Supporting bioactive honey production:

South Australia

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Foreword

Bioactive honey is considered healthy and, as a result, demands a higher price. The most common bioactivity that consumers are aware of in honey is its natural antibacterial properties. And of this antibacterial activity, the performance of the substance methylglyoxal (MGO) that is derived from the non-enzymatic conversion dihydroxyacetone (DHA) when the honey is ripened from the nectar collected from *Leptospermum* plants, has attracted the greatest interest.

South Australia has many areas requiring land rehabilitation, and offering a plant solution that appeals, to and adds value to the honey bee industry has many benefits. Domestication of plants for mass planting requires careful testing to understand its opportunities and limitations as well as ensuring the provision of a secure seed source. This project achieved this and opened a new opportunity for South Australian land care groups and farmers alike Land manager integration with the honey bee industry is the way forward to open new needed apiary sites to the growing honey bee industry. This will also help meet the demand for crop pollination, particularly in the growing almond industry in South Australia.

Dr Liz Barbour
CEO

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About the Authors

Dr Kate Delaporte is a horticulturist and researcher at The University of Adelaide's School of Agriculture, Food and Wine. She is the Curator of the Waite Arboretum and Waite Conservation Reserve and extensively works on the commercial development of Australian flora. Kate has a passion for supporting Australian flora in commercial production and urban forest systems, whether it be for honey, food, biodiversity, or ecosystem services. Kate is the lead in all aspects of this project and Tate's principal supervisor.

Tate Hancox is a passionate plant scientist who loves researching, collecting, and growing plants from across Australia and the globe. When the opportunity to complete his Ph.D. on *Leptospermum* for bioactive honey in South Australia arose, he jumped at the chance to work with such an interesting genus. Upon completion of his Ph.D., he hopes to be able to gain employment at the Royal Botanic Gardens, Kew in England. Tate's Ph.D. covered Key Activity 1 and 3.

Nick Timbs brings expert technical skills to all horticultural and bee-related projects at the University of Adelaide's School of Agriculture, Food and Wine. His background in intensive production horticulture, practical problem-solving skills, and high work ethic ensure he provides admiral support for both Kate and Katja through this work. Nick managed Key Activity 2 and 4.

Dr Katja Hogendoorn is a senior researcher at the School of Agriculture, Food and Wine. Her research focuses on the conservation of native bees and the sustainable use of bees as crop pollinators. At a more general level, she is interested in ecology and the evolution of bee behaviour and diversity. Katja aims to use her understanding of behaviour to improve management decisions for the improvement of bee health, biodiversity, and crop pollination services. Katja provided collegiate support throughout this project and input was shared in Key Activity 2.

Acknowledgments

Kev Webb (General Manager, Spring Gully Foods) for his unwavering enthusiasm for all things manuka and ongoing support.

Phil Ashby, for being the man on the ground in Wirrabara and caring for the plants.

Professor Peter Brooks and his team, University of the Sunshine Coast, Queensland, for nectar analysis

The CRC for Honey Bee Products and its CEO Dr Liz Barbour, for supporting this research

Executive Summary

This project was developed to answer key questions raised through industry conversations regarding the development of *Leptospermum* plantations for bioactive honey production in South Australia. Issues identified included the need for species/plant selection and variety improvement, information on optimal growing environments and impacts of inputs such as irrigation on plant growth, flowering, and nectar production, and how to deploy superior lines once developed. The CRC Project was subsequently formulated as a Ph.D. research project to investigate genetic diversity, species/plant selection, and variety improvement processes, and a field-based observational project to monitor environmental effects on plant growth, flowering, and nectar production. The Project report has been split into two parts; Part 1: Plant development and Part 2: Environment and plantations.

Part 1: Plant development focussed on the key questions of "how much genetic diversity is needed to establish a breeding program using *Leptospermum scoparium* and *L. continentale?*", and "how will these two species propagate by cuttings?". This was achieved by collecting provenances from different parts of the geographical range of both species, where those ranges were of similar environments to where future bioactive honey plantations might be established, and comparing them to each other using Diversity Arrays Technology sequencing (DArTseq). It was identified that there was enough genetic diversity within both collections to support a future breeding and development program, whether it be to use controlled pollination to produce superior genotypes for selection or seed orchards for deployment, or a combination of both. Two robust methods of cutting propagation were developed, one for both species; different genotypes were more or less capable of producing roots and thriving through the transplanting phase. Data suggests that there is no difference in root development, plant survival, time to flowering, and nectar flow between seed and cutting grown plants, once planted out in irrigated plantations. The two key findings are that there is enough genetic diversity within small populations of *L. scoparium* (Victoria) and *L. continentale* (South Australia) to support a breeding and development program to improve germplasm for plantation establishment, and that elite plants can be readily cost-effectively propagated by cuttings, providing superior material directly to growers aiming to establish high yielding bioactive honey plantations.

Part 2: Environment and plantations investigated how two key species would perform under natural and plantation conditions. Natural rainfall and temperature were recorded and compared across 7 sites and over 4 years for *L. scoparium*, and 5 sites over 4 years for *L. polygalifolium* and *L. continentale*. Other species were tested to a lesser extent. Regardless of provenance or species, plants establish and survive better with supplementary irrigation, particularly in areas where natural rainfall is low. Plant growth measurements were taken yearly, and it was evident that young plants establish quickly, growing tall, then once they reach flowering, the vertical growth slows and the plants become bushier. This supports more flowers for honey production. Plants can be negatively impacted by not enough water and too much water at any stage of establishment and growth. If the water supply is limited during high temperatures, plants will show signs of stress and potentially die.

The key finding was that irrigation (both timing of and volume) seems essential for not only plant survival, but flowering and nectar flow, while soil type and temperature have a lesser impact, with the proviso that extreme temperature events will impact plants. Plants suffer from few pests and diseases and natural soil nutrition is adequate, and plantings can be 'over grazed' by large herbivores once well established.

Nectar from individual plants in all sites was collected and assessed each year for DHA: Tsugar concentration; a degree of variability in nectar DHA: Tsugar concentration is present between individual plants across years, regardless of species, genotype, or site. These data suggest that while genetics will play an important role in producing elite plants, there are other influences at play. Various ideas are proposed for these influences; is the local environment (temperature and soil moisture availability) influencing nectar production; and is the testing method most appropriate, do we need clarification around in-field methodology, including specifying the stage of flower development at nectar collection and is the as the of the sampling and analysis process robust and repeatable?

In 2020, a seed orchard of selected *L. scoparium* genotypes was established at Wirrabara, South Australia. This is the first dedicated seed orchard for *L. scoparium* in South Australia and is an important ongoing resource for a future breeding and development program and deployment strategy.

This project produced important answers to key questions to support the establishment of a bioactive honey industry in South Australia, using key species *L. scoparium* and *L. continentale*; further work is required to progress beyond the first seed orchard and small-scale plantations, to enable those interested in producing bioactive honey to produce high yielding plantations.

Figure 1: *Leptospermum scoparium* flowers with a Blue Flower Wasp; Adelaide Hills, South Australia. T Hancox.

Leptospermum selection for bioactive honey in South Australia

Introduction

In a world where antibiotic-resistant bacteria are becoming more prevalent, alternate antibacterial sources must be investigated; one important source is bioactive honey. The most famous bioactive honey is Manuka honey, which is produced in Australia and New Zealand from *Leptospermum scoparium;* however, Australia also produces honey from other *Leptospermum* species, including *L. polygalifolium,* that have the same bioactive ingredient. Currently, the South Australian honey industry is investing in ways to improve the quality of bioactive honey produced to benefit the primary producer as well as the consumer.

Bioactive honey produced from *L. scoparium* and *L. polygalifolium* shares three common modes of antimicrobial activity (acidic, supersaturated solution of sugar and produces hydrogen peroxide upon dilution) with common honey as well as containing the fourth mode of antibacterial action. This antibacterial action is derived from a group of bioactive compounds, most importantly methylglyoxal(MGO), and is referred to as non-peroxide activity. MGO is produced from dihydroxyacetone (DHA) during honey maturation. DHA is found in the nectar of *L. scoparium* as well as other *Leptospermum* species.

In July 2015, a project to research the potential development of and make recommendations regarding the implementation of an industry based on honey from plants of the *Leptospermum* (Manuka) family in the Wirrabara and Bundaleer forests in the Jamestown regions commenced. The 2015-2016 PIRSA Project identified key questions regarding the establishment of bioactive honey plantations in SA; subsequently, these questions guided the thought processes behind both the PIRSA/SGF Mid North Forest Project and the CRC for Honey Bee Products Projects that followed between 2017 and 2022.

It became clear that understanding the influences of genetics and heritability, environment, climate and weather, and soil moisture on the production of DHA: Tsugar in *Leptospermum,* alongside the management of European honey bees in *Leptospermum* for honey production, is critical for maximising improvement programs for large scale bioactive honey plantations. Thus, our CRC Project, "*Leptospermum* selection to support a bioactive honey industry in South Australia" set out to investigate the practicalities of developing seed orchards for producing improved seed, by investigating the genetic diversity of key species; how different genotypes respond to clonal propagation; by recording how plants from these genotypes/species perform in different climates and document how DHA concentration in nectar is influenced by those climates. This information can feed into the future deployment program to ensure economical plantation establishment of genotypes that are best adapted to local climates, and that will produce adequate yields of DHA to support the production of bioactive honey.

Through these subsequent projects we sought to address the questions around genetic diversity, selection and propagation, in addition to the biggest environmental question "can *L. scoparium* produce harvestable nectar in an intensive planation situation, under South Australia's natural rainfall regime?".

Ph.D. Student Tate Hancox prepared a review of available literature in 2018 on the commencement of his Ph.D. research. This has been revised as of 29th of March 2022 and provides a review on mechanisms behind *Leptospermum scoparium* bioactive honey, *Leptospermum* in Australia and South Australia, previous work in *Leptospermum* genetics as well as propagation of *Leptospermum* using clonal methods as well as seed.

The genus *Leptospermum* is very diverse and has a wide distribution across Australia, and New Zealand as well as extending into southeast Asia (Thompson 1989). Previous attempts to document the genetic variation and

diversity within and between *Leptospermum* genera have focused on differences between phenotypes expressed when grown in the same environment, as a substitute for genetic diversity (Cook, Mark & Shore 1980; Greer, Muir & Harris 1991; Harris 2002; Primack 1980; Ronghua, Mark & Wilson 1984). *Leptospermum* species have been shown to exhibit genotypic variation with DHA in the nectar (Williams et al. 2014; Williams et al. 2018), essential oil composition (Brophy et al. 1998, 1999a, 1999b), flowering characteristics (Dawson, MI 1990; Dawson, Murray 2009; Primack & Lloyd 1980), seed shed (Harris 2002), leaf variation (Harris 2002; Ronghua, Mark & Wilson 1984), frost hardiness (Greer, Muir & Harris 1991), root anatomy (Cook, Mark & Shore 1980). *Leptospermum* also exhibits phenotypic plasticity (Burrell 1965; Lee, Mark & Wilson 1983; Price & Morgan 2006).

Previous molecular investigations into genetic diversity have been limited but recently more focus has been placed on this area. Research has utilised various molecular techniques to sequence the matK gene and the atpβ–rbcL intergenic spacer (Lam et al. 2002; O'Brien, Quinn & Wilson 2000; Wilson et al. 2001; Wilson et al. 2005) as well as chloroplast and nuclear ribosomal DNA (Binks et al. 2021). This has been useful to show that *Leptospermum* is a polyphyletic genus (Binks et al. 2021; O'Brien, Quinn & Wilson 2000; Wilson et al. 2001) suggesting that it comprises multiple genera that show convergent evolution. However, eastern Australian *Leptospermum* species have yet to have genetic diversity analysis completed.

Advances can be made quickly through the selection of desirable genotypes from the wild or cultivation. To achieve this, selections must be clonally propagated, which involves collecting, transporting, preparing, and planting material. Cuttings may be treated with rooting hormone and/or kept on heat mats to encourage root formation as well as placed into a mist propagation tent to maintain humidity and prevent desiccation (Cox 2018). Cultivation of the genus taking place since 1772 and many cultivars of *Leptospermum* are currently propagated from cuttings (Dawson, Murray 2009); however, published clonal propagation methods on *L. scoparium* and *L. continentale* are limited. Most methods for cutting propagation of *Leptospermum* are limited to comments or brief mentions (Seelye et al. 2001; Woodgyer 1995). Successful protocols have been developed for cutting propagation of *Kunzea pomifera*, a close relative (Page 2004). These methods were found to be similar to the anecdotal methods utilised at the Botanic Gardens of South Australia (Coulter 2019). More recently, Darby et al. (2021) developed a basic methodology but suggested further work was required to refine and improve the methodology. It was unknown how *L. scoparium* and *L. continentale* would be influenced by genotype, auxin application, and length of time in the mist propagation tent.

Objective

Develop a bioactive *Leptospermum* plantation program for South Australia

Key activities

1. Determine the genomic diversity of selected Leptospermum species within and between provenances and endemic locations for South Australia for breeding and seed orchard deployment

2. Investigate environmental influences on plant growth, flower development, flowering, nectar production, and DHA concentration. Characterise Leptospermum plant performance, soil structure, and soil moisture for site selection

3. Investigate deployment methods (seedling and cuttings) for the cost-effective establishment of superior selected Leptospermum material.

4. Establish a seed orchard to support a breeding program for high DHA/sugar levels, nectar production, and plants suited to the SA environment

Impact

Bioactive *Leptospermum* plantations are established in SA for a growing medicinal honey market.

Industry outputs

Identification of the parameters for bioactive *Leptospermum* establishment in South Australia

A *Leptospermum* seed orchard, acclimatised for South Australian conditions, was established to provide a reliable seed supply

Vegetative propagation methods were developed for rapid deployment of *Leptospermum* selections

Academic Outputs

Hancox, T, 2022, *Leptospermum* for bioactive honey production in South Australia: selection, genetic diversity, and propagation. Ph.D. thesis, University of Adelaide.

Hancox, TJJ, Binks R, Burton R, & Delaporte, K 2022, Genetic diversity and differentiation of *Leptospermum scoparium* in the Grampians, Victoria and *L. continentale* in South Australia. *In preparation*.

Hancox, TJJ, Burton R, & Delaporte, K 2022, Development of a robust method for propagation of *Leptospermum scoparium* J.R.Forst. & G.Forst. from semi-hardwood cuttings. *In preparation.*

Hancox, TJJ, Burton R, & Delaporte, K 2022, Development of a robust clonal propagation and transplant survival methods for Leptospermum continentale Joy Thomps. from semi-hardwood cuttings. *In preparation*.

Sites and Plant material

The Project covered a range of sites in South Australia, for both collection of material for KA1: Genomic and KA3: Propagation, and as field sites for KA2: Environment and KA4: Seed Orchard.

Material for KA1: Genomic and KA3: Propagation were soured from natural populations of *Leptospermum scoparium*, located in Western Victoria's "Southern Grampians/ Gariwerd" region and the coast of South Western Victoria. Three subsets were collected from commercially available seed sources. The material of *L. continentale* was collected from natural populations in South Australia. Table 1. lists the locations, provenances/regions, and the number of individuals used for each species.

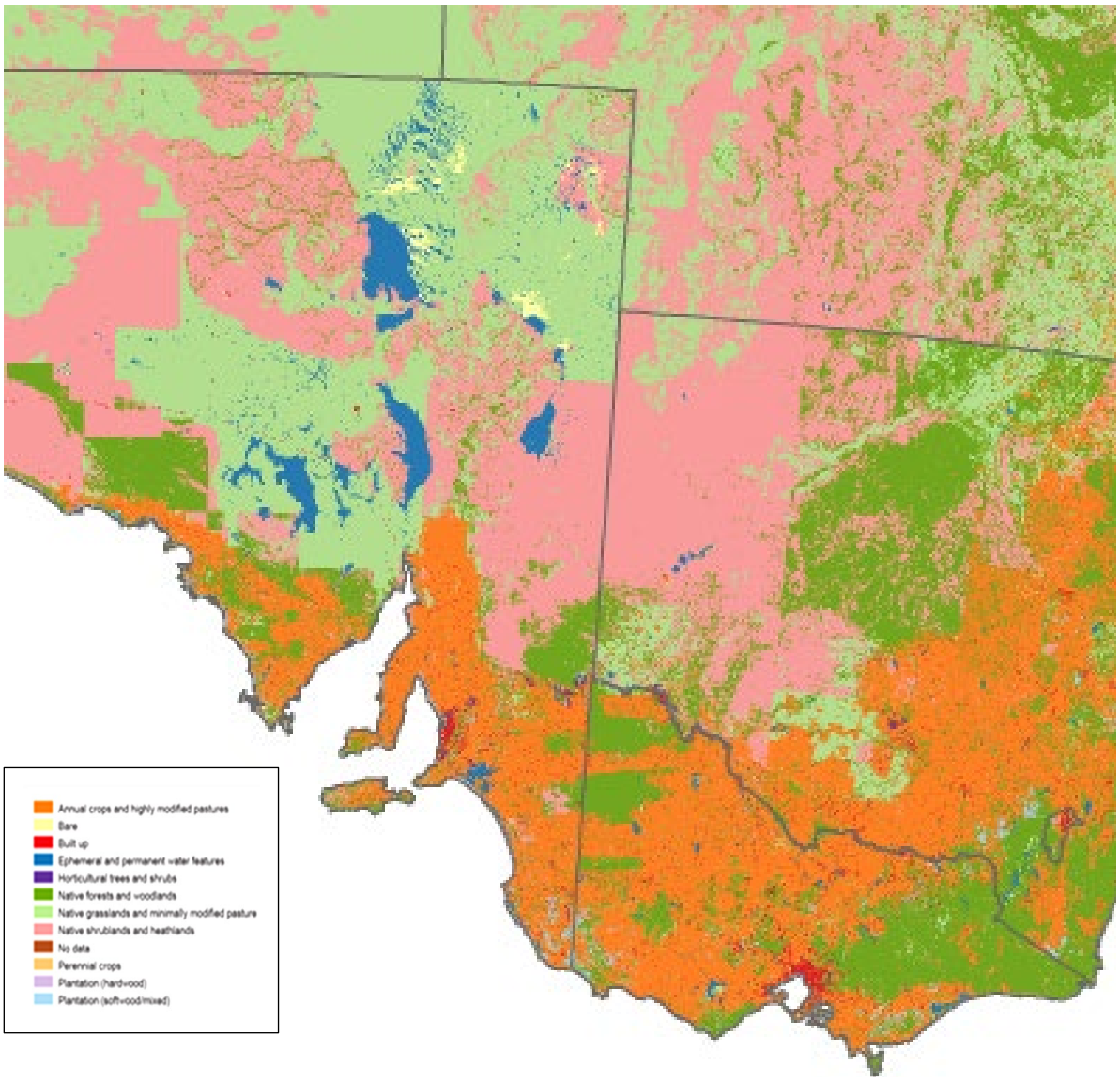

Figure 2: Detail of South Australia and Victoria covering the area covered by this project. Source: Australian Bureau of Agricultural and Resource Economics and Sciences, Integrated Vegetation Cover dataset 2009, used under CC BY 2.5

The primary genetic material for KA2: Environment and KA4: Seed Orchard, was sourced from natural populations of *Leptospermum scoparium*, located in Western Victoria's "Southern Grampians/ Gariwerd" region. Over 150 individual plants from 5 individual locations were sampled (Delaporte, 2017). The 20 genotypes with the highest DHA: Tsugar ratio (recorded in January 2016) were selected and propagated by cuttings. Subsequently, multiple clones of 13 genotypes have been planted at Wirrabara and Waite and used throughout the study (Vic – selected). Additional seed-derived genotypes originating from the Grampians populations have also been used (Vic – commercial 1 & 2) at Waite and Wirrabara. Other *L. scoparium* trial sites contained material derived from commercial sources, which identify the seed as originating from Tasmania (Tas – commercial 1 and 2) or New Zealand (NZ – commercial). The genotypes of *L. continentale* and *L. lanigerum* were sourced from natural populations in South Australia, either directly from plants or as seeds collected from natural populations and grown.

Table 1: Genetic diversity population study list of two key species of interest collected from South Australia and Victoria by region (# families)

South Australia	Victoria
L. scoparium	
Southern Grampians Collection Carlsens Road (18) Dunkeld North (20) McCutcheon's Road (7) Mallah Park (20) Victoria Valley A (20) Victoria Valley B (18)	*Coastal Victoria collection* Bay of Islands (20) Port Danger (20) RP (ex-Tasmania) (7) SS (ex-Tasmania) (7) Ex- New Zealand (cultivated selection) (1)
Initial analysis included all populations of *L. scoparium* listed. However, the results of the initial analysis suggested the two coastal Victorian populations were misidentified. These were removed from further analysis and future plant improvement programs until they can be correctly identified. The Tasmanian sourced plants were also removed from further analysis (in Tate's thesis) as they were drought sensitive even though their analysis was completed	
L. continentale	
Kangaroo Island (18) Kersbrook (19) Mylor (18) South East near Robe (18) South East near Mt Gambier (19)	
Populations of *L. continentale* were selected to represent the range of the species in South Australia. This collection was to obtain individuals from across the different environments to provide an understanding for further plant selection based on DHA nectar sampling. It also gives an estimate of the diversity within the species which is important for designing a future selection program.	

KA2: Environment focussed around seven field sites located throughout South Australia in areas where it was considered feasible to grow *Leptospermum* for honey production (Figure 1). Six of these sites were visited yearly over the 4.5 years of the CRCHBP (2018 to 2021) and data were recorded on plant growth and flowering, and nectar was collected each flowering for DHA analysis. The Wirrabara site was visited quarterly. Sites were located at Wirrabara (*L. scoparium*), Port Vincent (*L. polygalifolium*), Bridgewater (*L. scoparium* and *L. polygalifolium*), Mylor (*L. continentale* and *L. lanigerum*), Charleston (*L. continentale*), Macclesfield (*L. scoparium*) and Waite Campus (refer Table 1). We collated weather data (rainfall and maximum/minimum temperatures) from the closest Bureau of Meteorology station to each site for comparison.

KA4: Seed Orchard is planted at the Wirrabara field site in partnership with Spring Gully Foods and uses 12 genotypes from the "Southern Grampians/Gariwerd" collection and three from Vic – commercial 1 source where plants were grown at Waite and selected for growth performance and clonally propagated for the seed orchard. In addition, where possible, clonal plants from all collections are established at the Waite campus, Urrbrae for ongoing research, breeding, and development.

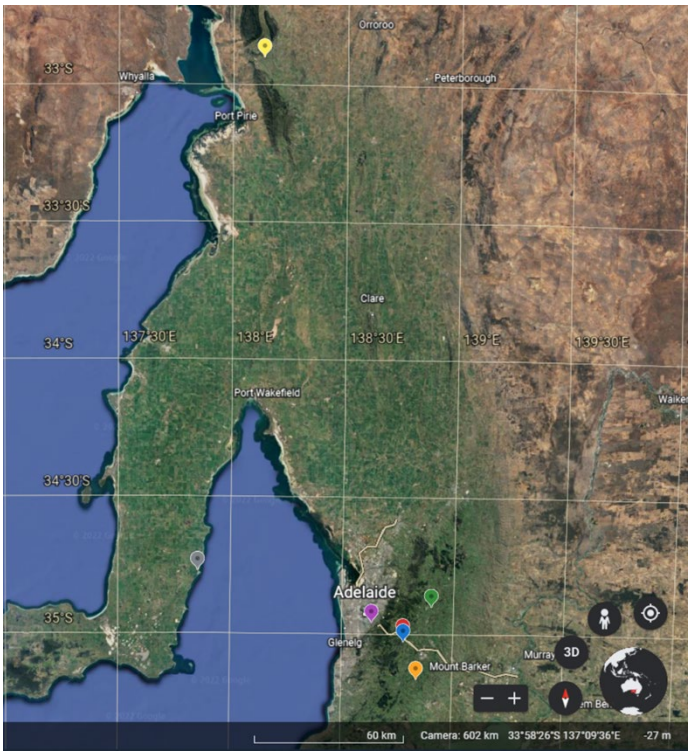

Figure 3: Seven locations in South Australia were selected as field sites for observations on plant growth and flowering, environmental impacts, and nectar DHA: Tsugar assessments over 2018-2022. Yellow = Wirrabara Forest Trial site, Grey = Port Vincent Trial Site, Green = Charleston Field Site, Purple = Waite Field Site, Red =Bridgewater Field Site, Blue = Mylor Field Site, and Orange = Macclesfield Field Site.

Table 2: South Australian field sites growing different species of Leptospermum under different conditions, visited yearly between 2018 and 2022; weather data, plant growth data, and nectar collected.

SA Field Site	Plant species	Source	Total # plants	general health	flowering	height & width	nectar tested
Port Vincent	*L. polygalifolium*	Vic - commercial 1	15	Y	Y	Y	12
Bridgewater	*L. polygalifolium*	Vic - commercial 1	10	Y	Y	Y	7
Waite	*L. polygalifolium*	State Flora	3*	Y	Y	Y	2
Bridgewater	*L. scoparium*	NZ - commercial	20	Y	Y	Y	10
Bridgewater	*L. scoparium*	Vic - commercial 1	10	Y	Y	Y	10
Wirrabara	*L. scoparium*	Vic - selected (13 gen)	209	Y	Y	Y	12
Wirrabara	*L. scoparium*	Vic - commercial 1	50	Y	Y	Y	3
Macclesfield	*L. scoparium*	Tas - commercial 2	19	Y	Y	Y	9
Charleston	*L. scoparium*	Tas - commercial 1 & 2	14	Y	Y	Y	4
Waite	*L. scoparium*	Vic - commercial 2	69*	Y	Y	Y	11
Waite	*L. scoparium*	Tas - commercial 1					3
Waite	*L. scoparium*	Tas - commercial 2					4
Waite	*L. scoparium*	Vic - selected (11 gen)					17
Mylor	*L. continentale*	indigenous	20	Y	Y	Y	18
Charleston	*L. continentale*	indigenous	5**	Y	Y	Y	2
Waite	*L. continentale*	indigenous	3**	Y	Y	Y	3
Mylor	*L. lanigerum*	indigenous	1**	Y	Y	Y	1
Waite	*L. lanigerum*	indigenous	2**	Y	Y	Y	1

* data combined for growth trials
** Plants tagged and assessed but population size too small for inclusion in growth trials

In addition, where possible, clonal plants from all collections are established at the Waite campus, Urrbrae for ongoing research. All commercial seed sources are from either commercial seed suppliers and grown by the project team, or from commercial nurseries, Provenances are identified by the state of origin only, Indigenous plants are from naturally established stands, except for the Waite site, where plants are purchased from local suppliers and are sourced from the Mount Lofty Ranges provenance.

All sites are irrigated except for Mylor and Macclesfield, and this has had an impact on plant growth and survival throughout the Project period.

Genomic study for the establishment of a breeding program

Collections of plant material, together with nectar analysis will occur across SA and environmentally compatible sites from western Victoria (8 populations). Material already established in trials (as above) will be included in the genomic study. The genomic work will be done in collaboration with Dr Rachel Binks in WA.

To assess genetic diversity, samples were collected from six wild populations of *L. scoparium* across the southwestern Grampians, Victoria, and five wild populations of *L. continentale* from across South Australia. These populations were selected because they grew in environments that experienced a similar climate to where plantations are intended to be established in South Australia. Using Diversity Arrays Technology sequencing (DArTseq), genetic diversity and differentiation within and among populations of both species were investigated using single nucleotide polymorphisms. The resulting dataset was analyzed to determine the number of genetic clusters as well as assess *the* genetic diversity within, and between, each species and population.

Results

The results of the genetic analysis have shown how many genetic clusters are present in the populations sampled of *L. scoparium* and *L. continentale*., and the distribution of the genetic diversity within, and between, each species and population. In addition, the analysis highlighted potential taxonomic issues between the two species that warrant further investigation.

Full details are documented in:

Hancox, T, 2022, *Leptospermum* for bioactive honey production in South Australia: selection, genetic diversity, and propagation Ph.D. thesis, University of Adelaide.

Hancox, TJJ, Binks R, Burton R, & Delaporte, K 2022, Genetic diversity and differentiation of *Leptospermum scoparium* in the Grampians, Victoria and *L. continentale* in South Australia. *In preparation*.

Environmental

Influences on plant growth, flower development, flowering, nectar production, and DHA concentration

Characterise *Leptospermum* plant performance, soil structure, and soil moisture for site selection

SA Environmental Study

Weather data for each site was retrieved from the Australian Bureau of Meteorology website, for the years 2018 to Dec 2021, with long-term averages provided for comparison where possible. Data considered of key importance are rainfall (monthly) and maximum and minimum temperatures which were taken from the closest formal weather data recording station. Weather data for years outside the project was collected from the Australian Bureau of Meteorology website; data availability is dependent on that station's operational capacity. Data are presented graphically.

Plant growth and flowering

Alongside the seedling-derived plantings at Bridgewater, Macclesfield, Charleston, Mylor, Wirrabara, and Port Vincent, a trial of selected, clonally propagated genotypes was established at Waite Urrbrae and Wirrabara (Table 1).

Plant growth data was collected manually at each site from between 1 and 209 individuals, most commonly between 10 and 20 (Table 1 for detail) randomly selected at the beginning of the project with the same plants assessed each year for the following

- height (cm) and width (cm) measured with a graded stick, growth stage (new growth + healthy, some new growth + ok, no new growth + poor health, dead),
- reproductive stage (not flowering, in the bud, flowering.
- other observations made and noted such as the volume of flowers and the formation of seed capsules), damage e.g., insect attack, herbivory (listed but not scored as no damage occurred).

The sites at Bridgewater, Macclesfield, Charleston, Mylor, Port Vincent, and Waite were assessed yearly during nectar collection (October-December) and the site at Wirrabara was assessed quarterly as well as during nectar collection (December-January). Figures 2, 3, 4, and 5 show *L. scoparium* at the Wirrabara field site in January 2022.

Data for height and width is represented graphically to highlight differences and similarities between species and sites. An observational commentary of the growth stage, reproductive stage, and damage are provided.

Figure 4: *L. scoparium* plants, Wirrabara Forest Trial site facing north; the combination of cutting grown plants and seed grown plants on irrigation.

Figure 5: *L. scoparium* plants, Wirrabara Forest Trial site facing south; the combination of cutting grown plants and seed grown plants on irrigation.

Figure 6: *L. scoparium* plants, Wirrabara Forest Trial site, showing different growth habits – short and wide and tall and thin

Figure 7: *L. scoparium* plant in full flower, Wirrabara Forest Trial site.

Nectar collection

Nectar for each site was collected during peak flowering, from the same individual plants. The method used throughout all our research follows that developed by Norton *et al* (2015) and updated by Williams *et al* (2018) where one sample is collected by washing nectar from 10 open flowers using 10µL Millipore water and collected into a vial for analysis by the University of Sunshine Coast team lead by Professor Peter Brooks. Duplicate samples were collected from each plant and data were shown as the average and the range for DHA: Tsugar (mg/kg) and estimated sugar mass (µg). Williams *et al* (2108) noted that a cut-off of 43µg for Tsugar mass was required, as samples with a low Tsugar mass distorted DHA: Tsugar values. Figures 6 shows European honey bees active in the Wirrabara site and honey on a comb from the hive; Figure 7 shows *L. scoparium* nectar samples in eh laboratory ready for analysis, and *L. scoparium* seed capsules maturing after flowering.

Results and discussion

The site and species selection

Numerous studies have shown that a multitude of *Leptospermum* species will produce very good quality bioactive honey (Cokcetin *et al,* 2019, Williams *et al,* 2018), however, not all species will grow and thrive in all parts of Australia. For example, *Leptospermum scoparium* is currently popular for large-scale plantations, however, the geographical range is predominantly in the cooler, wetter parts of southern Australia. It is therefore practical to understand the climate and site-specific requirements for different species when selecting plantation species and adapted provenances of those species. There are five species of *Leptospermum* native to South Australia, of which *L. continentale*, *L. lanigerum*, and *L. fastigiatum* have been recorded as having potential for bioactive honey production (Williams *et al* 2018). Of these three, *L. fastigiatum* has the highest mean DHA: Tsugar concentration, however, most of this species is in the far northwest of South Australia, and is highly adapted to arid environments, but difficult to access for testing or sampling. *Leptospermum lanigerum* is the second highest and is reported by Cokcetin *et al* (2019) as being a species of interest, however, in South Australia it is predominantly located along creek lines and swampy areas, potentially indicating that *L. lanigerum* has high water requirements and may not be conducive to large scale plantation production. The third species, *L. continentale*, has a widespread distribution and a moderate level of DHA: Tsugar recorded, providing opportunities for selection based on growth habit, environmental adaptability, and DHA concentration. Species selection for a South Australian bioactive Honey industry is further discussed in Delaporte *et al* (2022).

The focus species for this project were *L. scoparium* and *L. continentale*, as the two species most suited to wide areas of South Australia, and likely to be the most adaptable to plantation production. Other species included in this project are *L. polygalifolium* and *L. lanigerum*.

Hogendoorn *et al* (2022) worked in collaboration with our team and reported on how to site orchards and select species, based on climate envelope modeling (Key Activity 1: Situating the orchards and selecting the species). Their report provides a useful insight into using climate layers, bioclimatic layers, and spatial layers to evaluate how species respond to differences in soil and climate and provide an opportunity to fine-tune species selection and site selection.

During the work for their publication, Santos *et al* (2021), demonstrated that if water were not a limiting factor, *L. scoparium* should grow well in most regions of SA, and if water was restricted, then temperature and evapotranspiration become influential factors (Santos *pers comm*. 2019). Santos *et al* (2021) show the

combined factors of two climate layers, three bioclimatic layers, and one spatial layer on the potential distribution of *L. scoparium* refer to Figure 1 (Hogendoorn *et al,* 2022). The areas in which *L. scoparium* was predicted to grow to compare favourably to the natural distribution for *L. continentale*, such as the lower South East, Kangaroo Island, Fleurieu Peninsula and Mount Lofty Ranges, and the southern Eyre Peninsula, with the addition of South West of WA and Eyre Peninsula in South Australia. The study focussed on the seven *Leptospermum* species of most interest to producers of bioactive honey across Australia and did not specifically include *L. continentale*.

Leptospermum scoparium and *L. continentale* are adapted to seasonally wet conditions and prefer to grow in wetter soils. Over the course of the CRCHBP project, we undertook several approaches to determine how different species and provenances of natural adaptations to moisture and temperature would need to be considered when planning for an intensive plantation to produce high-grade bioactive honey.

Alongside the Bridgewater, Macclesfield, Charleston, Mylor, and Port Vincent plantation sites, a comparative trial of selected, clonally propagated genotypes was established at Waite Urrbrae and Wirrabara (Table 1). All sites, except for Macclesfield and Mylor, received some level of supplementary irrigation.

SA Environmental study

The *Leptospermum* species endemic to SA with bioactive honey are *L. continentale* (3,200ppm DHA), *L. fastigiatum* (1,360ppm), *L. lanigerum* (3,600ppm) (averaged DHA values from Williams *et al*, 2018). The remaining two species found naturally in SA, *L. coriaceum* and *L. myrsinoides* have been shown to have no bioactivity in their nectar (Williams *et al,* 2018). *Leptospermum laevigatum* is found in coastal areas of SA and is an introduced weed species from the eastern states of Australia. The distribution of these species across SA is varied, but with commonalities. Species can tolerate all soil types with a mostly neutral pH (5-7), are frost resistant to moderately sensitive, flower winter, spring, and summer, and prefer 500ml+ rainfall, which occurs mainly during the winter months. *L. scoparium* is widely distributed throughout southern Australia and Tasmania, as well as thriving in New Zealand. In contrast, *L. fastigiatum* is found broadly across central Western Australia and partially in the central desert regions of South Australia, and so would survive lower rainfall. An analysis of weather conditions recorded in the natural growing environment of this species indicates a preference for rainfall of at least 500ml distributed evenly throughout the year, 'wet feet' (grows in streams, soaks, and swamps), like acid to neutral soils (pH 5-7) and milder temperatures (Delaporte, 2016; Delaporte, 2017).

In addition to the desktop study by Santos *et al* (2021) we connected with seven field sites located throughout South Australia in areas where it was considered feasible to grow *Leptospermum* for honey production (Figure 1), and these sites were visited yearly over the 4.5 years of the CRCHBP (2018- 2022). Sites were located at Wirrabara (*L. scoparium*), Port Vincent (*L. polygalifolium*), Bridgewater (*L. scoparium* and *L. polygalifolium*), Mylor (*L. continentale* and *L. lanigerum*), Charleston (*L. scoparium* and *L. continentale*), Macclesfield (*L. scoparium*) and Waite (mixed species) (refer Table 1). For each site, data were recorded on plant growth and flowering, and nectar was collected for each flowering for DHA analysis. We also collated weather data from the closest Bureau of Meteorology station to each site for comparison and collection of real-time data from the Wirrabara site.

For all sites identified as having potential for large-scale bioactive honey production, we compared rainfall and temperatures with the overlay of the general requirement for *L. scoparium*. During a previous project, we reviewed the rainfall and temperature requirements for *L. scoparium* across its endemic regions compared to potential plantation regions in South Australia (Figure 8) (Delaporte, 2016; Delaporte 2017). The volume of rainfall is similar, however, the distribution of rainfall across the year may be the key factor in determining inputs such as irrigation. The recorded annual rainfall across all seven sites visited during the study period was variable from the long-term averages as might be expected over four years. The seven study sites differed in

their 10 years' mean annual rainfall from Port Vincent with 303 mm to Bridgewater (Adelaide Hills) with 1038ml and Wirrabara with 518ml (Figure 9). However, the distribution of the rainfall by month (observed and mean) again demonstrated both the variable nature of rainfall in a typically Mediterranean climate as well as the peak rainfall occurring in the winter months rather than a consistent level throughout the year, as might be found in Hobart in Tasmania or Melbourne Vic, which represent regions where *L. scoparium* occurs naturally.

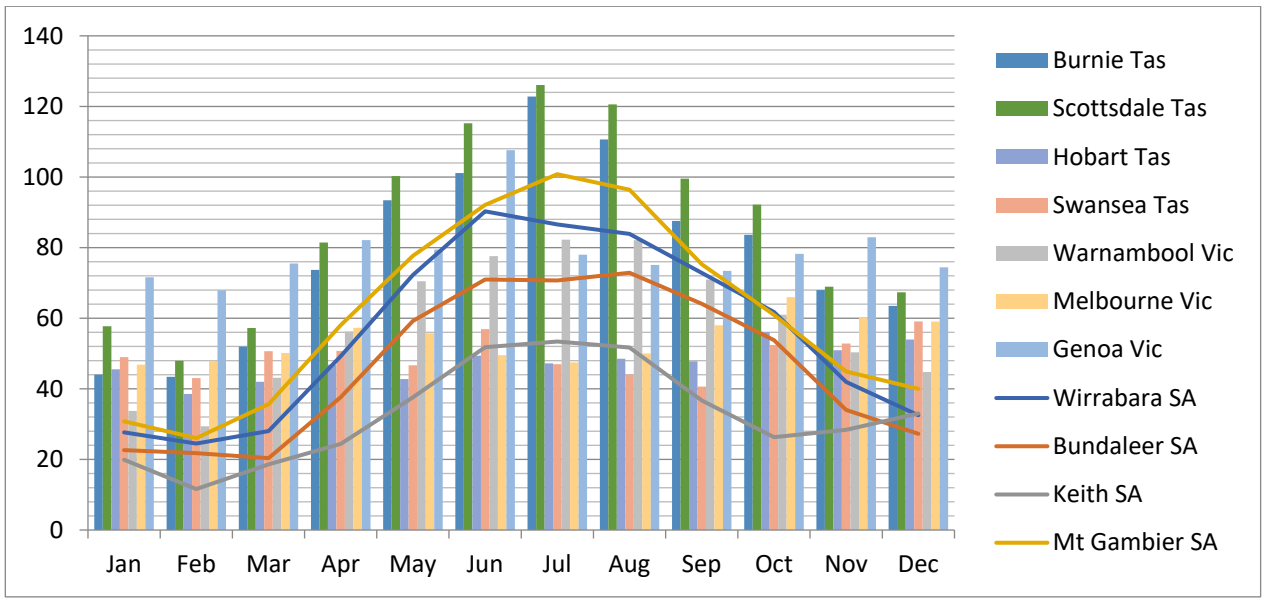

Figure 8: Mean monthly rainfall average for *L. scoparium* endemic vs potential locations, (from Delaporte, 2016). Y axis = mm rainfall.

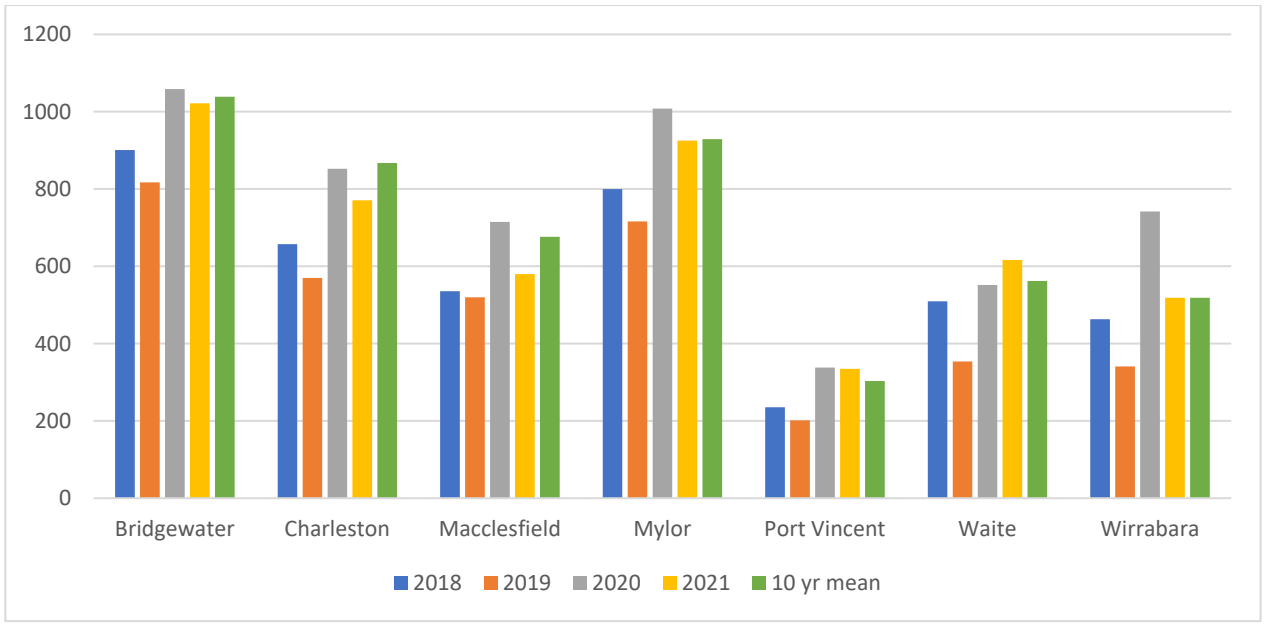

Figure 9. Annual rainfall for the study period and 10-year mean rainfall for all study sites: Bridgewater, Charleston, Macclesfield, Mylor, Port Vincent, Waite, and Wirrabara. Y axis = mm rainfall.

Figures 10 to 16 present the monthly rainfall averages for the seen field sites, plus 5-year means, 10-year means, and greater, where data is available. These figures illustrate the variability present in year-to-year rainfall as well as deviations from long-term trends. The data showed that for the first three years of the study period, July rainfall was well below average for all sites, as was rainfall in March and September or October. These are critical rain periods for plant survival, and impact plant growth and flowering.

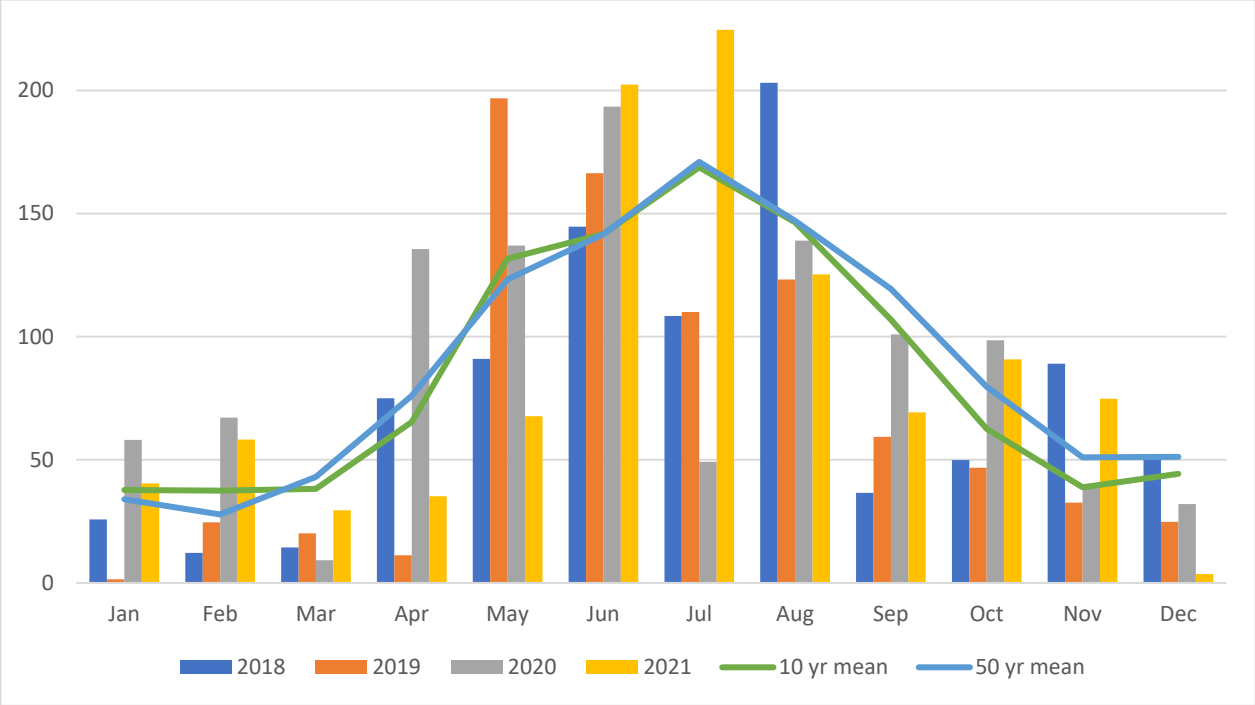

Figure 10: Mean monthly rainfall for the study period, 10 years mean and 50-year mean rainfall for Bridgewater. Y axis = mm rainfall.

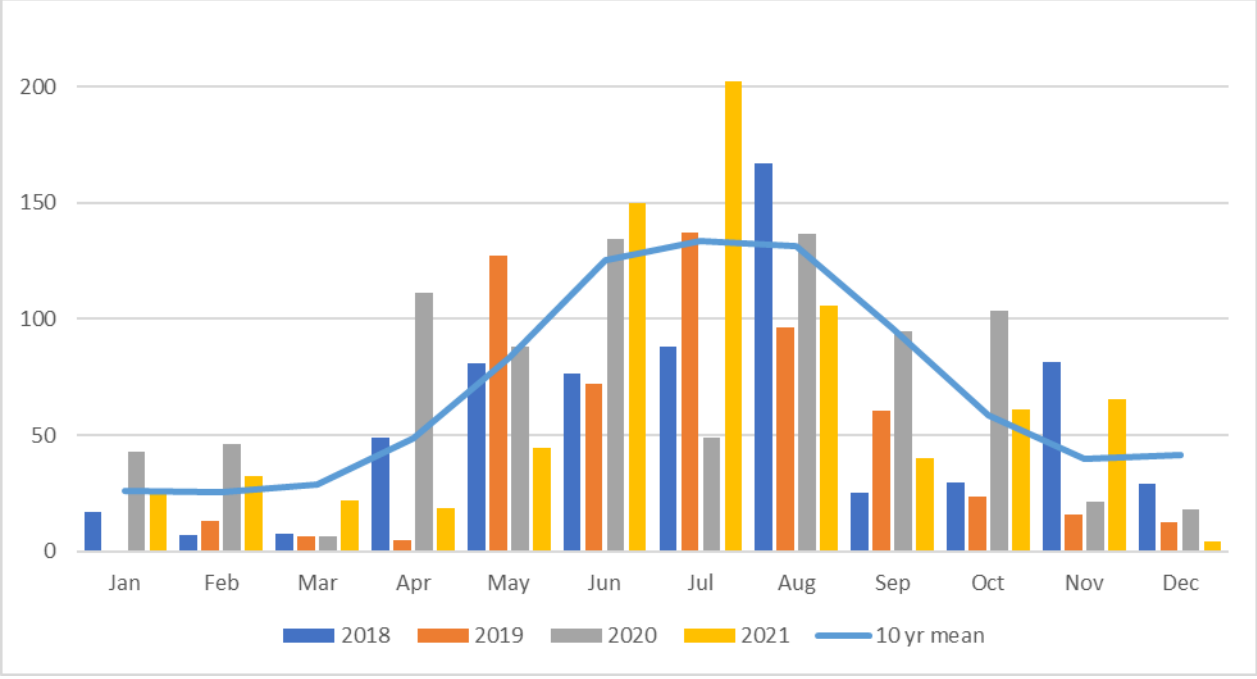

Figure 11: Mean monthly rainfall for the study period and 10-year mean rainfall for Charleston. Y axis = mm rainfall.

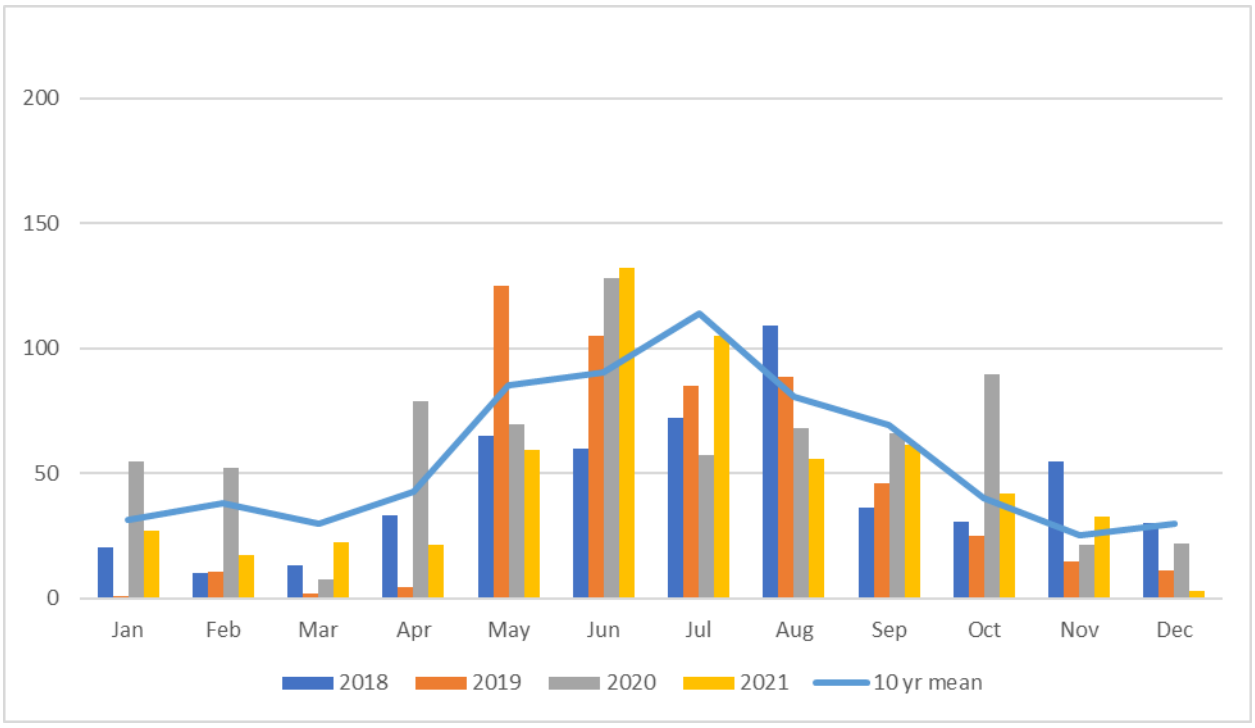

Figure 12: Mean monthly rainfall for the study period and 10-year mean rainfall for Macclesfield. Y axis = mm rainfall

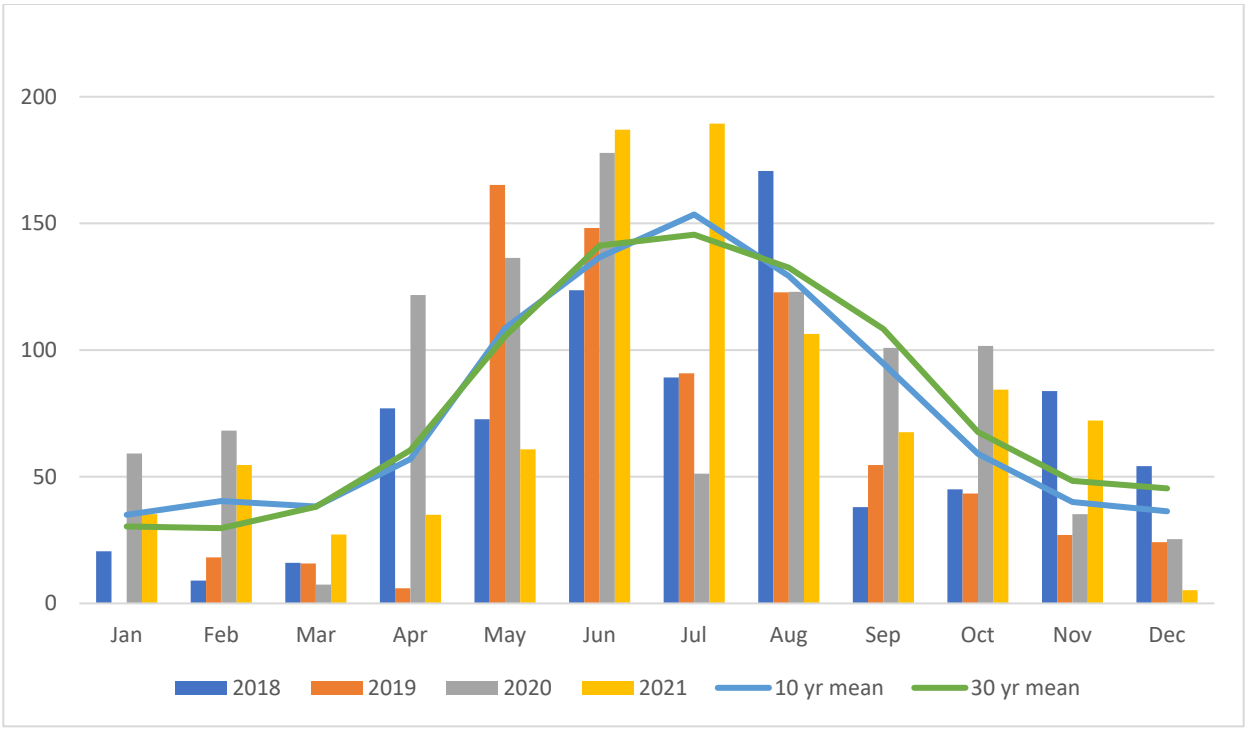

Figure 13: Mean monthly rainfall for the study period, 10-year and 30-year mean rainfall for Mylor. Y axis = mm rainfall

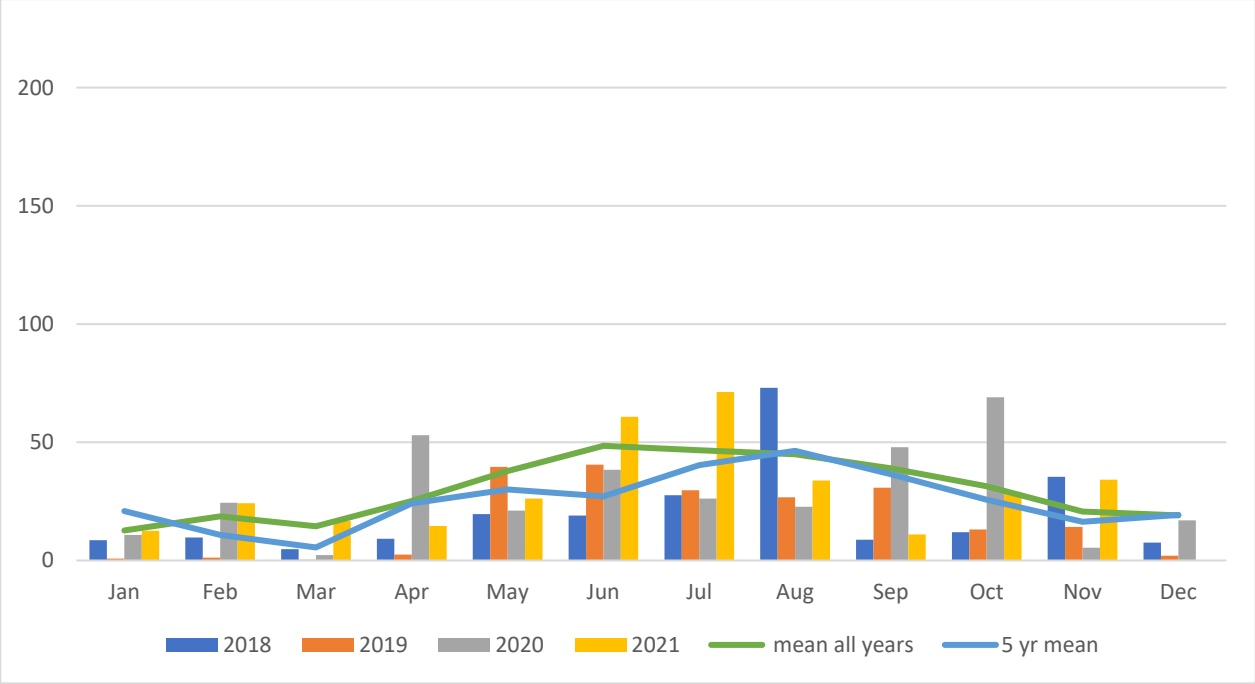

Figure 14:Mean monthly rainfall for the study period and 5-year mean rainfall for Port Vincent. Y axis = mm rainfall.

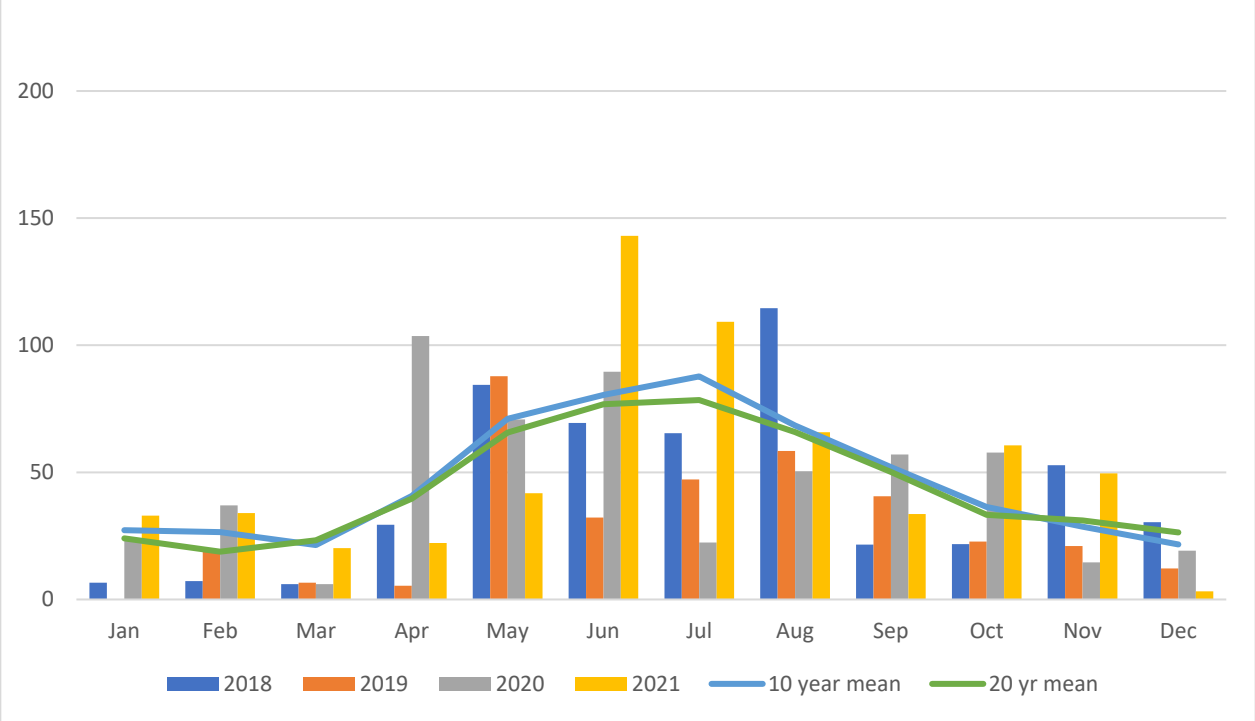

Figure 15: Mean monthly rainfall for the study period, 10-year and 20-year mean rainfall for Waite. Y axis = mm rainfall

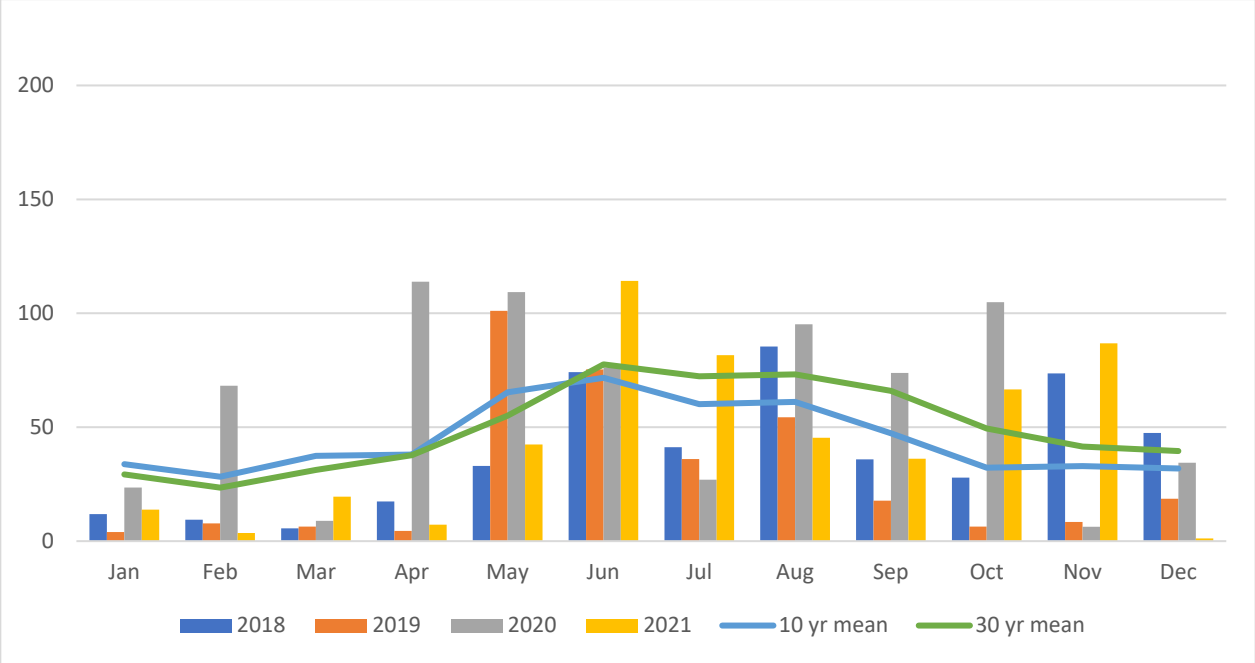

Figure 16: Mean monthly rainfall for the study period, 10-year and 30-year mean rainfall for Wirrabara. Y axis = mm rainfall.

Maximum and minimum temperatures did not deviate from the medium- and long-term averages as much as the rainfall, however, the analysis does not highlight actual maximum temperatures that were experienced during the month. For example, for the four sites represented here, both January 2019 and December 2019 showed much higher average monthly maximum temperatures than the average, and this was the result of a few very hot days increasing the average for the month (Figures 17 – 20).

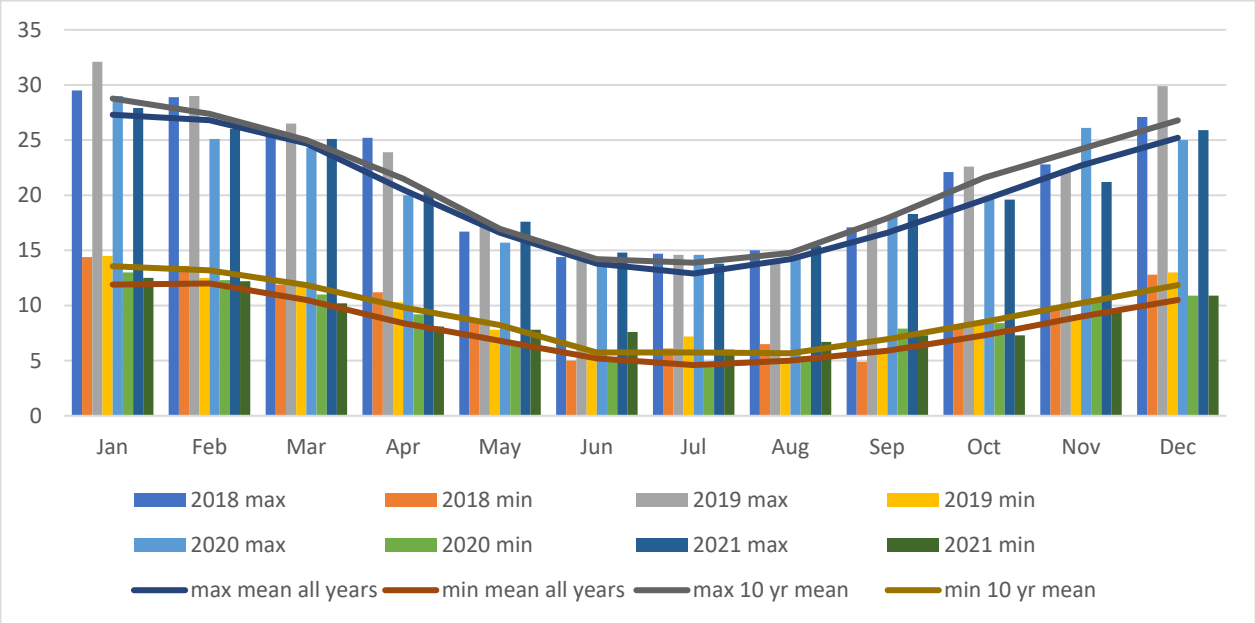

Figure 17: Bridgewater average monthly maximum and minimum temperatures and mean max/min temperatures over 'all years' and 10-year mean. Y axis = °C.

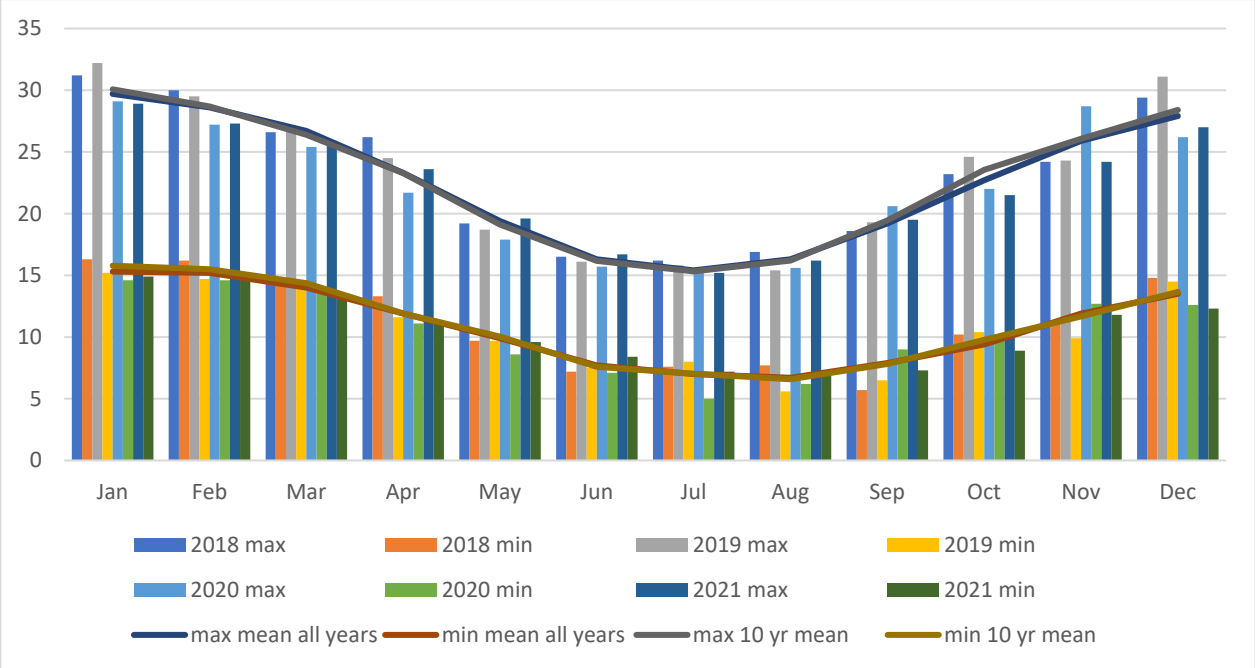

Figure 18: Port Vincent average monthly maximum and minimum temperatures for the study period, and mean minimum and maximum temperatures for 10 years and 'all years. Y axis = °C.

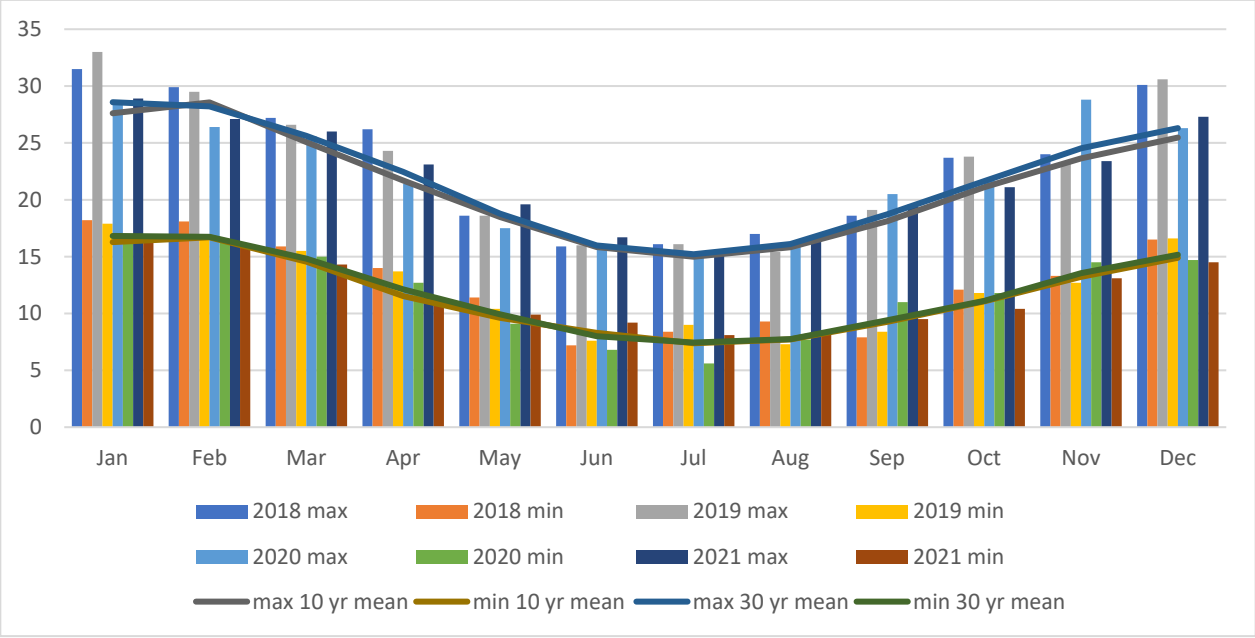

Figure 19: Waite (Kent Town) average monthly maximum and minimum temperatures for the study period, and mean minimum and maximum temperatures for 10 years and 30 years. Y axis = °C.

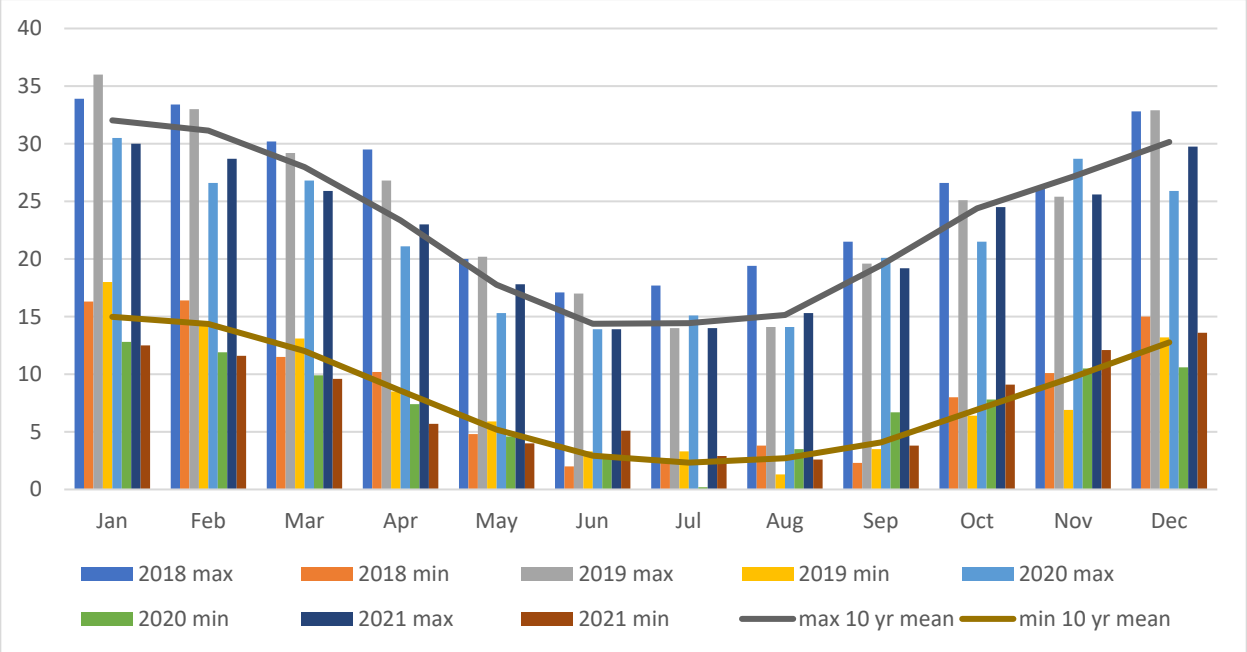

Figure 20: Wirrabara average monthly maximum and minimum temperatures for the study period, and mean minimum and maximum temperatures for 10 years. Y axis = °C.

Minimum and maximum temperatures, as individual day temperatures, monthly averages, and long-term means, are important considerations when establishing a plantation, as extremes of hot and cold can negatively impact plants at critical growth and production stages, particularly in conjunction with a period of very high or very low rainfall. Whilst rainfall can be supplemented with irrigation, it is difficult to control the ambient temperature and minimise its impacts on plants.

Plant growth and flowering

Observations have been conducted either quarterly or yearly (during flowering). Plants at all sites showing new growth all year round, and the most active period for new growth is mid-winter/spring/early summer. The active new growth becomes the flowering site for the next flowering season; flower buds are located in the current season's growth. Flowering occurs from October through to February, depending on the original provenance and progression of summer heat. Over watering, under watering and poor water quality can all negatively impact growth during any season.

Figures 21 and 22 provide the average height for plants assessed at each of the 7 sites. Figure 21 shows the variability in vertical growth, depending on plant age, and how specific sites can impact plant growth e.g., Port Vincent vs Bridgewater *L. polygalifolium* – same provenance and age and both irrigated, but at different rates and different soil types are impacting growth. Figure 22 illustrates that *Leptospermum* plants grow fast vertically until flowering and then slow and continue to grow horizontally. This is evident in the "Wirrabara *L. scoparium* Vic – commercial 1" plants, which started the trial as seedlings <20cm in 2018 and grew to over 120cm in 3 years with the first flowering occurring in 2020, whereas the "Wirrabara *L. scoparium* Vic – selected" plants started the trial at ~100cm and finished at ~150cm and flowered first in 2019/20.

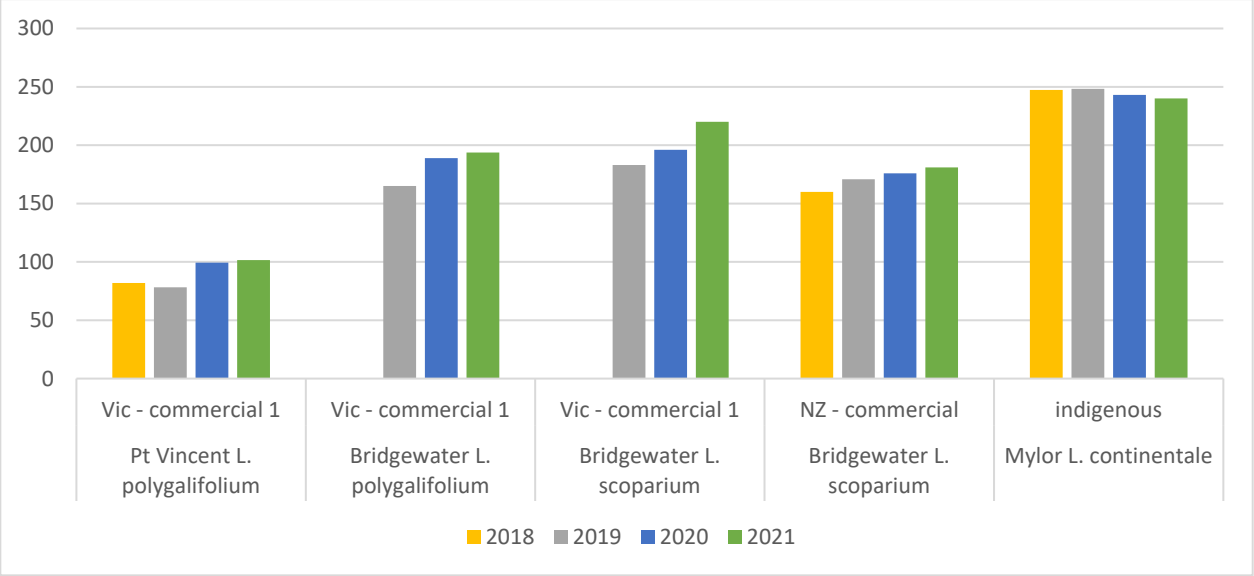

Figure 21: Comparison of the average height between 2018 and 2021; *Leptospermum scoparium* (Bridgewater), *L. polygalifolium* (Port Vincent and Bridgewater), and *L continentale* (Mylor). Y axis = cm

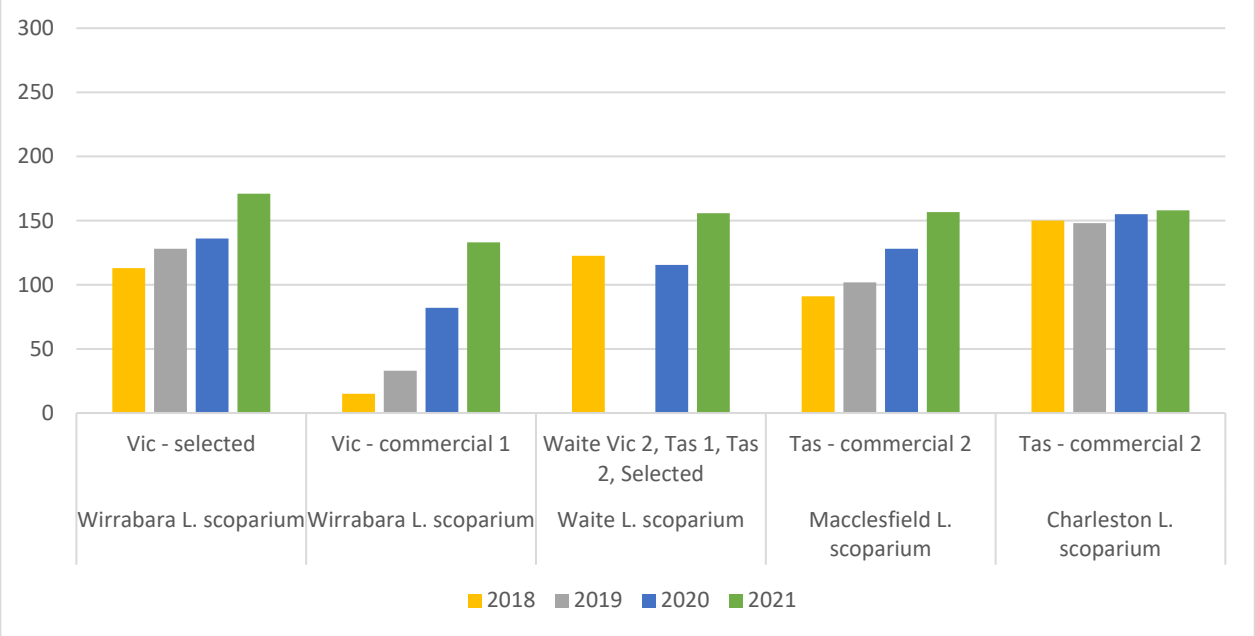

Figure 22:Comparison of the average height between 2018 and 2021; *Leptospermum scoparium* Wirrabara, Waite, Macclesfield, and Charleston. Y axis = cm

Figures 23 and 24 provide the average width for plants assessed at each of the 7 sites, and both differ from height in that once the plants reach flowering age, they become wider rather than taller. Both figures show that *Leptospermum* plants grow steadily horizontally, regardless of the time of first flowering. Interestingly, even though plant height varies from ~100cm in 2021 to ~250cm in 2021, plant width is more consistent with ~150cm to 200cm measured across all sites.

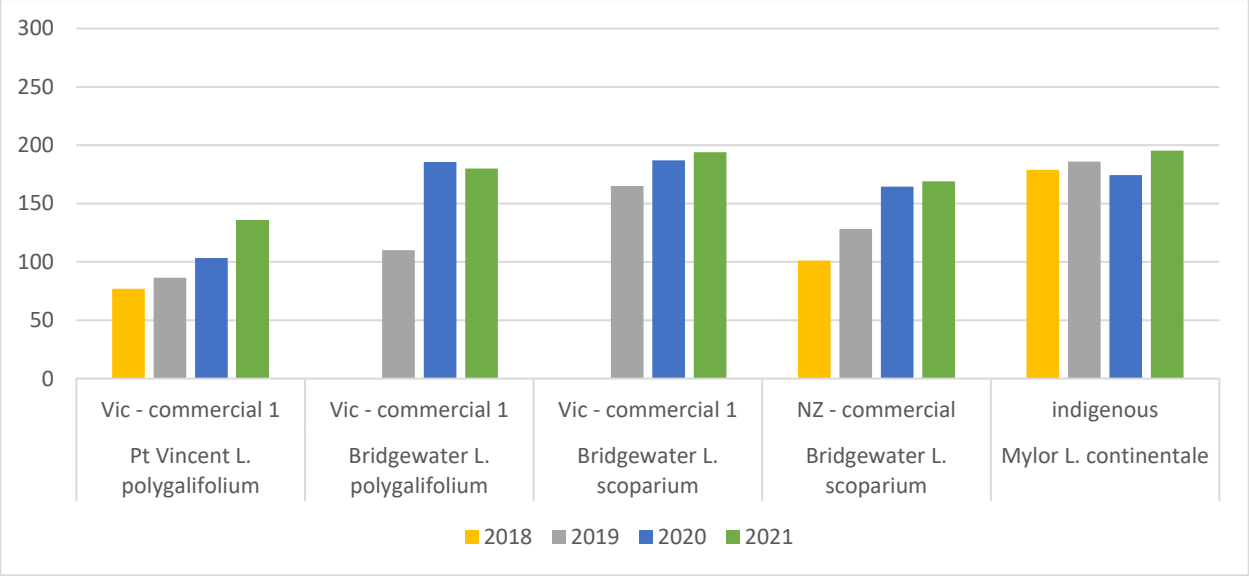

Figure 23: Comparison of the average width between 2018 and 2021; *Leptospermum scoparium* (Bridgewater), *L. polygalifolium* (Port Vincent and Bridgewater), and L continentale (Mylor). Y axis = cm

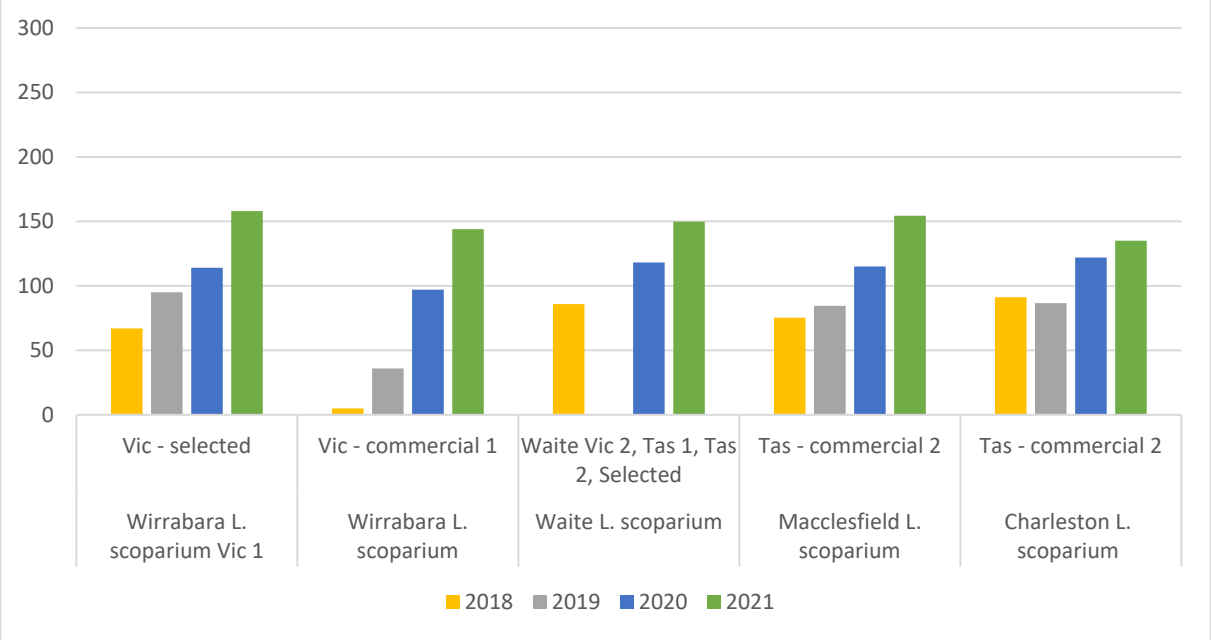

Figure 24: Comparison of the average width between 2018 and 2021; *Leptospermum scoparium* Wirrabara, Waite, Macclesfield, and Charleston. Y axis = cm

Considerable plant growth data was collected over the 4 years of this project. 'Site x Species' height and width data is presented graphically in the Appendix for all species at all sites. *Leptospermum scoparium* is reported to reach between 2 and 5m in height with a similar width but can also grow into a tree 15m in height; while *L. polygalifolium* may reach 1-5m high, *L. continentale* 1-4m height, and *L. lanigerum* 2-5m high (Grieg, 1999). From this information, we expect that the plants observed in this trial are not fully mature in terms of their growth and could at least double in size if ideal conditions for growth are present.

Flowering generally began in the second year after planting seedlings (usually the third year after germination) and once the plants were fully flowering the following year, vertical growth slowed down. For cutting grown plants, flowering occurred once the correct growth was present e.g., flower buds form on current seasons growth, therefore the plants need to have had a growth spurt before the next spring flowering season. Anecdotal evidence suggests that trimming seedlings before planting can initiate flower buds a year earlier than otherwise, however, this was not tested in this project. As plant vertical height slows once flowering occurs, any plant management needs to be considerate of this and adjusted according to ongoing plantation output requirements.

Observations on plantation maintenance were made and these reflect common sense and practice when establishing any plantation:

- good weed control at planting and for the first two years will benefit establishment and subsequent plant growth.

- Regular watering seems crucial to ensure plant survival and maintain growth; more water produces more growth but too much water while plants are small and establishing can be detrimental to both establishment and survival; the ongoing impact of irrigation volume and timing on flowering and nectar flow should be determined as the plants get older.

- Large and small herbivores can be an issue when plants are still small (e.g., kangaroos, deer, rabbits, and sheep). There is the option to graze-over establishing plantations from ~6 months (once *L. scoparium* has gone 'prickly').

- Very few invertebrate pest issues were observed –webbing caterpillars (natural pest of Leptospermum) and ants in drier years possibly looking for liquid nectar.

- Overall, no evidence of any nutrient issues at any sites, e.g., at Wirrabara – no fertilizer has been added.

- Plant spacing is personal/site preference, depending on how the site will be managed for honey production and bee movement, e.g., denser plantings will develop into hedgerows earlier than wider spacings, influencing pruning of plants to maintain new growth and optimal flowering.

Soil structure was considered; at each site, the soil was considered appropriate for Leptospermum and varied from silty loam (pH 5.5-7) at Waite to sandy loam over rock at Port Vincent (N. Timbs, pers. comm.). The impact of soil type on plant growth was not documented, however, anecdotally, sites with lighter soils held less residual soil moisture compared to sites with heavier soils, which may be less suitable for moisture-loving Leptospermum species.

Due to the periodic nature of the rainfall in South Australia, with warm dry summers where very little rain falls and evaporation is high, irrigation is essential for the establishment of *L. scoparium* and *L. continentale*. At sites where plants rely entirely on rainfall, individual plants die. Before this project commenced, a young plantation of over 400 plants at Keith died due to a prolonged dry spell in Jan-March, following above-average rainfall from July to October. Observations of the root systems of the deceased plants showed that they were well developed and complex after 6 months, but there was simply no soil moisture to keep them alive. Thus, ensuring adequate soil moisture for plant survival is essential, however, the role of soil moisture in flower development, floriferousness, nectar flow during flowering, and volume of nectar, remains to be elucidated. Hogendoorn et al (2022) undertook some research into this aspect and this is outlined in the Nectar Collection section.

Nectar collection and testing

Nectar for each site was collected during peak flowering, from the same individual plants each year. The method used throughout all our research follows that developed by Norton et al (2015) and Williams et al (2018) and analysis performed by the University of Sunshine Coast team led by Professor Peter Brooks. Duplicate samples were collected from each plant and data was shown as the average and the range for both DHA: Tsugar (mg/kg) and estimated sugar mass (µg) (refer to Table 2). Due to the nature of the plants and seasons, we were not always able to collect a sample to analyse from all specimens every season.

Over the course of the CRC Project, we have sampled nectar from a wide range of species; *Leptospermum scoparium* (the Grampians, commercial seed sources originating from SE Tasmania), *L. polygalifolium* (commercial seed sources, provenance unknown), *L. lanigerum* (SA indigenous), and *L. continentale* (SA indigenous). From this testing, we found that DHA: Tsugar results can vary immensely – even individual plants can have a wide range of values over different years. This may be caused by one of three reasons or a combination of all – it is possible that the sampling method used is not robust and reliable, that the age of the flower at nectar collection influences DHA concentration, as reported by Clearwater et al (2018) and Clearwater et al (2021), and that environmental impacts in the lead up to and during flowering, such as volume of rainfall, relative humidity, and soil moisture, are influential on nectar flow.

Hogendoorn et al (2022) investigated the impact of irrigation on nectar flow and subsequent honey production. Through observation of honey bee activity on irrigated and non-irrigated plants, they found that the irrigated plants attracted 10.2 times more honey bees than non-irrigated plants. There was a significant effect on year, probably due to plant size, but not time of day for observation. More bees visited larger bushes on irrigation, but the time of day was not important. To support this evidence of irrigation increases visitation, Hogendoorn et al (2022) undertook a small greenhouse experiment to assess the impact of irrigation vs non-irrigation on the volume of nectar production. Plants were watered or not watered, and nectar was collected on day 8 and day 16. On day 8, flowers from watered plants produces significantly more nectar by volume, than those plants that were not irrigated, with a factor of 2.7 difference. Hogendoorn et al (2022) discuss their results in relation to Clearwater et al (2018) and surmises that their data demonstrates the importance of adequate soil moisture during flowering to produce nectar and attract honey bees; moisture can be environmental from planting in creek beds, or through supplementary irrigation.

Figure 25: *L. scoparium* buds and flowers. The open flower shows the inside of the flower on which the bee lands. The nectar accumulates at the base of the anthers with the stigma prominent in the middle of the flower.

Table 3: DHA: Tsugar (mg/kg) from 2-18 plants at each of the seven South Australian field sites growing different species of *Leptospermum* under different conditions, visited yearly between 2018 and 2022. Refer to Table 1 for the numbers of plants tested for each Site x Species x Source combination.

		DHA:Tsugar (mg/kg) Average	Range (min to max)	DHA:Tsugar (mg/kg) Average	Range (min to max)	DHA:Tsugar (mg/kg) Average	Range (min to max)	DHA:Tsugar (mg/kg) Average	Range (min to max)
SA Site	Source	2018/19		2019/20		2020/21		2021/22	
Leptospermum. polygalifolium									
Pt Vincent	Vic - commercial 1	2,643	0 - 5,704	4,816	0 - 10,340	4,885	713 - 14,995	1,861	0 - 4,818
Bridgewater	Vic - commercial 1	na	na	2,535	1,276 - 4,905	3,286	1,920 - 4,586	641	0 - 3381
Waite	State Flora	na	na	15,969	13,467 - 19,221	18,427	13,126 - 25,990	11,356	10,275 - 12,437
Leptospermum. scoparium									
Bridgewater	NZ – commercial	729	0 - 2,725	1,228	376 - 2,959	445	0 - 1,985	249	0 - 966
Bridgewater	Vic - commercial 1	na	na	3,087	1,153 - 9,003	2,403	0 - 6,380	1,023	0 - 5,252
Wirrabara	Vic – selected	na	na	2,027	1,238 - 2,942	3,577	1,928 - 4,499	2,241	0 - 1,180
Wirrabara	Vic - commercial 1	na	na	na	na	3,809	3,650 - 4,078	514	0 - 1,081
Macclesfield	Tas - commercial 2	na	na	969	0 - 1,596	6,663	3,320 - 12,028	1,048	368 - 1832
Charleston	Tas - commercial 1 & 2	na	na	426	0 - 2,027	1,705	1,406 - 2.311	665	375 - 1,462
Waite	Vic - commercial 2	2,554	2,262 – 2,847	3,896	2,267 - 8,479	1,059	0 - 3,327	na	na
Waite	Tas - commercial 1	731	640 - 822	976	640 - 1,421	820	0 - 2,039	287	0 - 959
Waite	Tas - commercial 2	623	587 - 658	2,076	0 - 8,818	na	na	426	0 - 867
Waite	Vic – selected	na	na	3,713	2,362 - 6,448	4,711	725 - 10,060	1,548	335 - 3,815
Leptospermum continentale									
Mylor	Indigenous	na	na	3,474	0 - 8,477	8,126	4,011 - 16,117	3,389	0 - 9,734
Charleston	Indigenous	na	na	3,535	3,179 - 3, 892	4,828	3, 012 - 6, 565	462	0 - 1001
Waite	Indigenous	na	na	3,699	2,904 - 4,495	6,272	5,877 - 7,189	1,144	377 - 1,772
Leptospermum lanigerum									
Mylor	Indigenous	na	na	8,562	7,939 - 9,184	8,363	8,280 - 8,445	3,751	3,646 - 3,856
Waite	Indigenous	na	na	8,693	7,936 - 9,451	na	na	na	na

Table 4: Est Sugar Mass (µg) * from 2-18 plants at each of the seven South Australian field sites growing different species of *Leptospermum* under different conditions, visited yearly between 2018 and 2022. Refer to Table 1 for the numbers of plants tested for each Site x Species x Source combination.

		Est Sugar Mass (µg) Average	Range (min to max)	Est Sugar Mass (µg) Average	Range (min to max)	Est Sugar Mass (µg) Average	Range (min to max)	Est Sugar Mass (µg) Average	Range (min to max)
SA Site	source	2018/19		2019/20		2020/21		2021/22	
Leptospermum polygalifolium									
Pt Vincent	Vic - commercial 1	100	39 - 179	22	9 - 30	43	9 - 95	145	37 - 259
Bridgewater	Vic - commercial 1	na	na	103	24 - 212	65	20 - 98	55	22 - 132
Waite	State Flora	na	na	245	92 - 442	454	146 - 780	544	494 - 594
Leptospermum scoparium									
Bridgewater	NZ - commercial	71	31 - 137	148	71 - 195	37	24 - 46	121	42 - 190
Bridgewater	Vic - commercial 1	na	na	96	42 - 150	79	21 - 192	57	4 - 167
Wirrabara	Vic - selected	na	na	77	26 - 151	100	69 - 170	20	0 - 65
Wirrabara	Vic - commercial 1	na	na	na	na	73	63 - 78	110	2 - 169
Macclesfield	Tas - commercial 2	na	na	104	24 - 173	28	16 - 43	75	27 - 123
Charleston	Tas - commercial 1 & 2	na	na	35	19 - 55	64	47 - 71	101	46 - 148
Waite	Vic - commercial 2	106	92 - 120	36	24 - 55	70	23 - 163	na	na
Waite	Tas - commercial 1	375	361 - 389	74	41 - 123	130	35 - 289	82	6 - 209
Waite	Tas - commercial 2	203	154 - 203	217	10 - 607	na	na	125	5 - 331
Waite	Vic - selected	na	na	62	14 - 117	141	16 - 257	112	13 - 333
Leptospermum continentale									
Mylor	indigenous	na	na	210	74 - 392	46	19 - 103	93	10 - 219
Charleston	indigenous	na	na	67	54 - 79	19	15 - 28	15	10 - 20
Waite	indigenous	na	na	87	83 - 91	89	34 - 179	65	10 - 136
Leptospermum lanigerum									
Mylor	indigenous	na	na	211	205 - 216	32	31 - 33	93	88 - 98
Waite	indigenous	na	na	96	76 - 116	na	na	na	na

*We note that some values of Tsugar mass are below the cut-off of 43 µg as proposed by Williams *et al* (2018) and thus some data may be unreliable.

The clonal populations established at Waite and Wirrabara enabled a comparison between sites for DHA: Tsugar, to ascertain the level of variability within a clone between years and sites. Variability was high, as is demonstrated with all plants sampled regardless of origin (Table 2). There is an indicator of a strong environmental influence in the 2021-22 flowering season, as all values were much lower than 2020-21, which is generally higher than either 2019-20 or 2021-22.

A subset of five genotypes is presented in Figure 25, illustrating the variability across three years compared to the original sample taken in 2016 (purple). Genotypes S and T were relatively uniform for DHA values between sites in the same year, but genotypes E, H and M varied considerably. Genotypes E and M produced much higher DHA: Tsugar values in 2020-21 compared to the original value from 2016.

The variability within genotypes between years is present in the estimated mass sugar values (Figure 26).

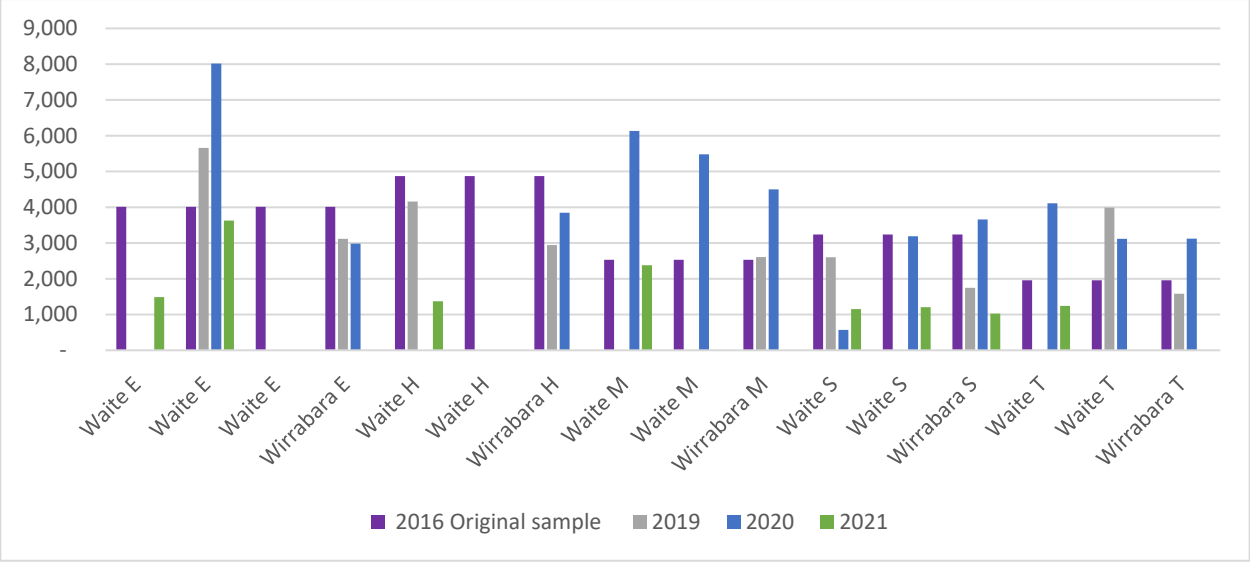

Figure 26: Comparison of the average DHA: Tsugar values for 5 selected genotypes, growing in a clonal population at Waite and Wirrabara. Y axis = DHA:Tsugar (mg/kg)

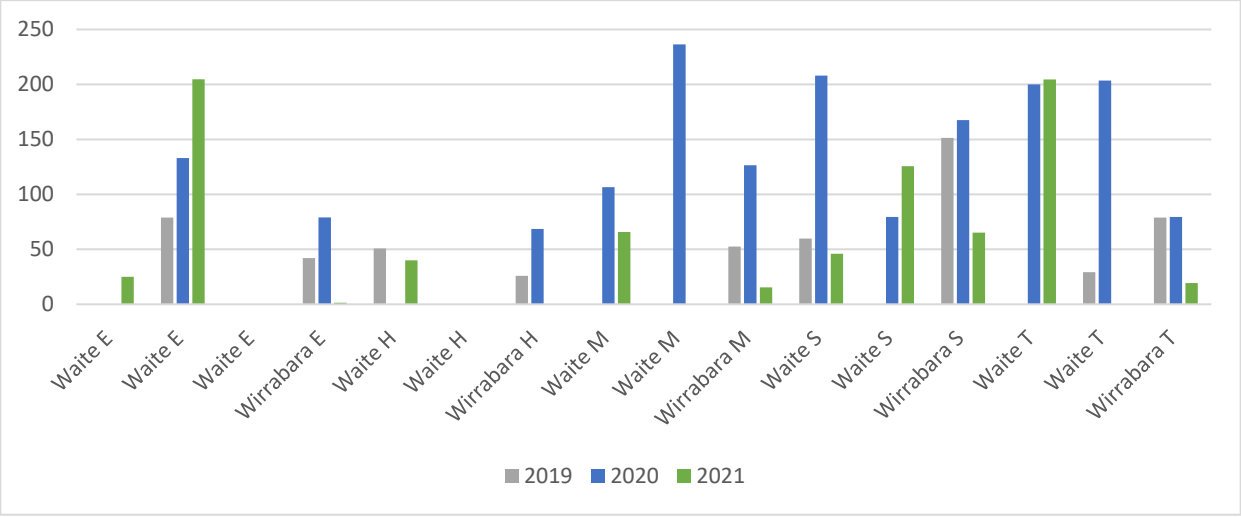

Figure 27: Comparison of the average Est Sugar Mass values for 5 selected genotypes, growing in a clonal population at Waite and Wirrabara. Y axis = Est Sugar Mass (µg)

Propagation for cost-effective deployment

To assess the commercial benefit of rooted cuttings, a propagation trial will be established to determine the conditions required to optimise rooting success and root quality for field survival.

A series of propagation experiments were established to develop methodologies for *L. scoparium* and *L. continentale* propagation from semi-hardwood cuttings. These experiments investigated the effect of auxin application (type and concentration), genotype as well as the length of mist propagation time in the tent before being transferred to potting media.

This process involved creating and planting approximately 9,000 cuttings and collecting data on 8,000 cuttings over three and a half years from four genotypes of *L. scoparium* and ten genotypes of *L. continentale*. When transplanted, data was collected on the number of surviving cuttings, those that had formed roots and roots per cutting. Some cuttings had formed callus and showed necrosis, leaf drop, and the presence of fungus. For approximately 3,500 cuttings with roots, data were recorded on the survival of each plant, the number of shoots, and growth after six weeks from planting. Of these cuttings, 396 plants were used to assess the growth rate for *L. continentale* after transplanting. The data created from these experiments were analyzed using GenStat (VSN International, 2021).

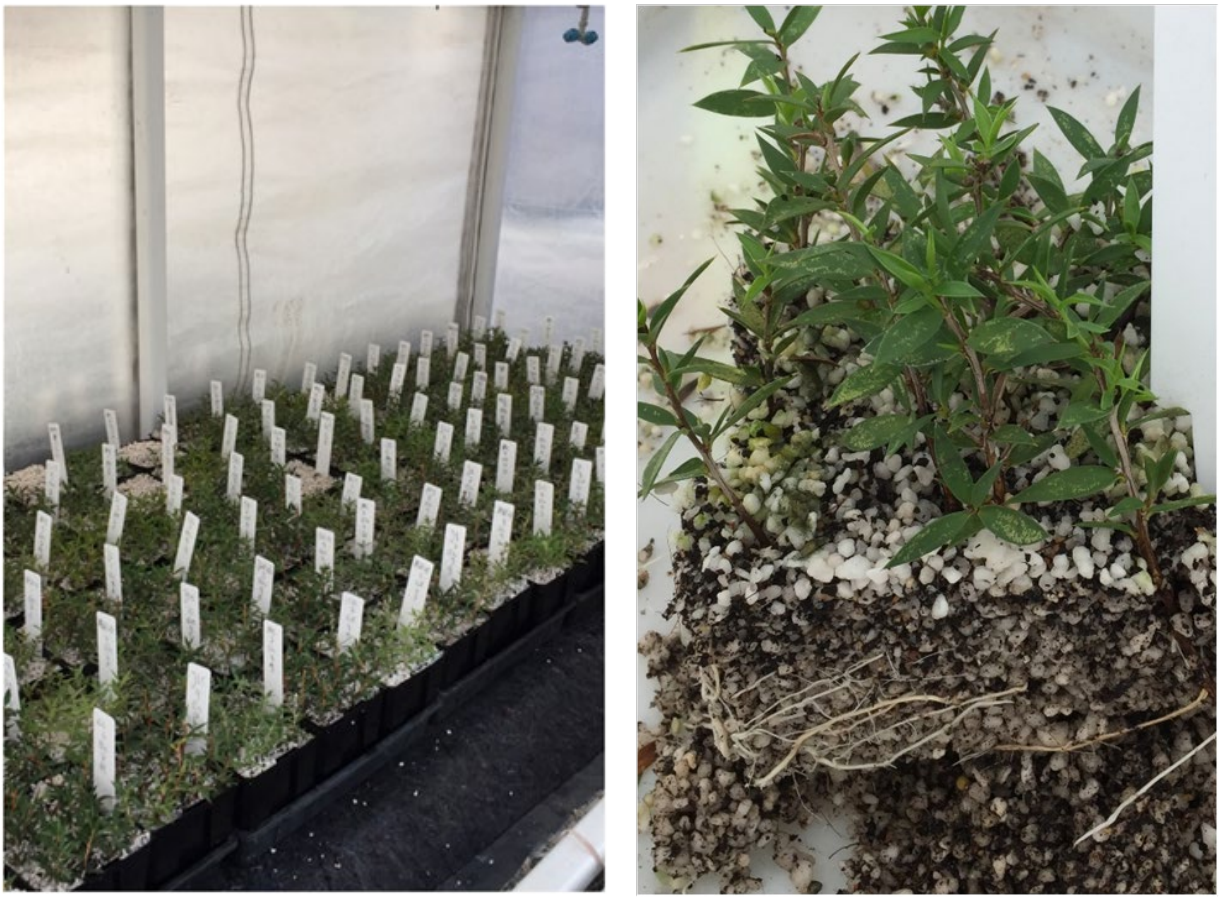

Figure 28: *Leptospermum scoparium* cuttings in the propagation tent at the University of Adelaide Waite Campus. Tate Hancox. (left) and the roots produced by the cuttings (right)

Figure 29: Assessment of the root growth from the cuttings

Results

The results from the *L. scoparium* and *L. continentale* propagation experiments showed genotype, auxin application, and length of time in the mist propagation tent had a significant effect on the ability to propagate each species. Information from the earlier experiments helped refine the methodology and helped to highlight the importance of stock plant health on propagation. These experiments also showed *L. continentale* can successfully be propagated from wild material and that transplant survival for this species is influenced by genotype and length of time spent in the mist propagation tent. It was also found that planting media can have a significant effect on transplant survival.

Details of the results are recorded in the two following publications:

Hancox, TJJ, Burton R, & Delaporte, K 2022, Development of a robust method for propagation of *Leptospermum scoparium* J.R. Forst. & G. Forst. from semi-hardwood cuttings. In preparation.

Hancox, TJJ, Burton R, & Delaporte, K 2022, Development of a robust clonal propagation and transplant survival methods for *Leptospermum continentale* Joy Thompson. from semi-hardwood cuttings. In preparation.

Seed orchard establishment

Supporting a breeding program for high DHA/sugar levels, nectar production, and plants suited to the SA environment

The genotype list for the seed orchard was selected from the genotypes planted at Waite and Wirrabara, propagated by cuttings, with mother stock plants sourced from the "Southern Grampians/Gariwerd" collection (genotypes B, C, D, E, H, J, L, M, P, Q, R, S, and T) and three from "Vic – commercial 1" seed source, being genotypes403, 406 and 407 that performed well at Waite (Table 1). The final selections were developed by reviewing observed and measured growth data as well as nectar DHA values from KA2: Environment.

The orchard comprised six cutting-grown clones each of 15 genotypes selected from all plants available at the time of selection, ensuring that all plants within genotypes are identical clones. Plants were planted in a randomised design, to ensure that all genotypes were able to pollinate equally and randomly with any other of the 15 genotypes, but not themselves. Plants were established with irrigation at ~14L/week and spaced at 3 x 3m.

Figure 30: European honey bees in a hive; honey on the comb; Wirrabara Forest Trial site

Figure 31: *L. scoparium* nectar samples in the laboratory at Waite, ready for DHA analysis; *L. scoparium* seed capsules in the field, January 202 Wirrabara Forest Trial site.

Results and discussion

In winter 2020, a seed orchard was planted at the Wirrabara Forest Block 9 site in collaboration with Spring Gully Foods (Table 4). A seed orchard is an intensively managed plantation of specifically arranged trees for the mass production of genetically improved seeds to create plants or seeds for the establishment of new forests. The rationale behind a seed orchard is that by planting multiple units of superior genotypes together in proximity, the resultant seed will be the offspring of only superior selected genotypes, and the offspring should be as good as, if not better than, the parents for the characters of interest. Seed orchards are a common method of mass-multiplication for seed production and can be made up of clonal populations of selected genotypes or seed-derived progeny of superior trees. At this time, rather than developing superior lines through controlled breeding, it was determined that a seed orchard using previously selected genotypes to be established to provide improved seed for future breeding, development, and deployment.

The seed orchard comprised 90 individual plants from 15 genotypes. Selected plants were propagated by cuttings, with mother stock plants sourced from the "Southern Grampians/Gariwerd" collection (genotypes B, C, D, E, H, J, L, M, P, Q, R, S, T) and three from "Vic – commercial 1" seed source, being genotypes403, 406 and 407 that performed well at Waite (Table 1). The final selections were made by reviewing observed and measured growth data as well as nectar DHA values from KA2: Environment Nectar Collection and Testing.

The orchard comprised six cutting-grown clones each of 15 genotypes selected from all plants available at the time of selection, ensuring that all plants within genotypes are identical clones. Plants were planted in a randomised design, to ensure that all genotypes were able to pollinate equally and randomly with any other of the 14 genotypes, but not themselves. Plants were established with irrigation at ~14L/week and spaced at 3 x 3m.

In January 2022, 56 individual plants from all genotypes had flowered across the site and seed was available for harvest. It is expected that more plants will flower in the 2022/23 season and greater volumes of seed will be produced. Six plants were reported as deceased, randomly across genotypes.

Table 5: Randomised layout of the Wirrabara *Leptospermum scoparium* Seed Orchard 2020.

R x C	*1*	*2*	*3*	*4*	*5*	*6*
1	E	B	D	M	P	407
2	P	406	E	406	L	406
3	D	J	P	B	H	C
4	Q	S	H	R	406	H
5	L	P	C	D	Q	E
6	S	R	L	Q	B	P
7	J	H	R	H	407	403
8	B	403	S	L	C	R
9	C	407	407	407	S	J
10	406	M	Q	J	403	D
11	R	D	M	403	R	L
12	H	L	406	S	D	M
13	407	Q	403	C	E	S
14	M	E	J	P	M	B
15	403	C	B	E	J	Q

Implications and recommendations

Genetic diversity for future breeding and seed orchard establishment

Genetic analysis of the *L. scoparium* plant material collected and used in this project suggests there is sufficient diversity to progress breeding. Understanding the structure of the population will enable the future crossing of cultivated individuals from different genetic clusters. The wider the genetic difference should result in a greater gain and produce diverse progeny from which to select new elite varieties.

In the case of *L. continentale*, whilst enough diversity was captured in the assessed populations, the plants were not adequately tested for nectar quality, and there is presently an insufficient plant number to commence a breeding program. This can be resolved with further nectar testing of wild plants and subsequent collection of high-yielding lines from similar populations used in the genetic analysis. Once these new plants reach flowering age, a similar breeding strategy to *L. scoparium* can be implemented.

Whilst this was a small and intense study, future research could include the evaluation of other characteristics important to honey production from plantations, such as the flowering period, DHA: Tsugar production over time in various environments, and the suitability of other sites and climates in South Australia. In addition, a further taxonomic revision of *L. scoparium*, *L. continentale,* and other closely related species is required to clarify placement within the *Leptospermum* alliance (refer to Binks *et al*, 2021).

Vegetative propagation

With the selection of elite plants, this project identified vegetative propagation methods that can be easily scaled numbers to assist the industry to produce plants for further plantings. Initially, selections from the wild are a greater challenge to bring into cultivation; and so, their propagation assessment should not occur until they acclimatised in the nursery under ideal conditions.

Repeating the propagation experiments will add confidence to the vegetative ability of each *Leptospermum* species. Although currently over 300 cutting-derived plants are growing and flowering well in two different sites, in terms of plantation establishment this is a small number. Continuous improvement to offer economical gains in the plant-production system will be necessary for the industry to adopt vegetative propagation.

The advantage of clonally propagated plants is that measured genetically inherited traits are directly transferred via the cuttings into the newly established plantations. They also have value to researchers to assess how selections grow, flower and produce DHA under different environmental conditions (irrigation, soil, climate, etc.). Further research is necessary to identify how selections perform to ensure only the best selection is provided to the industry. Clonal propagation will also be important for the establishment of seed orchards, enabling superior lines to be planted after testing to fast-track elite seed production.

Environment

Over the project period of four and a half years, we undertook observations of both weather/environment and plants, to provide a deeper understanding of the conditions required to support the economic production of bioactive honey from *Leptospermum* species. We focused on regions in South Australia that are most likely to establish *Leptospermum* plantations, based on long-term weather data (rainfall and temperature) and matching to natural environments, as well as considering the availability of supplementary water for irrigation, land value, alternative land uses, and proximity to key processing facilities. In those seven locations, we established plantations (Wirrabara and Wait or made observations on sites recently established (Bridgewater, Charleston, Macclesfield, and Port Vincent), or used a site where local species are naturally occurring (Charleston and

Mylor). Our other focus was species and genotype selection, where we monitored individual plants from different species across the project for growth, flowering, and DHA production.

We were able to establish clonal populations at two sites of selected genotypes and sample DHA from clones at both sites. DHA: Tsugar readings from clonal individuals of five selected genotypes were sampled for three years, and the results were variable, indicating that local environments were playing a part as well as overarching climatic conditions. For example, Genotypes E, H, and M varied considerably in their DHA values between sites in the same year, but Genotypes S and T were relatively uniform. There is an indicator of a strong environmental influence in the 2021-22 flowering season, as all values for the genotypes were much lower than either 2019-20 or 2020-21. Interestingly, Genotypes E and M produced much higher DHA: Tsugar values in 2020-21 compared to the original value from 2016, raising the possibility that any of the selected genotypes could perform better than previously under different environmental conditions. The variability observed in the DHA: Tsugar and Est Sugar Mass raise the possibility that the sampling method used has inherent variability and needs review. These findings, of variability within the same plants and genotypes but also consistency in others, means that elite lines can still be found with further selection and breeding. The 2020-21 nectar collection season produced very high values for DHA: Tsugar across most sites and genotypes. When drilling deeper into the environmental data for the 20-21 nectar sampling season (which occurs between October and January depending on the site), the recorded rainfall for October 2020 was very much above average across the whole of South Australia, while November and December were lower (refer figures 10 to 16). The high October rainfall could have provided a near-optimal amount of soil moisture for plants coming into flowering, then the following drier conditions resulted in clear weather for flower production and nectar flow.

It is worth noting that Douglas (2019) summarised several key findings from the recent 8-year New Zealand manuka improvement program, which bear similar findings. They reported that good plantation design, plant selection, and matching provenance/selection with the site (soil, aspect) and local weather, will aid plant strong growth and nectar production. They state that the timing of flowering and level of nectar production can be predicted between locations when a superior selection is grown but is influenced by the season at each site and that climate factors drive both sugar content and DHA concentration in nectar. Flowering must coincide with good weather for bee activity, which is not usually a problem for *L. scoparium* in South Australia with our mild springs, however, the recent pattern of warmer drier spring weather will likely impact nectar flow if soil moisture is inadequate. From their summary, other important findings are soil type affects plant growth, flowering time, and duration, but not nectar quality; response to water stress varies between selections; and plantations need multiple selections, where each one is tailored to different microclimates and to extend the flowering season.

While the observations of *Leptospermum* plant growth and flowering across seven sites in South Australia have provided excellent baseline information to support the establishment of bioactive honey plantations in South Australia, it is evident that there are still areas of concern that require further evaluation and investigation. Soil moisture appears critical, and it needs to be determined whether it is required at particular times, or generally available at consistent levels throughout the year. Can plantation heat/cold/evaporation be managed by inter-planting and between row planting with other plants (avoiding those that flower at the same time as the *Leptospermum* species)?

We recommend ongoing investigations into:

- Expanding the site survey to more species and genotypes in more sites across South Australia
- The role of soil moisture in plant growth, flowering, nectar flow, and DHA: Tsugar concentration
- Environmental conditions such as high heat during flowering and its impact on timing, flower longevity, nectar flow, and bee foraging

- The method for testing DHA: Tsugar to optimize reliability and reproducibility
- The role of inter-planting and edge planting to manage plantation micro-climates and support bee health without impacting the purity of the subsequent honey produced
- Evaluating genotypes/species for high performance in different environments under different soil moisture regimes
- Evaluating the role of arbuscular mycorrhizal fungi (AMF) in *Leptospermum* growth, flowering, and nectar production
- The foraging and management of European honey bees in large-scale plantations, particularly about off-target species nectar foraging.

Seed orchard

The seed orchard will provide Spring Gully Foods with a readily available supply of seeds for the planting of future elite seed orchards and bioactive honey plantations. As the seed orchard has reached reproductive maturity, ongoing observations can be made to link flowering and nectar production through different seasons with different genotypes.

It may become apparent that some clones are more adaptable to the conditions at the field site (borderline for *Leptospermum* survival if unirrigated) and thus could be selected for propagation by cuttings and used to develop new lines for specific sites and conditions. These superior clones will be available for targeted combinations through controlled pollinations, to produce progeny with specific characteristics. These progenies could be used for the identification of molecular markers (and putative gene regions) linked to high DHA production by screening progeny of a cross between a high DHA and a low DHA-producing plant. This information can be used to progress the breeding and improvement program.

The seed orchard can be used to assess nectar flow by volume and DHA: Tsugar concentrations over time, within and between flowering seasons, as well as bee foraging activity and honey production under reasonably controlled conditions.

The Wirrabara Seed Orchard is a valuable resource that provides a starting point for a *Leptospermum* breeding and development program. We have observed that seedling-derived plants reach flowering at 3 years of age and cutting grown plants may achieve these 1-2 years after propagation. Seed takes 6-12 months to reach maturity on the plant before it can be harvested. This site is therefore ready for the next steps to further the breeding and development program, and we recommend that:

- The seed orchard is continuously maintained with irrigation and weed control
- Seed is collected regularly; the seed is planted, and progenies were grown for testing and selection, removing underperforming clones and lines.
- All plants are assessed yearly for nectar volume, DHA: Tsugar ratios, and total sugar mass, to develop information about the heritability of DHA: Tsugar ratio and nectar production volumes.

- Controlled crosses are undertaken between selected lines and use the progenies to identify molecular markers linked to DHA production
- Provide elite material for interested parties under arrangement with industry owners
- Use the replicated randomized trial to investigate the role of soil moisture and irrigation on numerous aspects of plant growth, flowering, and nectar production.

To build on the first seed orchard and progress the breeding and development program, we also recommend that:

- Observations are made each year to select high-performing genotypes from within existing and future plantations (outside of this site) for adding to this seed orchard or creating new seed orchards in new locations.
- Incorporate high-performing genotypes from other DHA-producing *Leptospermum* species that are adapted to drier environments to assess natural hybridisation or undertake controlled crosses to produce hybrid lines for evaluation.
- Provide elite material for interested parties under arrangement with industry owners.
- An agronomic investigation plan is developed to provide clear direction for future research into environmental conditions and soil types to suit different selections, in different locations.

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Appendix

Height and width data for seven sites and three species

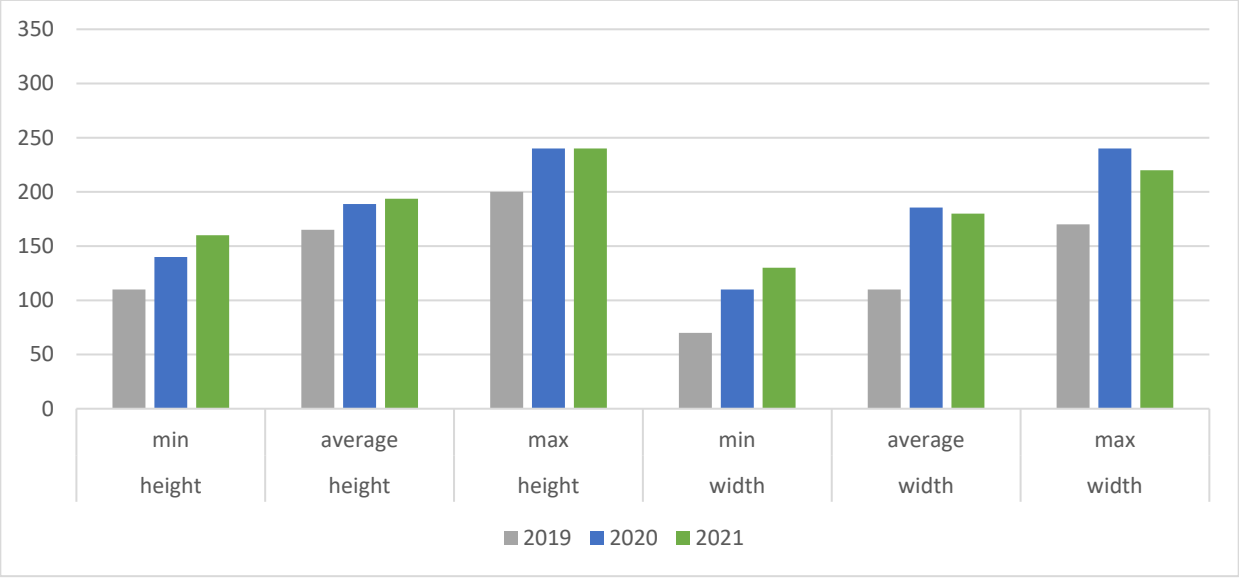

Figure 32: Bridgewater *L. polygalifolium* Vic – commercial 1: height and width, minimum, average, and maximum recorded over 3 years. Y axis = cm.

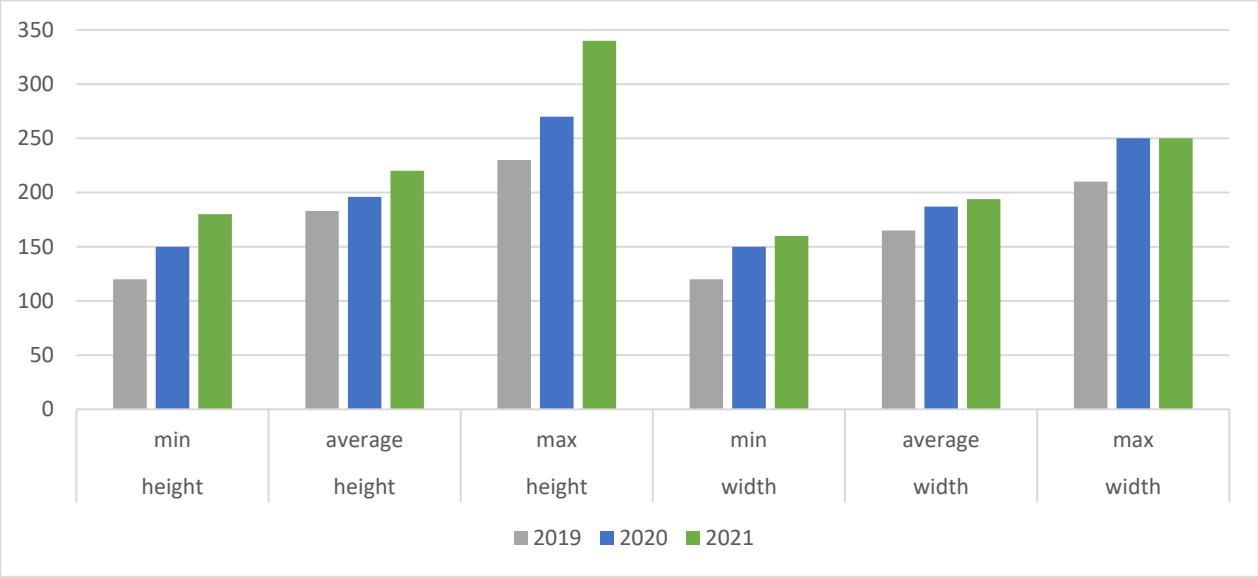

Figure 33: Bridgewater *L. scoparium* Vic – commercial 1: height and width, minimum, average, and maximum recorded over 3 years. Y axis = cm.

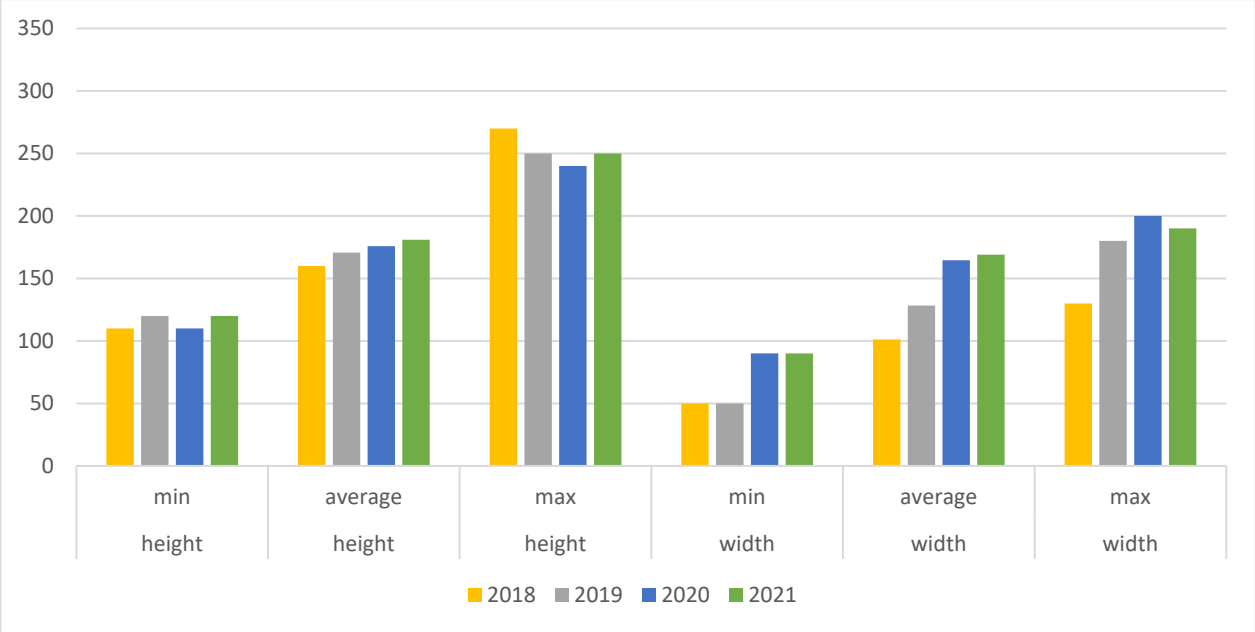

Figure 34: Bridgewater *L. scoparium* NZ – commercial: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

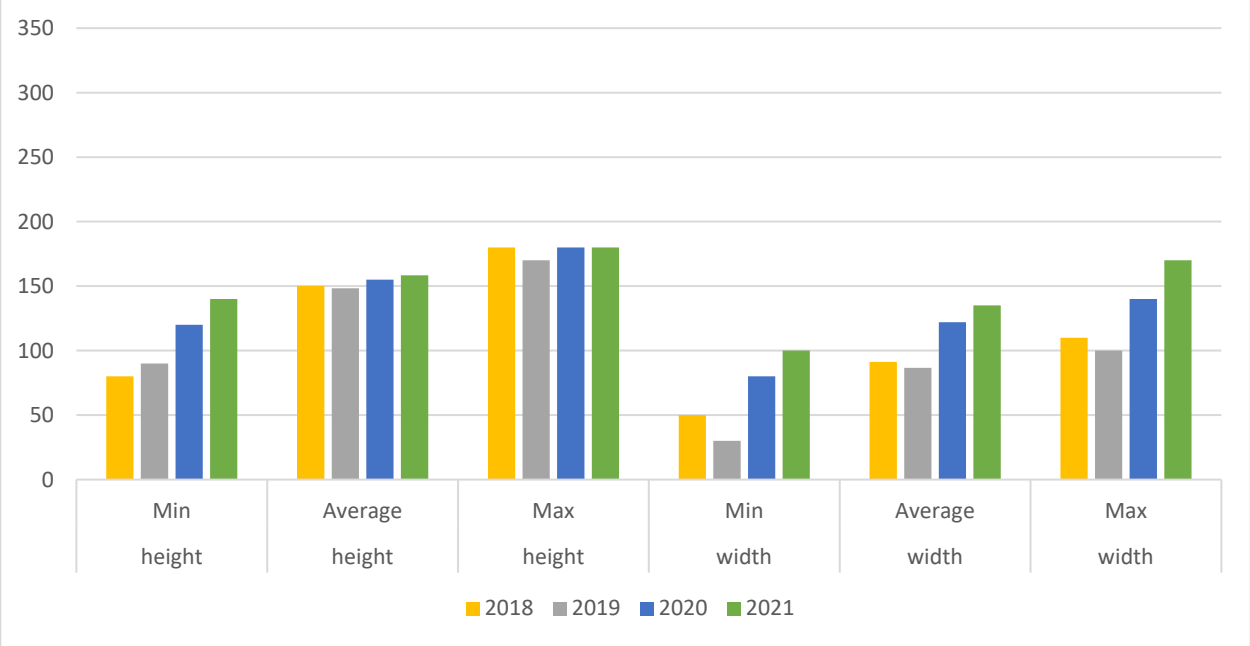

Figure 35: Charleston *L. scoparium* Tas – commercial 2: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

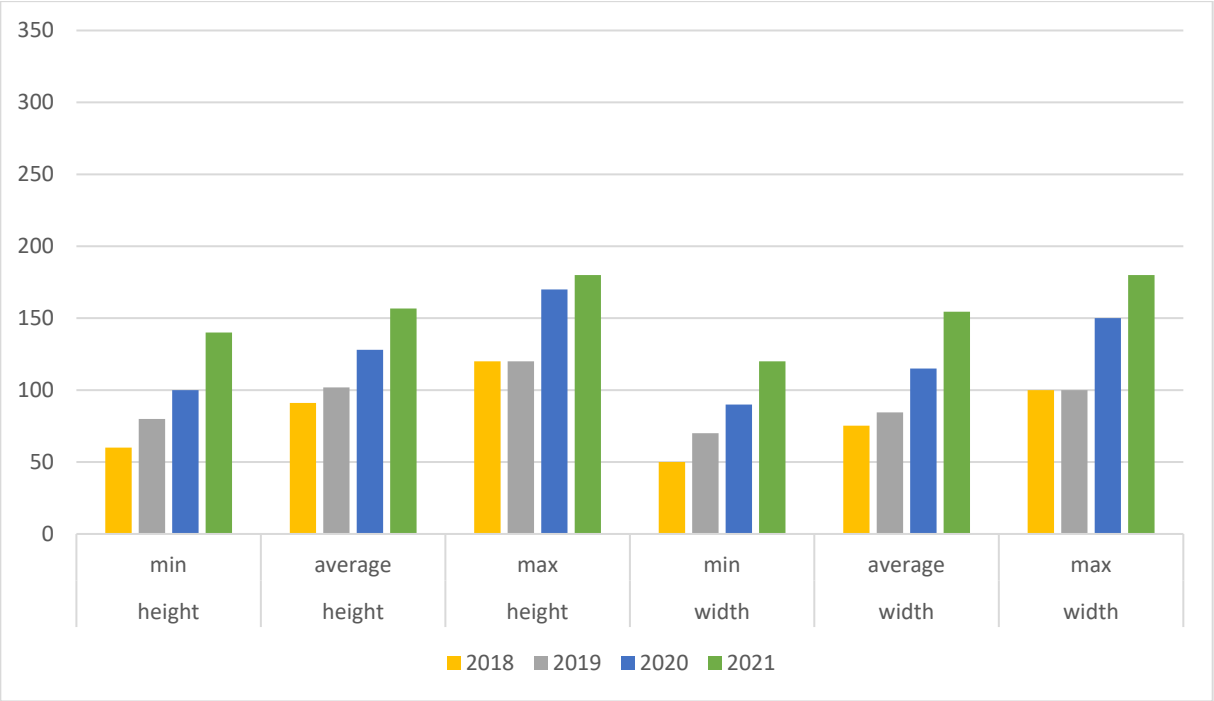

Figure 36: Macclesfield *L. scoparium* Tas – commercial 2: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

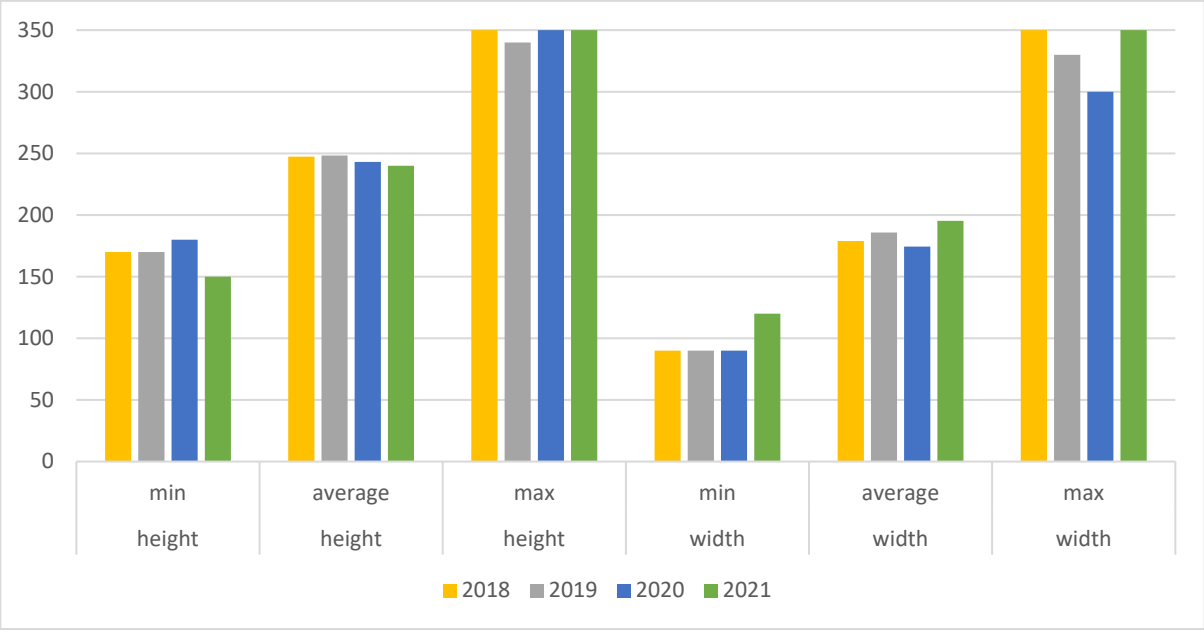

Figure 37: Mylor *L. continentale* – indigenous: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

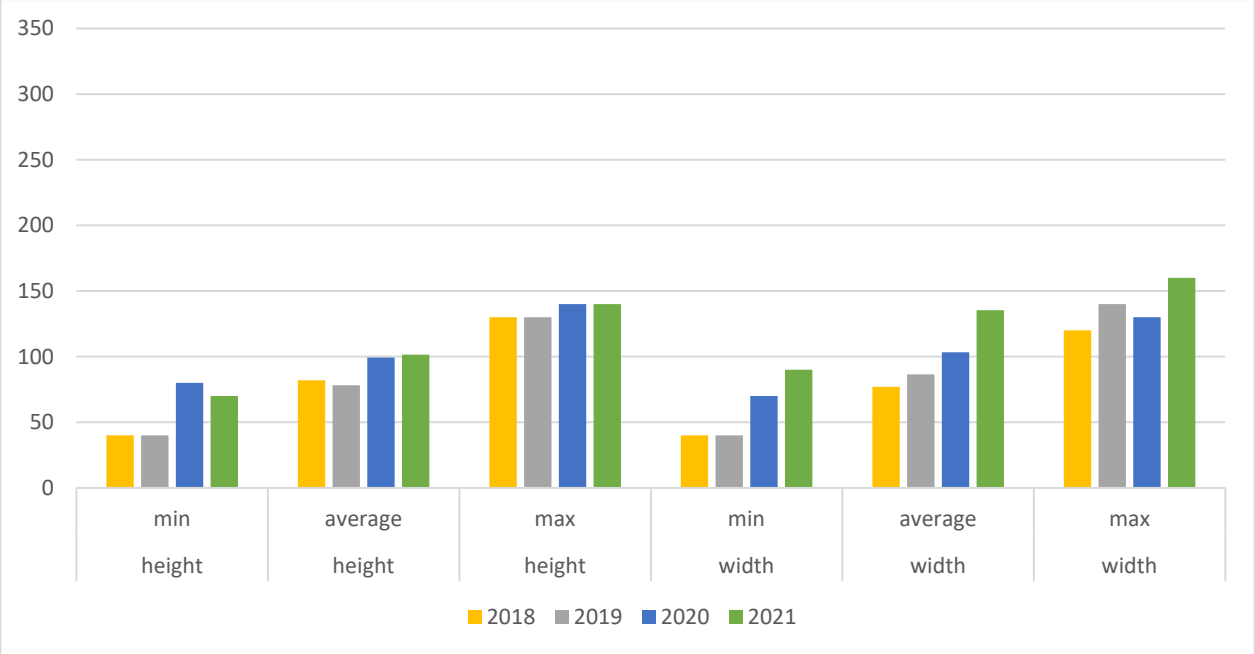

Figure 38: Port Vincent *L. polygalifolium* Vic – commercial 1: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

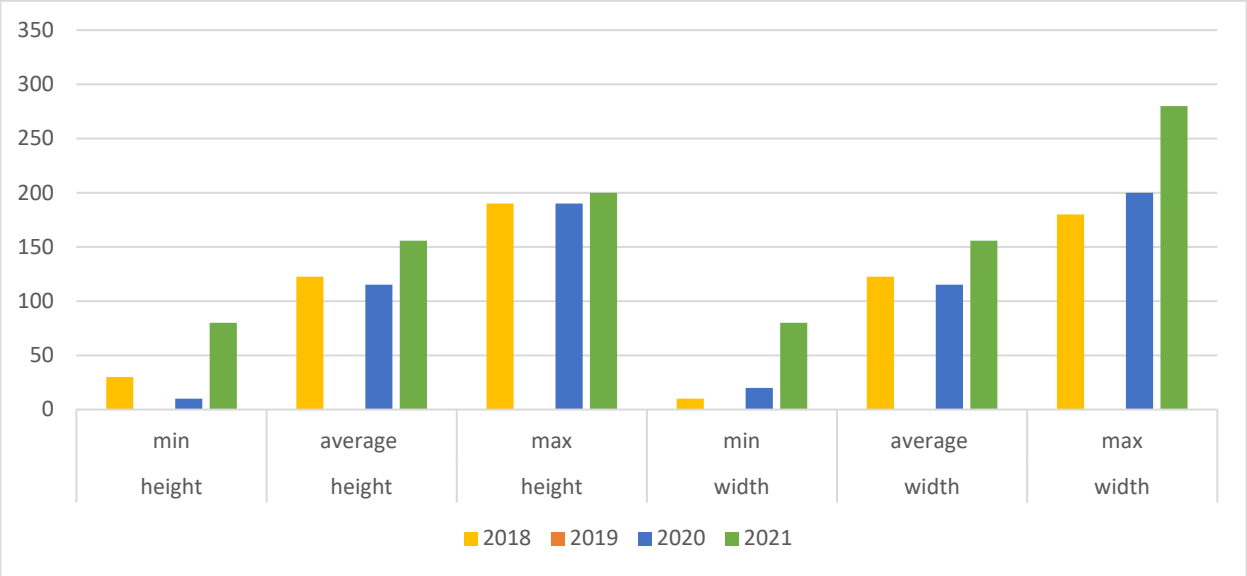

Figure 39: Waite *L. scoparium* Vic – commercial 1 and Vic – selected combined: height and width, minimum, average, and maximum recorded over 3 years (data not available for 2019). Y axis = cm.

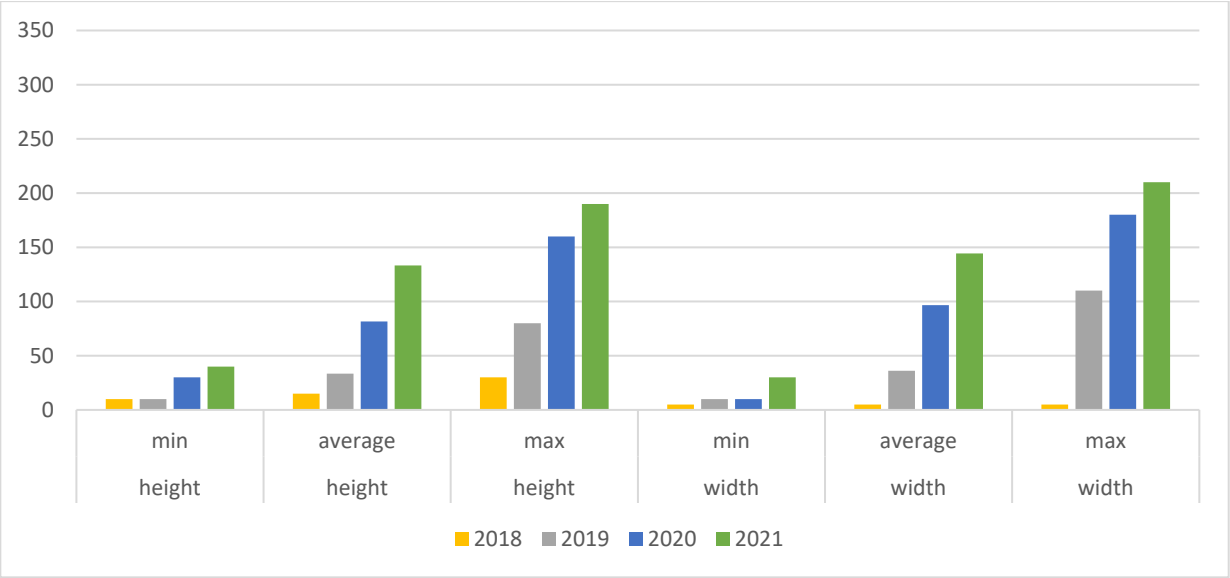

Figure 40: Wirrabara *L. scoparium* Vic – commercial 1: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

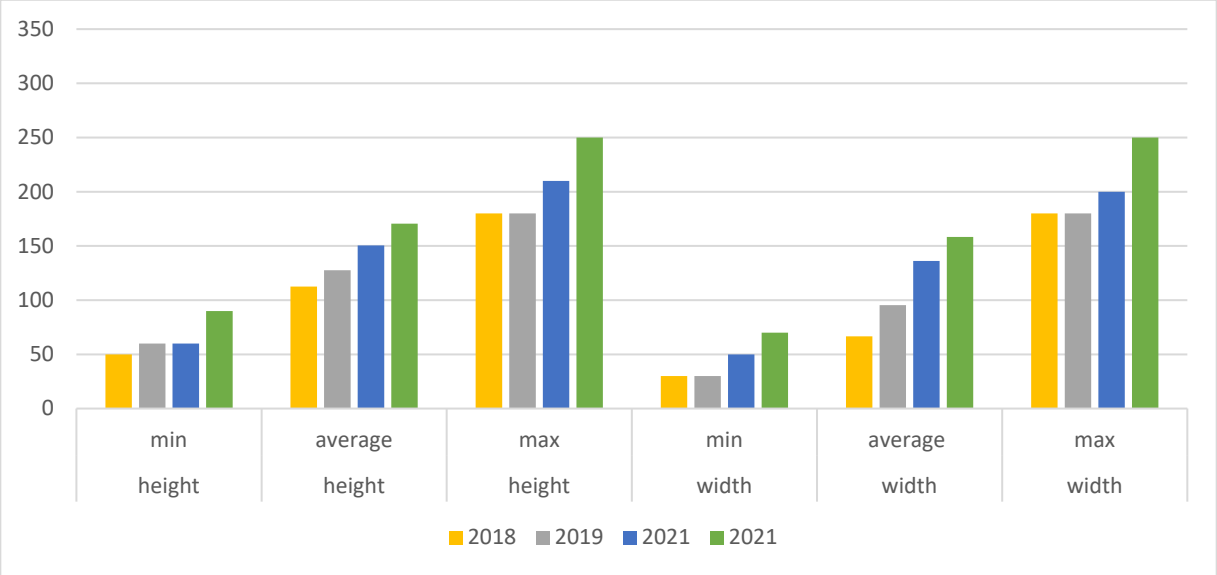

Figure 41: Wirrabara *L. scoparium* Vic – selected: height and width, minimum, average, and maximum recorded over 4 years. Y axis = cm.

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