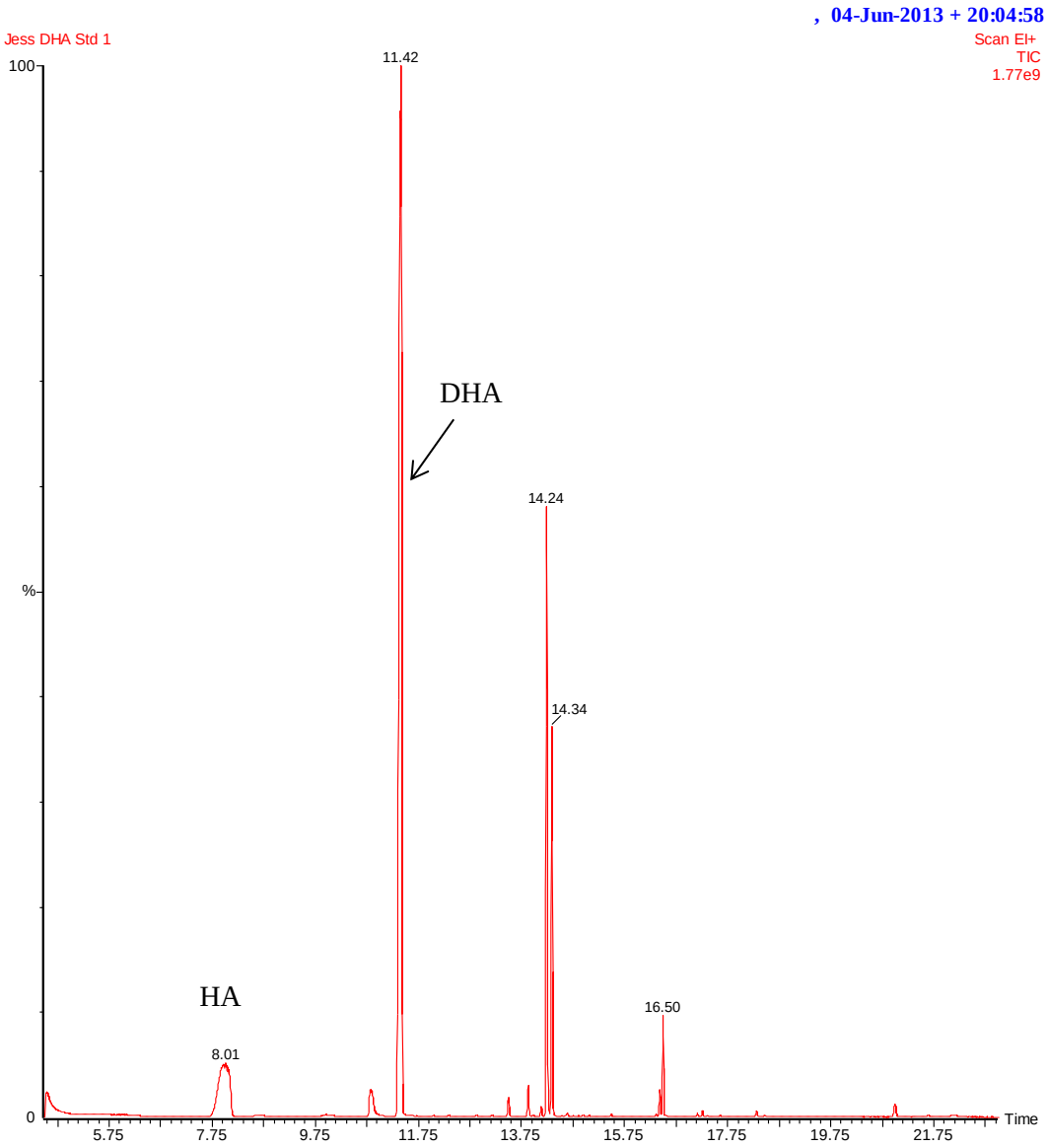
6.3.2 Expanded Fructose Region from Full Chromatogram



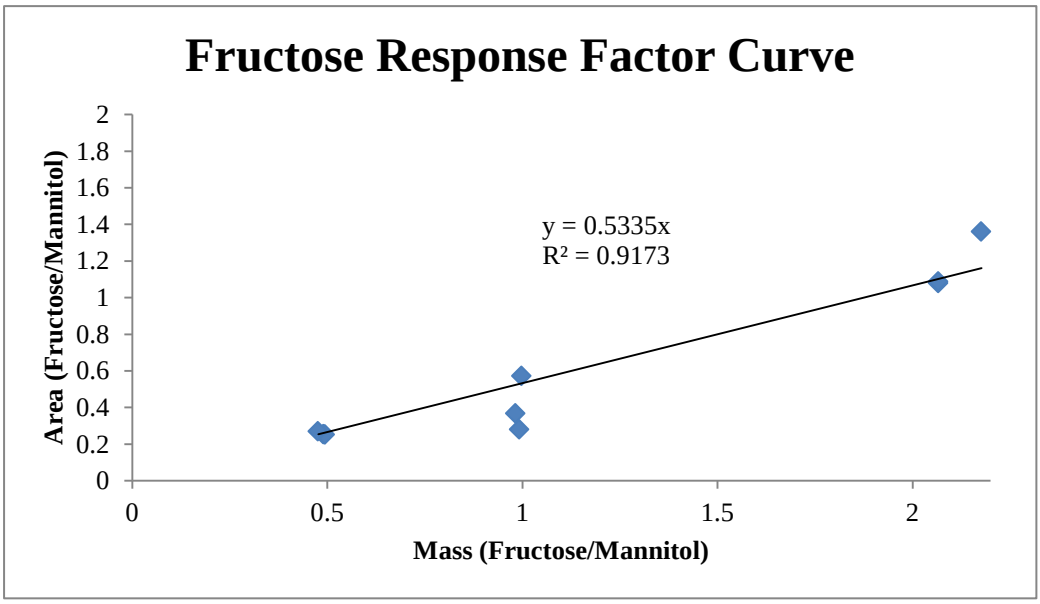
6.3.3 Expanded Glucose and Mannitol region from Full Chromatogram

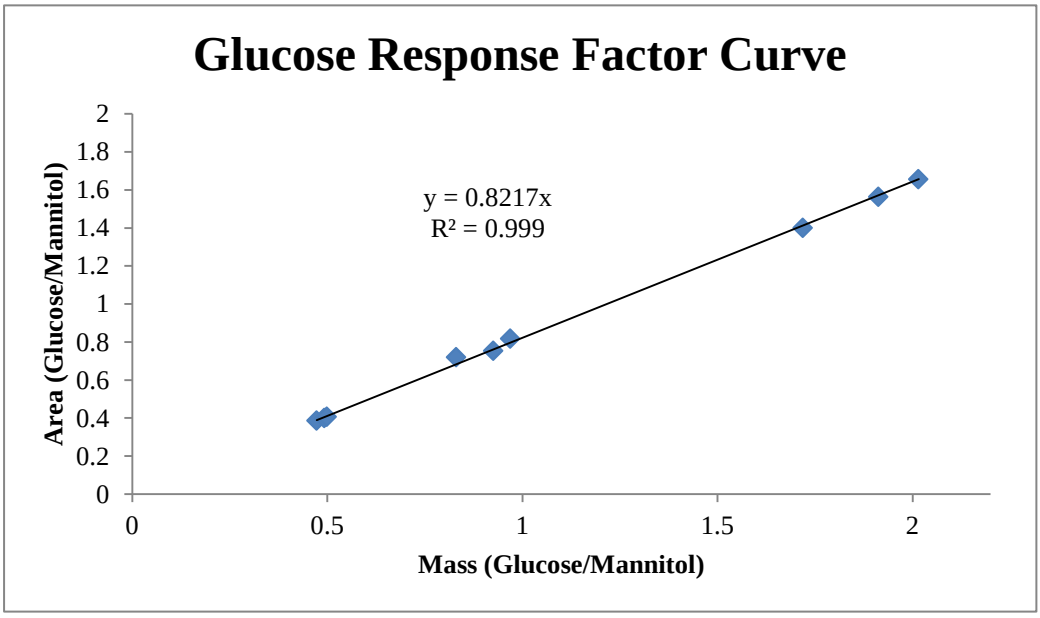


6.3.4 Full Gas Chromatogram of HA and DHA



6.4 Sugar Response Factor Curves

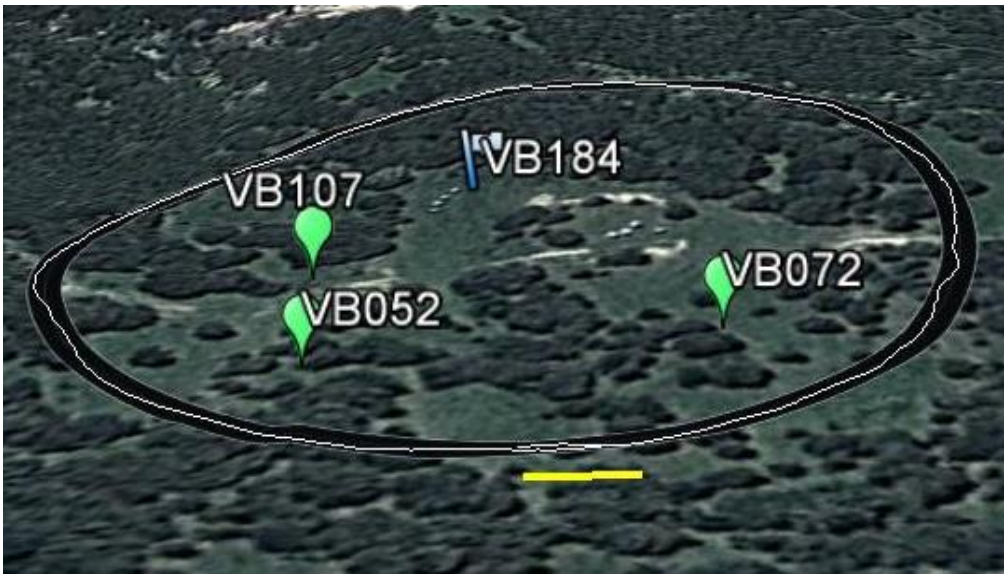




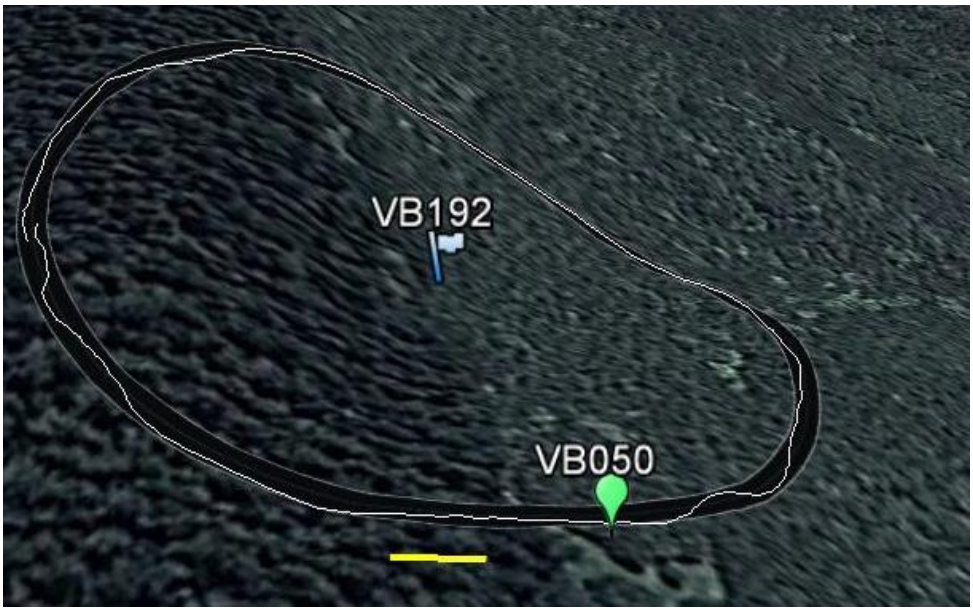
6.5 East Cape Sites

The yellow scale marker represents 10m. The black circle indicates the 50m radius in which trees were sampled. The original tree is shown by the flag icon whereas the 2011 trees are shown by a green icon.

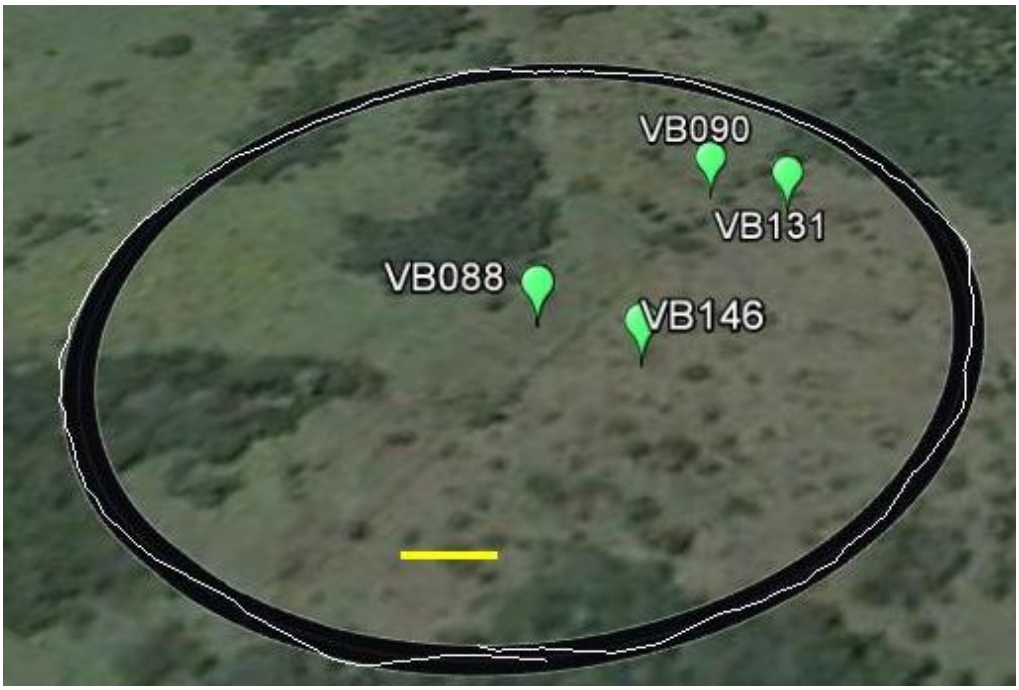
6.5.1 Site 1



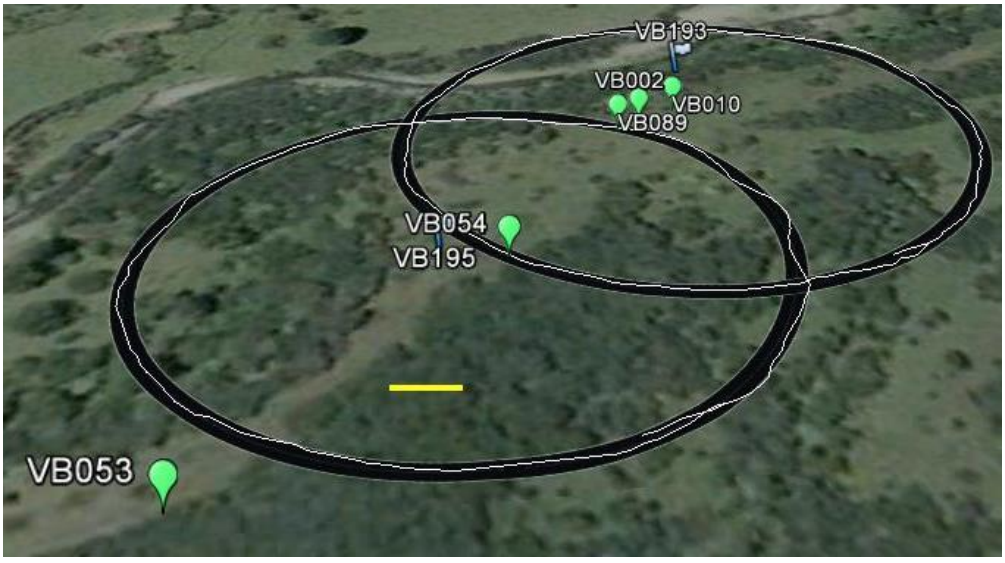
6.5.2 Site 2



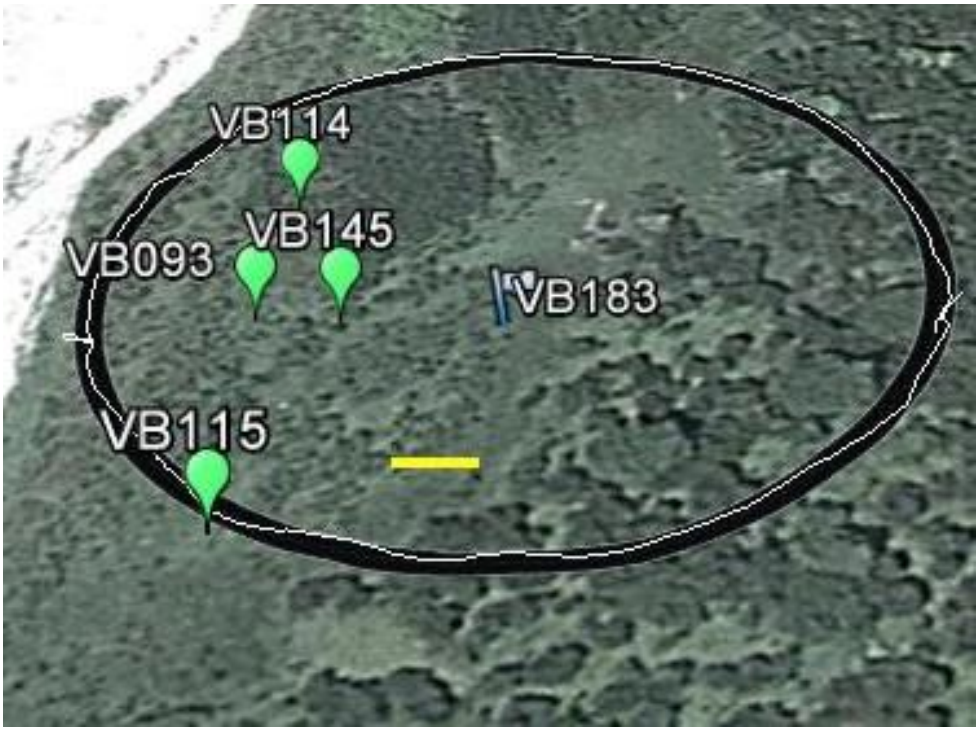
6.5.3 Site 3



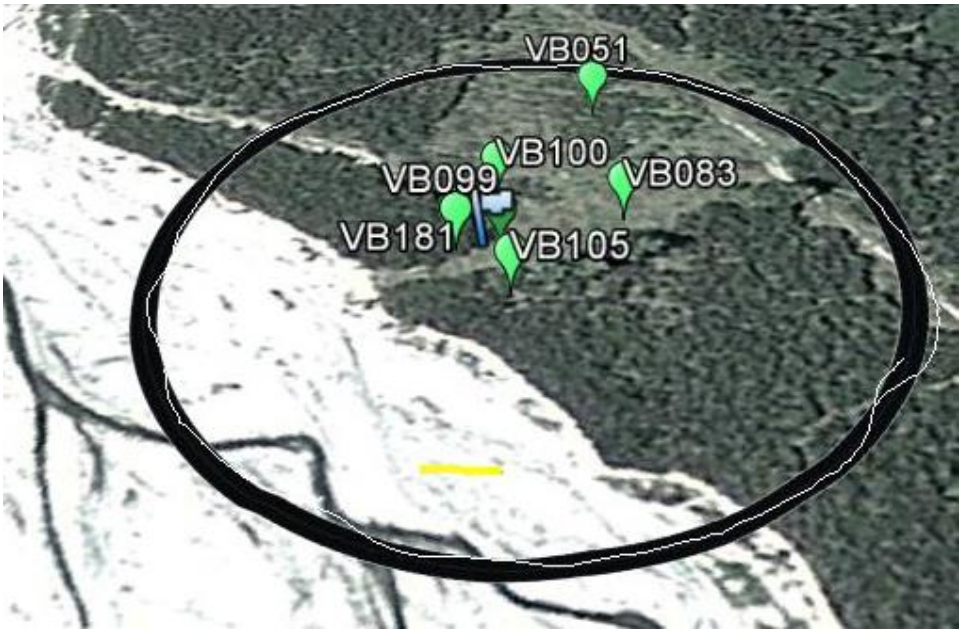
6.5.4 Site 4 and 5



6.5.5 Site 6

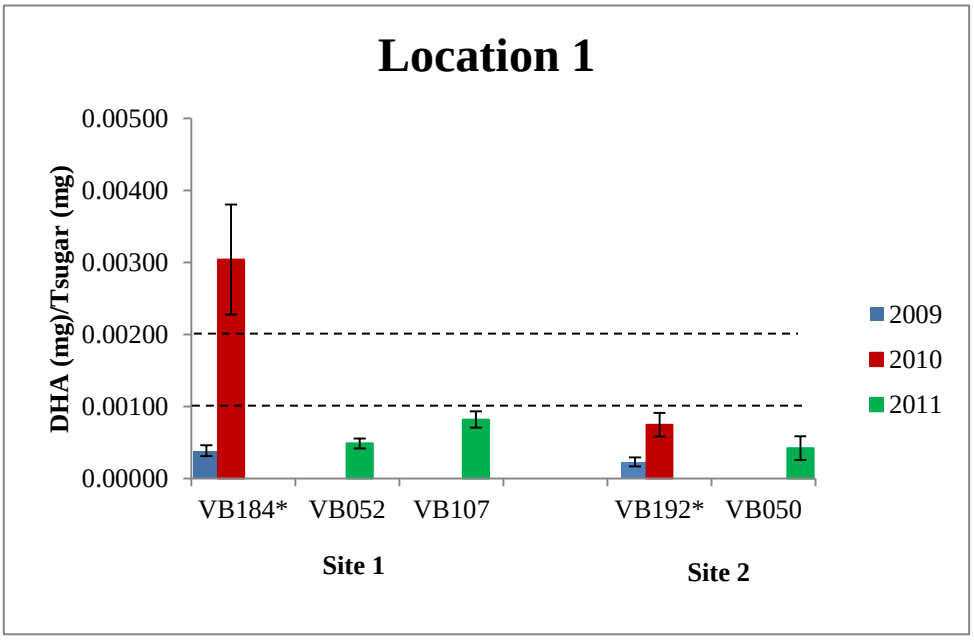


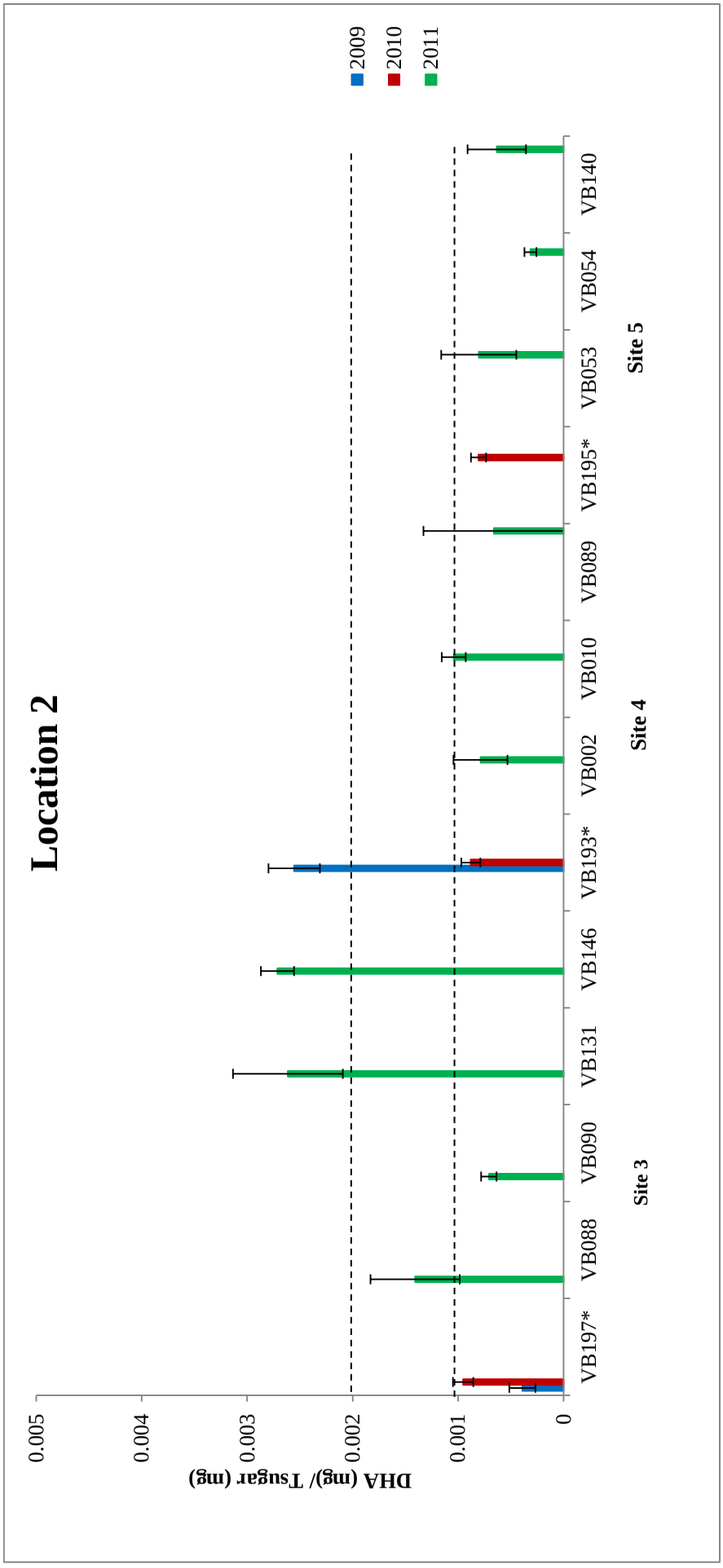
6.5.6 Site 7

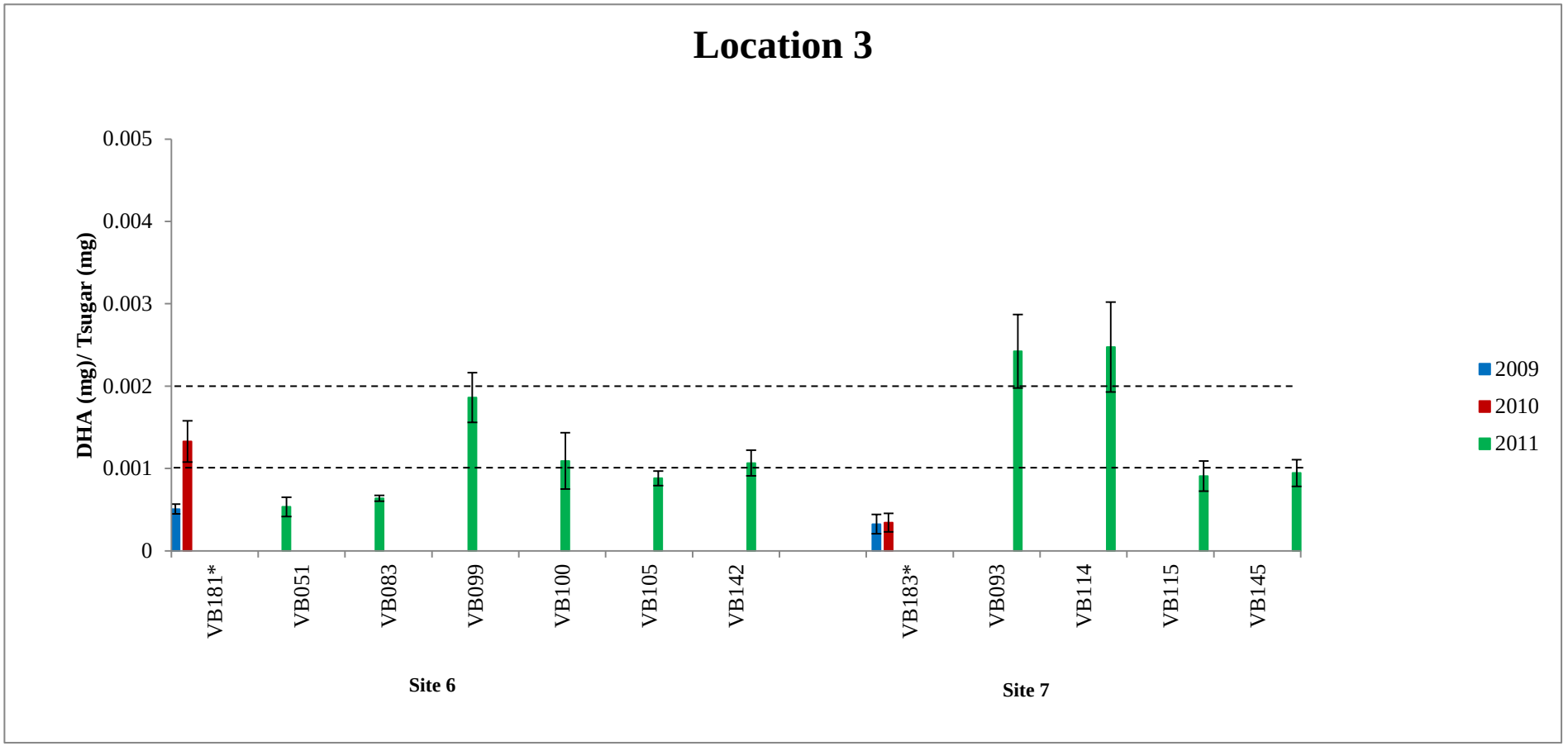


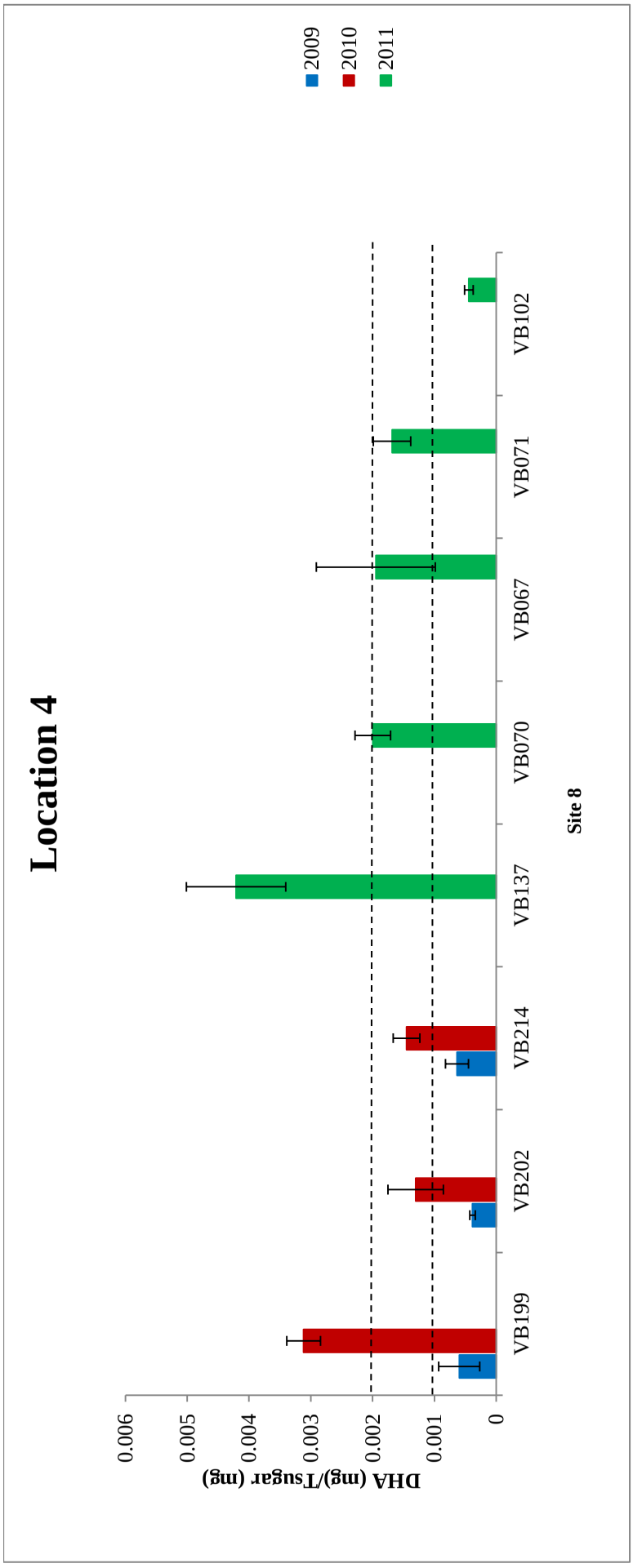
6.5.7 Site 8

6.6 East Cape Annual Variation




Location 2
2009
2010
2011
DHA (mg)/ TSugar (mg)
0.005
0.004
0.003
0.002
0.001
0
VB197*
VB088
VB090
VB131
VB146
VB193*
VB002
VB010
VB089
VB195*
VB053
VB054
VB140
Site 3
Site 4
Site 5


Location 3
DHA (mg)/ Tsugar (mg)
0.005
0.004
0.003
0.002
0.001
0
VB181*
VB051
VB083
VB099
VB100
VB105
VB142
VB183*
VB093
VB114
VB115
VB145
Site 6
Site 7
2009
2010
2011


Location 4
2009
2010
2011
DHA (mg)/Tsugar (mg)
0.006
0.005
0.004
0.003
0.002
0.001
0
VB199
VB202
VB214
VB137
VB070
VB067
VB071
VB102
Site 8

6.7 Tsugar values for Cultivars with NQ DHA

The absolute Tsugar values are defined as low (<0.200 mg), moderate (0.200-0.500 mg) and high (>0.500 mg).

	Cultivar Code	**Tsugar (mg)**	**Classification**
Original Study	425	0.40720	Moderate
	431	0.31715	Moderate
	432	0.25962	Moderate
ABG Study	230	0.10059	Low
	411	0.06360	Low
	412	0.07662	Low
	432	0.12722	Low