Preliminary investigations into new opportunities for the production of premium and medicinal honey

by Kevin Foster, Megan Ryan, Daniel Kidd,
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Foreword

With the growing number of beekeepers and decline in native floral resources, new pollen and nectar sources are needed to sustain the continued health of the beekeeping industry in Australia. With fewer traditional native flora apiary sites, sustaining bee health through quality nutrition is a constant challenge for the industry. This project investigated a solution that would benefit agricultural systems more generally: annual and perennial nitrogen-fixing legumes, mostly clover (*Trifolium*) species. These species are important ruminant livestock forage sources across southern Australia, with many adapted to low-rainfall environments, particularly those with Mediterranean origins. Many of these species also produce copious nectar, and when foraged by a bee produce honey with distinct complex flavour profiles. Planting bee-friendly pastures comprising annual and perennial legume forages could help supplement native floral resources. Selecting a mix of legume species that together provide prolonged flowering periods could aid floral continuity under variable conditions.

There are five key messages from this report: (1) the extensive literature review revealed that pasture legume species are well-suited to providing nutritious and melliferous floral resources for the beekeeping industry across southern Australia; (2) enclosed shade houses can be used as experimental plots for the collection and analysis of monofloral pollen, nectar and honey, as long as the ratio of bees to floral resources is suitable; (3) there was a wide range of flowering times among the cultivars/selections examined, with flowering for *T. incarnatum* 'Caprera' early selection commencing in July, and continuing into February for *B. bituminosa* and *O. viciifolia*; (4) nectar sugar and pollen crude protein data indicate the suitability of pasture legumes as a floral resource for bees, consistent with the literature for *T. repens* and *T. pratense*; (5) the cultivars/selections had similar honey and nectar quality but differed significantly in chemical composition, antioxidant activity and antibacterial activity – some had particularly favourable qualities, such as high antioxidant activity indicators.

This project was funded by the Australian Government through the Department of Agriculture, Water and the Environment and is part of the 'Promoting the Importance of Bees' program. It is an addition to AgriFutures Australia's diverse range of research publications, and it forms part of our National Challenges and Opportunities Arena, which aims to identify and nurture research and innovation opportunities that are synergistic across rural industries. Most of AgriFutures Australia's publications are available for viewing, free download or purchase online at www.agrifutures.com.au.

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Contents

Tables

Figures

Executive summary

What the report is about

Beekeeping in Australia has thrived on our unique native flora and mild climate, resulting in the healthiest honey bees in the world and the cleanest honey bee products available. However, stresses are becoming evident, with demand for insect pollination growing and the loss of spring flora impacting honey bee colony strength and subsequently pollination services.

Pasture legume species are recognised as a quality floral resource as they provide crucial crude proteins for bee health. These exotic legume species can provide supplementary floral resources for apiarists in Australia while also providing a valuable animal fodder resource with their complementary nitrogen-fixation ability, reducing the need for manufactured nitrogen fertiliser. Annual and perennial pasture legume species can also be supplementary forage resources for endemic bees and other flower visitors increasingly impacted by climate change and fire.

This project was designed to provide a selection of bee-friendly pasture plant species suited to southern Australia, with information on their flowering windows and quality of pollen and nectar, for beekeepers, agriculture pollination services and pasture managers. Honey profiles, bioactivity and medicinal characteristics were explored for their commercial value.

Who is the report targeted at?

This report is written for all those interested in the beekeeping industry, industry association members in each Australian state, professional apiarists, and the increasing number of people and farmers who keep bees but do not depend solely on beekeeping for their livelihoods. The report will also benefit seed producers, pasture seed companies and agricultural consultants.

Apiarists and farmers will be the project's main beneficiaries as many pasture legumes are prolific nectar and pollen producers and can be used for both bee-friendly pastures and ruminant livestock forage. This report is also targeted at people who wish to develop honey products with potential medicinal properties or commercial pollen production for health foods or supplementary feeding.

Where are the relevant industries located in Australia?

The relevant industries in Australia are located in the states with the strongest beekeeping industries. Australia's principal honey-producing area is the large band of temperate land stretching from southern Queensland to central Victoria, including the Australian Capital Territory. Western Australia and South Australia are also significant honey-producing states. Tasmania is the smallest producer.

Background

Australia has huge potential for many locally adapted and commercially available annual and perennial legume forages to be sown on farms or near horticultural and viticultural crops, to improve the consistency and diversity of nectar and pollen supply to the beekeeping industry. Developing different uses for apiary products could also assist the Australian beekeeping industry. The use of honey from legumes as a therapeutic agent is an exciting prospect for the beekeeping industry and the demand for specialist pharmaceutical honey products is gaining momentum worldwide. Researchers overseas are developing treatments and understanding the factors that provide the medicinal activity.

Aims/objectives

The project objectives were to:

1. Conduct a desktop literature review to identify annual and perennial legumes adapted to southern Australia that are best-suited to producing pollen and nectar with bioactive properties.

2. Conduct field-based trials that practically demonstrate to the pasture-based and beekeeping industries the farming practices required to produce premium-grade honey.

3. Assess honey quality produced from commercial legume cultivars for bioactivity and medicinal characteristics.

Methods used

A systematic literature review was conducted on the body of peer-reviewed scientific research on pasture legumes and their interaction with *Apis mellifera* (European honey bee). A steering committee and industry advisory panel was established with an enthusiastic and diverse group of representatives from across Australia to help guide the scientific and technical direction of the project. Discussions ensued with seed producers, commercial and amateur apiarists, agricultural consultants, and current and former bee researchers, including those at the CRC Honey Bee Products.

Thirteen pasture legume cultivars or selections from nine species (including two *T. pratense* cultivars previously developed for pharmaceutical use) well-suited to southern Australian growing conditions were sown in enclosed shade houses at The University of Western Australia (UWA) Shenton Park research facility to assess the monofloral characteristics of bee-collected nectar and honey. The species in the enclosed shade houses had a wide range of flowering maturities and were chosen as they are suited to key soil types and rainfall zones across southern Australia. There was some important methodological development regarding the provision of bees to pasture legume species in small, enclosed shade houses and the techniques for extracting flower nectar and pollen.

The bee colony size maxima that could be sustained in the small, enclosed shade house plots could only be estimated. Nectar was sampled from the florets of most of the pasture species to determine both volume and quality (sugar concentrations) using high-performance liquid chromatography. Pollen was removed by hand from individual florets of each species and analysed for crude protein. In the field, honey and nectar were collected from seed crops of *T. michelianum* (balansa clover) and *T. resupinatum* (Persian clover) from our commercial partner. These two legumes were selected as they are highly attractive to bees and perform well across a range of soil conditions and climates (Rob Bell, pers. comm.). We also sampled one small plot of *Hedysarum coronarium* (sulla) grown for seed increase at the UWA Shenton Park research facility.

Due to the limited amount of nectar and honey produced in the enclosed shade houses, not all cultivars/selections could be tested for all parameters. Total phenolics content of honey and/or nectar for nine cultivars/selections was determined using a modified and validated UV-Vis analysis, and antioxidant activity was measured by the (2-diphenyl-1-picrylhydrazyl) and ferric reducing antioxidant power assays. Nine samples of either honey or nectar from six legume cultivars/selections were screened for antibacterial activity against four pathogens.

Results/key findings

The extensive literature review indicated that many pasture legume species are well-suited to providing nutritious and melliferous floral resources for the apiculture industry in Australia. The reviewed pasture legumes have physiology and attributes adapted to key soil types and rainfall zones, and are mutually beneficial to both ruminant livestock and bees. In addition, these pasture species have vast adaptability to a range of potential planting zones in Australia. Critically, for an apiarist, clover crops provide a predictable window for floral resource availability for bees.

For the annual and perennial legumes species grown in the enclosed shade houses and in the field, there was, as expected, a large range of flowering maturities. Flowering started in early July for *T. incarnatum* (crimson clover) cultivar 'Caprera' early selection, and continued into February for *B. bituminosa* and *O. viciifolia* (sainfoin). Pollen that was hand-collected from the flowers was high in crude protein, ranging from 33–47%. Sucrose was the greatest contributor to nectar sugars, with *H. coronarium*, *T. incarnatum* and a cultivar of *T. pratense* producing the highest amount per flower head. Nectar volume per floret was somewhat proportional to flower size, with the highest volume in the larger flowers of *H. coronarium* and the lowest volume in the smaller flowers of *T. vesiculosum* (arrowleaf clover).

A subset of honeys and nectars from the enclosed shade houses exhibited a range of bioactivity profiles (antibacterial vs antioxidant); some had high antioxidant activity. The nectar and honey of several clovers had noticeable antibacterial activity against a range of pathogens. Nectar or honey of *O. viciifolia, T. vesiculosum* and *T. resupinatum* had the highest total phenolic content values (gallic acid equivalents (GAE) >30 mg/100 g).

Implications for relevant stakeholders

Annual and perennial legumes can provide good continuity and predictability of nectar supply for the beekeeping industry. There is also the potential for new and unique honey types from these novel floral sources, which may improve the market value of their honey products. In addition, there is an opportunity for beekeepers to diversify and develop products specifically for their medicinal properties or pollen production for health foods or supplementary feeding.

Recommendations

1. Extend the current work to a multi-year project to investigate the potential for production of high-quality honey from annual and perennial pasture legumes at the commercial paddock scale.

2. Investigate the utility of strategic mixes of pasture legume cultivars to extend the flowering window and produce nectar and honey with desirable characteristics.

3. Identify the best management practices for bees in pasture production systems currently used for ruminant livestock production or commercial seed production.

4. Examine the impact of farming practices (such as crop nutrition) on pollen, nectar and honey quality and quantity.

5. Commence preliminary investigations into other commercially available pasture legume species with existing cultivars and promising floral characters, to broaden the diversity and adaptation range of bee-friendly pasture options for apiarists.

6. Investigate the potential of bee-friendly pasture legume cultivars for developing new and unique honey types with medical or pharmaceutical significance.

7. Produce a ‘bee-friendly pasture picker’ tool and ute guide to help farmers choose the appropriate bee-friendly pasture mixes for soil and rainfall zones across southern Australia, and an agronomic package for farmers outlining how to best manage bee-friendly pastures for pasture and honey bee benefit.

8. Facilitate further discussions between the pasture seed industry and the beekeeping industry to identify common goals.

Introduction

Many exotic annual and perennial pasture legume species have been developed as commercial cultivars for use as forage in pastures across southern Australian agricultural regions. These species include *Trifolium* (clover) species and other legumes. Despite their overseas origins, they are well-adapted to Australian climatic conditions and soil types, and are therefore able to produce flowers and set seeds under a range of seasonal conditions. The annual forages are self-seeding and designed to re-establish year after year without repeated sowing. Because of the importance of these pasture legume species to our agriculture systems, an Australian seed store of species and varieties has been maintained.

Among the annual and perennial forages, there is wide range in flowering maturation timing, and some have deep roots that allow them to remain green and flowering well into summer. Most forages flower during spring, but if mown or grazed, some species can extend their flowering duration into summer. In addition, when pollinated by bees, many forages set more seed, benefiting pasture seed producers or farmers who want to provide nutritious and dense pastures for winter and spring grazing by ruminant livestock.

These forages have co-evolved with honey bees and hence offer a floral morphology that is functionally well-suited to bee pollination (Palmer et al., 2009), with anthers and stigmas often uncovered in the flowers. Flowers are usually racemes or panicles, with a high number of reward centres per planting area. Clover honey is produced from flower nectar and is popular for its sweet, smooth, mild flavour and light amber colour. However, the honey crystallises quickly due to its high glucose-to-fructose ratio and has not been explored for its health benefits in Australia.

Forage species such as *Trifolium incarnatum* (crimson clover), *T. michelianum* (balansa clover), *T. glanduliferum* (gland clover), *T. vesiculosum* (arrowleaf clover), *T. pratense* (red clover), *T. hirtum* (rose clover) and *T. fragiferum* (strawberry clover) are considered valuable crops for honey bees (SARE, 2015), particularly as they are a reliable spring food source when bee colonies are building for pollination services and to capture the remaining honey season.

Despite the potential benefit of legume forages for agriculture and honey bee health, most remain unassessed and not exploited specifically for pollen or nectar in Australia. Thus, it is likely that these legume forages represent an under-utilised resource for beekeepers. The opportunity to add value by breeding these forage plants for medicinal honey is attractive.

Project objectives

1. Conduct a desktop literature review to identify annual and perennial legumes adapted to southern Australia that are best-suited to producing pollen and nectar with bioactive properties.

2. Conduct field-based trials that practically demonstrate to the pasture-based and beekeeping industries the farming practices required to produce premium-grade honey.

3. Assess honey quality produced from commercial legume cultivars for bioactivity and medicinal characteristics.

Chapter 1. Desktop literature review to identify annual and perennial legumes adapted to southern Australia that are best-suited to producing pollen and nectar with bioactive properties

Literature review methodology

A systematic literature review of the body of scientific research in peer-reviewed journals on pasture legumes and their interaction with *Apis mellifera* was conducted between July and December 2020. The review spanned studies from 1950–2020 due to the availability of articles and records. Search terms for flora included ‘pastures’, ‘pasture legumes’, ‘clover’, and ‘legume’, in conjunction with ‘annual’ or ‘perennial’, and multiple species names identified within the search and proposed through consultation with industry, the project advisory panel, Department of Primary Industries and Regional Development (DPIRD) and specialists in pasture legume research at The University of Western Australia.

Search terms also included ‘pollen’, ‘pollination’, ‘nectar’, ‘flower’, ‘flowering’, ‘phenology’, ‘morphology’, ‘ontogeny’, ‘physiology’, ‘yield’, ‘productivity’, ‘growth’, ‘rain’, ‘soil’, ‘tolerance’ and ‘climate’ in conjunction with the flora terms presented. The search was not restricted to a particular geographical location, but available data were interpreted for presentation within the literature review. Search terms for fauna included ‘*Apis mellifera*’, ‘bee’, ‘honey bee’, ‘European honey bee’, ‘pollination’, ‘morphology’, ‘health’ and ‘attraction’. Platforms for the search included Google Scholar, UWA OneSearch and a private collection on Mendeley, ‘Australia NZ Bee Research Compilation’, compiled by the CRC for Honey Bee Products.

Annual and perennial pasture legumes are a high-quality floral resource for apiarists: a review

Introduction

The Australian beekeeping industry traditionally relies on native forests and coastal plains as forage resources for European honey bees (*Apis mellifera*). However, floral resources required for bee health, work capacity and honey production are declining in Australia and worldwide (Potts et al., 2010; Goulson et al., 2015). Human intervention is largely responsible for these losses due to urbanisation, globalisation and a changing climate (Cunningham et al., 2002; Batley and Hogendoorn, 2009; Winfree et al., 2009; Chauhan et al., 2015; Carrié et al., 2017). In addition, the ever-increasing global population is increasing agricultural demand for essential crop pollination services and honey production (Aizen and Harder, 2009). The evolution of pasture legumes suggests that they will provide suitable floral resources for the Australian apiary industry.

Increasing floral resources for apiculture with pasture species

Worldwide, up to 75% of fruit, vegetable and seed crops require animal-mediated pollination, predominantly by honey bees (Klein et al., 2007). Australian food crops were introduced primarily from Mediterranean regions in Europe, resulting in a dependence on honey bees to service many horticultural, oilseed and biofuel crops, and pasture legumes (Paton, 1993). The estimated value of honey bee pollination to Australian agriculture is $10–15 billion (Hein, 2009; Karasinski, 2017), with an additional $90 million attributed to honey and beeswax production (ABARES, 2016). However, the viability of this valuable industry has come under increasing threat from external pressures, such as logging and increasing bushfire frequency, which have reduced the abundance and diversity of native floral resources in Australia. This decline in reliability and accessibility of native nectar and pollen sources is a significant issue confronting the beekeeping industry (Clarke, 2008).

Consideration of alternative plant species to supplement native floral resources is needed to ensure the long-term viability of the apiculture industry in Australia. Pasture legumes are considered desirable to enhance the floral resources for bees in agricultural systems in Europe and North America (Palmer et al., 2009; Decourtye et al. 2010).

Pasture legume species have been adopted across Australian agriculture as part of annual cropping systems (fodder, ley, silage, hay), cover crops in horticulture (water management, erosion control, weed control, nutrient input) and perennial agricultural systems (erosion control, buffer for spray drift, beautification and wild forage) (Peoples et al., 2009), but not considered for their use in the apiculture industry. However, advanced plant breeding programs in Australia, using germplasm primarily sourced from the Mediterranean basin, have led to a suite of locally adapted pasture varieties suitable for a range of soil types, grazing options, environments and tolerance to pests and diseases (Table 1) (Nichols et al., 2013), which could provide bees with a veritable buffet.

Pasture legumes in Australian agricultural systems

Pasture legumes were introduced to Australia as high-quality forage to increase the productivity of ruminant livestock production systems (Puckridge and French, 1983). Used as ley, phase and forage pastures across temperate Australian agriculture (Figure 1), these species provide agricultural benefits to Australian agricultural systems (Nie et al., 2016). Further to their use in agriculture, the annual production of an abundance of flowers from pasture legumes suggests a dual-purpose role. Access to commercially grown legume pasture species offers a potential floral resource to the apiculture industry, with some of these species already known as broadly attractive to bees (Decourtye et al., 2010).

Aerial-flowering pasture legumes are generally honey bee pollinated (Goodwin et al., 2011; Rundlöf et al., 2014) (Table 1). Bee pollination of annual clover crops (*Trifolium* spp.) increases seed set for commercial production (Blake, 1958; Hawkins, 1965; Palmer-Jones et al., 1962; Rodet et al., 1998; Cecen et al., 2007; Vleugels et al., 2019), but it is unknown whether pasture species could provide apiculturists with a mutually beneficial opportunity to maintain hive health – preceding or following their use for other pollination services – and produce high-quality honey.

Clovers are a predominant species in multifloral honey, indicating bee preference for these crops in an agricultural setting (Primorac, 2008). The capacity of Australian-adapted pasture legumes to provide a melliferous and bee healthy floral resource is not widely understood and warrants further investigation.

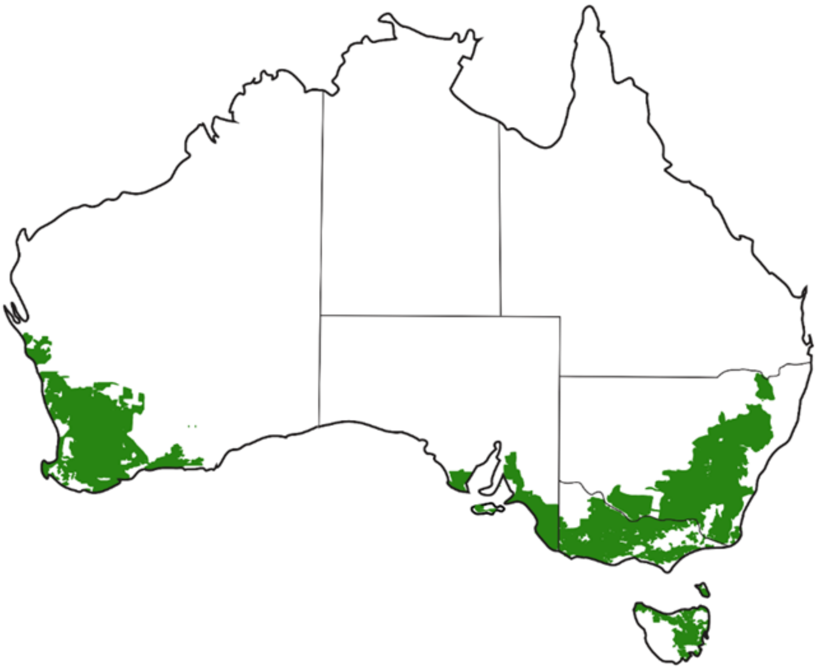

Figure 1. Zones suitable for planting leguminous pasture species in Australia (green).

Pasture legumes offer spatial stability

Pasture legumes provide the potential for spatial stability of floral resources. Australia's high honey production is only achieved because beekeepers migrate their hives from one favourable area to another throughout the year. However, this practice exposes bee colonies to increased stress, pests and diseases (Traynor et al., 2016). Furthermore, increasing drought, fire and logging may affect native plant survival (Costa e Silva et al., 2019) and flowering events (Campoe et al., 2020) for many years. Indeed, floral resources available to apiarists from Australian forestry tend to flower on two- to four-year cycles (Somerville and Nicholson, 2005). Subsequently, a range of pasture species that offer reliable annual flower resources across the Australian landscape commensurate with apiculture would be ideal.

Pasture species have vast adaptability to a range of potential planting zones in Australia (Table 1). Southern Australian cropping systems originated with European settlement but have become some of the most innovative, low-input and extensive systems worldwide (Connor, 2004; Ryan and Kirkegaard, 2012; Sadras et al., 2016). Due to the genotypic and ecophenotypic variability offered by leguminous pastures, numerous species and cultivars are suitable for various geographic environments across southern Australia (Figure 1), where the apiculture industry is located. Encouraging further adoption of pasture species in sustainable agricultural systems, with economic reciprocity through honey production or fee-paying services, is worthy of exploration.

Climate extremes are becoming progressively unpredictable (Alley, 2004). The increased fire frequency, drought frequency and changing temperatures significantly affect tree mortality and stand structure in Australian native forests (Evans et al., 2013; Etchells et al., 2020). Fire activity can lead to long-term losses in floral resources and essential nitrogen from the system (Goergen and Chambers, 2009), which, together with native forest logging of reproductively mature trees, makes apiary site security tenuous.

Australian native flora is adapted to a dry climate, but prolonged periods can adversely affect flowering and nectar production, and the ability of beekeepers to sustain colonies within a defined space in the landscape (Manning, 1993). Subsequently, annual and perennial pasture legumes can be predictable and responsive to apicultural needs relative to slow-growing silviculture.

Table 1. Summary of commercially available aerial leguminous pasture species and their typical planting suitability to temperate Australia.

Scientific name and common names	Life cycle	Feed type	Sand	Loam	Clay	pH	Tolerances				Rainfall range (mm)	Pest susceptibility	Disease susceptibility
							Waterlogging, low to high (1–9)	Drought	Salinity	Frost			
Trifolium glanduliferum Gland clover	Annual	Hay, grazing				4.5–8.0	5	Yes	No	Mild	350–600	Lucerne flea, lucerne aphid	None observed
Trifolium hirtum Rose clover	Annual	Hay, grazing				5.5–8.2	2	Yes	No	Yes	400–700	Mild tolerance	Not noted
Trifolium incarnatum Crimson clover	Annual	Hay, silage, grazing				5.0–7.0	2	Mild	No	Yes	>500	Blue-green aphid and red-legged earth mite (RLEM)	Crown & stem rot, fusarium wilt & fusarium root rot, bean yellow mosaic virus
Trifolium michelianum Balansa clover	Annual	Hay, grazing				4.2–8.0	9	No	Mild	Yes	350–800	RLEM, lucerne flea, blue-green aphid	Root rots if wet
Trifolium purpureum Purple clover	Annual	Hay, silage, grazing				4.5–8.0	6	Yes	No	Mild	>550	RLEM, blue-green aphid, native budworm	Chocolate spot, Phoma blackstem, bean yellow mosaic virus, cucumber mosaic virus
Trifolium resupinatum Persian clover	Annual	Hay, silage, grazing				5.0–8.0	8	No	Mild	Yes	>500	RLEM, lucerne flea	Leaf & stem rust, Clover rot
Trifolium vesiculosum Arrowleaf clover	Annual	Hay, silage, grazing				4.5–7.5	2	Yes	No	Mild	>350	RLEM, lucerne flea, blue-green aphid	*Phytophthora*, bean yellow mosaic virus
Hedysarum coronarium Sulla	Biennial	Hay, silage, ley, grazing				6.5–8.7	4	Moderate	No	Yes	500–900	Lucerne parasitic seed wasp	*Sclerotium rolfsii*, *Rhizoctonia solanum* AG 2-2, *Phytophthora medicaginis*, powdery mildew
Bituminaria bituminosa Tedera	Perennial	Grazing, hay				4.7–8.5	4	Yes	No	No	200	RLEM, aphids, budworm	*Phoma herbarum*, phytoplasma, root knot nematodes
Medicago sativa Lucerne	Perennial	Hay, ley, grazing				5.5–8.0	3	Yes	Mild	No	350–1200	Numerous mites, aphids, weevils, worms, thrips, snails, moths, wasps, mirids	Numerous viruses, rots, rusts, mildews, wilts
Trifolium fragiferum Strawberry clover	Perennial	Hay, grazing				5.5–8.0	9	No	Yes	Yes	600–1000	Aphids, RLEM	Not noted
Trifolium pratense Red clover	Perennial	Hay, silage, cattle grazing				5.5–7.0	5	Mild	No	Yes	>600	RLEM, pea aphid, blue oat mite, cut worms, bud worms, mirids, thrips	Root rot (*Phytophthora*), clover rot, crown rot, rust, powdery mildew
Trifolium repens White clover	Perennial	Hay, silage, cattle grazing				4.5–8.0	6	No	Mild	Yes	>700	RLEM, lucerne flea, corbies, cockchafers, nematodes, web cut and budworms, reticulated slug	Clover rot, wart disease, mosaic viruses, clover yellow vein virus, phyllody

Pastures offer phenological variability

Continuous flowering events are ideal for maintaining honey bee nutrition for bee productivity and hive health. Despite moving colonies between apiary sites, feed gaps often remain during winter, early spring and late summer (Somerville, 2000). Artificially feeding honey bees with sugar solutions or pollen substitutes is expensive, with much still to be understood on their benefit. Cheaper options to fill the feed gap are to use agricultural crops (e.g. early sown canola or lucerne), weed species (e.g. Patterson's curse), or horticultural species. However, these options can expose bees to chemicals, may be ecologically undesirable, offer minimal compensation, offer inadequate bee nutrition, or produce low-value apiary products (Gilpin et al., 2019; Somerville, 2005). Further floral limitations may occur when removing or controlling previously relied upon introduced invasive plant species (Chapman et al., 2008).

Advanced research in pasture physiology in Australia has developed techniques (e.g. management, breeding, vernalisation) to manipulate pasture crop phenology (Liu, 2007, Hayes et al., 2020). Providing a more consistent food source helps maintain large colony populations and reduces the need for or length of 'rebuilding phases' when hives are inactive (Decourtye et al., 2010). The opportunity to fill this feed gap using pasture legumes merits further exploration.

There are a variety of pasture legumes that can be used to capitalise on various phenologies (Table 2). Annual clovers generally flower during spring, but some species (mainly perennials) flower in late winter or, if mown or topped, in early summer (Johansen et al., 2019). The extension of available floral resources warrants further investigation to provide the apiculture industry with an alternative to bridge the feed gaps available from native sources.

Pasture species attractiveness and nutrition for bees

Many aerially seeding pastures bred in Australia (from Mediterranean origins) have flowers that are conspicuous to bees with easy access (Table 2). Pasture legumes belong to the subfamily Papilionoideae of the flowering plant family Fabaceae, where many taxa have a Papilionoideae flower form, familiarly associated with peas (Tucker, 2003). Inflorescences are usually racemes or panicles, indicating a high number of reward centres per planting area. Consequently, pollinators are more likely to visit plants offering more floral resources (Kunin, 1997). This floral abundance may be a key driver of bee behaviour (Pernal and Currie, 2002), broadly indicating mass flowering leguminous pasture species with high flower and floret numbers are suitably attractive to bees.

Understanding of floral signalling and pollinator reward, and their impact on honey bee behaviour and nutrition, is advancing. However, this field is complicated by the variability in quality and quantity of plant reproductive response to flower age, floret age in multiple flowers, plant age, time of day, time through season, genotype, phenotype, plant management (e.g. soil structure, nutrition, water availability, planting density), climate (e.g. diurnal temperatures, humidity, photoperiod), abiotic and biotic stress, and previous pollinator visitation (Boelt et al., 2015; Parachnowitsch and Mason, 2015). Unfortunately, limited examples examine these confounding factors in pasture species in the literature. Some include:

- Flower number per plant, nectar sugar concentration and volume per flower in *Trifolium pratense* varied with differing application rates of nitrogen, phosphorus and potassium (Shuel, 1957).
- High variability in nectar volume between day/night temperatures in *Trifolium repens* (Jakobsen and Kritjansson, 1994).
- Different flowering times due to temperature and photoperiod in *Hedysarum coronarium* and *Trifolium resupinatum* (Iannucci et al., 2008).

Although many pasture species are predominantly allogamous, even autogamous species such as crimson clover (*T. incarnatum* L.) can improve seed set through bee-facilitated tripping (Bohart, 1960). However, the exact morphological and chemical traits that make flowers attractive to bees is not well-explored in temperate pasture legumes (Suso et al., 2016).

Table 2. Summary of known floral morphology and phenology of commercially available aerial leguminous pasture species in Australia.

Common and scientific names	Life cycle	Flower colour	Flower protrusion (mm)	Growth habit	Pollination requirement (x = cross pollination)	Seed #/kg	Seed yield (kg/ha)	Potential pollinated florets	Typical flowering window (purple) or re-flowering after cut (grey)							
									A	S	O	N	D	J	F	M
Trifolium glanduliferum Gland clover	Annual	Light to dark pink	30–50	Semi-erect	Allogamous	1.40E+06	200–700	1.00E+09								
Trifolium hirtum Rose clover	Annual	Light to dark pink	50	Semi-erect	Allogamous	2.50E+05	250–700	1.75E+08								
Trifolium incarnatum Crimson clover	Annual	Bright red	50	Semi-erect to erect	Autogamous (improves with x)	3.2E+05	1000–1200	3.84E+08								
Trifolium michelianum Balansa clover	Annual	White to pink	100	Prostrate	Allogamous	1.20E+06	300–800	7.20E+08								
Trifolium purpureum Purple clover	Annual	Purple to pink	80	Erect	Allogamous	5.9E+05	500–900	5.31E+08								
Trifolium resupinatum Persian clover	Annual	Pink to violet	75	Semi-erect to erect	Autogamous (improves with x)	1.50E+06	150–300	4.50E+08								
Trifolium vesiculosum Arrowleaf clover	Annual	White to pink tinge	50–120	Semi-erect	Allogamous	8.00E+05	1400	1.12E+09								
Hedysarum coronarium Sulla	Biennial	Red to violet	30–160	Semi-erect to erect	Autogamous (largely allogamous)	2.0E+05	100–250	5.00E+07								
Bituminaria bituminosa Tedera	Perennial	Light blue to violet	20–100	Erect	Autogamous (some x)	5.00E+04	50–400	2.00E+07								
Medicago sativa Lucerne	Perennial	Blue, purple	40–80	Erect	Allogamous	4.40E+05	100–600	2.64E+08								
Trifolium fragiferum Strawberry clover	Perennial	Pink	20–35	Prostrate	Allogamous	8.00E+05	40–300	2.40E+08								
Trifolium pratense Red clover	Perennial	Red to rose purple	50–75	Erect	Allogamous	6.00E+05	250–600	3.60E+08								
Trifolium repens White clover	Perennial	White to pinkish	7.5–50	Prostrate, stoloniferous	Allogamous	1.60E+06	200–800	1.28E+09								

Flowering window months defined as August (A), September (S), October (O), November (N), December (D), January (J), February (F), March (M).

Signalling

The signalling stimuli of flowers is complex. While cues are usually studied independently for their attractiveness to bees (Leonard et al., 2011), there is strong evidence that all stimuli interact to affect floral attractiveness to honey bees (Giurfa et al., 1995; Dötterl and Vereecken, 2010; Jung et al., 2017). In pasture species that may not reflect an individually desirable trait such as large flower size (Benitez-Vieyra et al., 2014), evidence suggests that dense patches of flowers (as in pasture species, Table 2) attract bees (Giurfa et al., 1996; Spaethe et al., 2001). While pasture flowers such as *T. pratense* are comparatively small *en masse*, they are highly attractive to bees (Rundlöf et al., 2014; Montero-Castaño et al., 2016).

A range of aerially flowering pasture legumes of all colours that suit planting in Australia (Figure 1) should be considered when reviewing suitability for bee attractiveness. While honey bee perception is located predominantly in the ultraviolet, blue and green parts of the colour spectrum, there is evidence that the difference between the background green of foliage and the reflection spectrum of the flower (in this case, ultraviolet and pink/purple) allows for bee visualisation in pasture species such as *T. pratense* (Gumbert, 1999; Chen et al., 2020). Furthermore, there are abundant observations that bees effectively forage on red and pink flowers from pasture species, including many *Trifolium* spp. (Koçyiğit et al., 2013) and *H. coronarium* (Di Bella et al., 2015). Honey bees prefer ultraviolet-reflecting flowers (Johnson and Andersson, 2002); photographic endeavours showed ultraviolet reflectance in the tips of white clover flowers (Blum, 2014). Understanding the management effects on signalling properties from specific pasture species would add to the toolkit for pastoralists looking to service the apiary industry or apiarists looking to manage their plantings.

Reward

Honey bee nutrition is the reward for plant pollination (Vaudo et al., 2015). However, there is difficulty with the interpretation of available data on leguminous pasture species. First, the two species (*T. repens* and *T. pratense*) with worldwide significance and featured in the literature are adapted to high-rainfall, high-fertility zones. They do not dominate pasture populations in Australian zones suitable for temperate leguminous pasture species. There is much opportunity to understand the nectar and pollen rewards, and subsequent hive health and honey quality, within the suite of species adapted to southern Australia (Nichols et al., 2007). Second, the challenge lies in the confounding nature of reward calculation, including the variability in comparative measurement methodologies and plant offerings by phenotype and management found in the literature. We present a summary of the data in Table 3.

Trifolium (clover) species have above-average quality pollen, most with crude protein around 25% (Somerville, 2005). Pollen reward is assessed predominantly in terms of crude proteins and lipids (collected from pollen traps) (Di Pasquale et al., 2013). Without adequate pollen resources, colony health, size and longevity suffer, reducing the potential for honey bees to provide pollination services and produce honey and related products (Keller et al., 2005). Protein content varies across melliferous genera and species, ranging from 2–60% when collected directly from flowers (Roulston et al., 2000).

Reports of protein content in pollen of *Trifolium* spp. are reasonably consistent (Table 3). However, prediction of the suitability of protein content in pollen for honey bee health is confounded by subspecies, bee age, colony age, colony size, colony health, brood size, social structure, time in season, climate and other dietary components, including the nutritional contribution of a diverse diet (Basualdo et al., 2013; Di Pasquale et al., 2013). Nevertheless, pollen morphology studies indicate that within a pollen sample with multiple species origins, the representative contribution from pasture legumes such as *Trifolium* spp. and *H. coronarium* is high (Girard et al., 2012; Zerrouk et al., 2013). This suggests that when wild foraging across mixed swards, the attractiveness of these species to honey bees is high.

The literature is inconsistent with bee preference for sugar ratios in nectar. *T. repens* and *T. pratense* produce nectar concentrations (Table 4) in the optimal range of 35–65% (Kim et al., 2011; Pamminger et al., 2019). Honey bees have been shown to prefer nectars with a balanced ratio of these sugars (Wykes, 1952), hexose dominance (Dafni et al., 1988), sucrose dominance (Bachman and Waller, 1977), sucrose, fructose and glucose (Waller, 1972) or no preference (Southwick et al., 1981). Thus, the interpretation of ratios in Table 4 is difficult. *T. pratense* has relatively more total nectar sugar and higher volumes of nectar per inflorescence than *T. repens* (Table 4).

With potentially more florets per hectare (Table 2), the data suggests a more significant potential reward for bees from *T. pratense*. It is difficult to predict honey bee preferences for sugar ratios in nectar and measure nectar in small flower morphologies in general (Morrant. et al., 2009), which possibly accounts for the range in reported results (Table 4).

Table 3. Pollen protein reported as crude protein or protein content (per cent dry mass) in *Trifolium* species (clover mixture), *Trifolium repens* (white clover), *Trifolium pratense* (red clover) and *Trifolium michelianum* (balansa clover).

Species	Crude protein (% dry mass)	Pollen protein content (% dry mass)	References
Trifolium spp.	23.1 b		Forcone et al., 2011
	24.0 b		Liolios et al., 2015
	26.0 b		Somerville, 2001
Trifolium repens	24.7 b		Rayner and Langridge, 1985
	25.4 a		Pernal and Currie, 2001
		35.0 a	Hanley et al., 2008
Trifolium pratense	25.9 b		Somerville and Nichol, 2006
		40.8 a	Hanley et al., 2008
	22.1 b		Forcone et al., 2011
Trifolium michelianum	23.4–27.2 b		Somerville and Nichol, 2006

Bee leg pollen (foraging bees) (a), bee leg pollen (pollen trap) (b)

Table 4. Nectar sugar amount, composition, concentration and volume in *Trifolium repens* (white clover) and *Trifolium pratense* (red clover).

Species	Total nectar sugar per flower (mg)	Sucrose:fructose: glucose ratio in nectar	Total sugar concentration in nectar (%)	Nectar volume (µL)	References
Trifolium spp.			~38		Pamminger et al., 2019
Trifolium repens		1.0:1.0:1.0*			Smith and Johnson, 1969
				0.10 a	Morse, 1980
		1.5:1.0:0.7		0.20–1.70 c	Southwick et al., 1981
			29	1.10 c	Szabo and Najda, 1985
	0.04–0.61	6.0:2.0:2.0	60–74		Jakobsen and Kritjansson, 1994
			53–74	0.22–0.75 a	Bissuel-Belaygue et al., 2002
	0.01				Hanley et al., 2008
Trifolium pratense			50–70	9.40 b	Swanson and Shuel, 1950
	2.00–3.50		57–64	3.05–5.04 c	Shuel, 1957
			64.8	9.60 c	Szabo and Najda, 1985
	0.05–0.15				Fussell, 1992
	0.05				Hanley et al., 2008
		~20.0:2.0:1.0		20.90 c	Somme et al., 2015

Volume calculation based on per floret (a), 100 florets (b), inflorescence (c). * after boron addition.

Pasture species supporting pollination services

Pollination services are needed for the productivity of multiple horticultural commodities (Cunningham et al., 2002) and to set seed or improve seed set in crops (Free, 1965; Woodcock and Canadian Pollination Initiative, 2012; Lundin et al., 2017) (Table 2). However, declining floral forage options negatively impact pollination services due to bee malnutrition (Hein, 2009). Additionally, some horticultural crops offer poor floral resources for bees, further increasing the need for supplementary feeding (Ish-Am and Eisikowitch, 1998). Providing more floral resources will support pollination services, increase hive resilience to stress and improve pasture seed production profitability. A mutually beneficial relationship for pastoralists/horticulturists and apiarists in terms of crop yield, honey production and colony maintenance is of great value to agriculture and merits consideration.

Leguminous pasture floral resources can offer off-season hive build-up and maintenance (early and late) and bee nutritional forage balance and dietary diversity during pollination activity (Behmer, 2009; Di Pasquale et al., 2016). Specific examples include late-flowering *T. pratense* increasing queen bee and male densities (Rundlöf et al., 2014), high flower density in *T. pratense* related to higher bee visitation in cover crops (Ellis and Barbercheck, 2015) and *Trifolium* species recommended as part of a balanced diet (Filipiak, 2019). Pasture species are likely to offer genotypic and phenotypic variance of reward that meets bee health requirements (Tables 3 and 4). More research is recommended to establish the benefits of co-flowering species on pollinator health and landscape management and conservation (Cunningham et al., 2002; Carvalheiro et al., 2014).

Honey

Trifolium spp. honey, commonly known as clover honey, is considered a premium product worldwide (Abell et al., 1996). However, the marketing of multifloral or unifloral honey from leguminous pastures such as clover is limited in Australia. Chemometric classification of honey quality includes physicochemical and sensory profiles, with sugar, moisture, mineral, acid and storage standards in most regulated countries (Bogdanov et al., 1999). Clover honey exceeds these standards and is reportedly similar to eucalyptus honey, with less mineral content and slightly less acidity (Ciappini et al., 2016). Clover honey also has high processing tolerance and storage capacity (Wang et al., 2004; Rogers et al., 2014). Honey quality is also assessed on a sensory basis (Anupama et al., 2003). Clover honey, for example, is often lighter in colour, fetches a higher price premium (Boffo et al., 2012) and may have higher flavonoids than dark honey (Bogdanov et al., 2004).

Honey products promote many beneficial human responses due to bioactive constituents, including antibacterial, antimicrobial, antiviral, antioxidant, anti-inflammatory, antidiabetic, antiulcer and anti-atherogenic activity (Gomes et al., 2010; Fedorova et al., 2011; Almasaudi et al., 2016). Honeys marketed with pharmaceutical benefits are prized as monofloral (Ciappini et al., 2016). Mass-flowering pasture legumes offer the capacity to yield such honey from hives situated in the middle of large-scale crops already planted in agricultural systems (Montero-Castaño et al., 2016). Desirable bioactive properties in honey from pasture legumes have been noted (Lu et al., 2013; Abbas, 2014; Jerković et al., 2016) but are under-explored in Australian-adapted pasture legume species.

Future opportunities

Pasture species adapted and already grown in Australia undoubtedly provide opportunities for increased and reliable floral resources to support pollination services and the production of honey and honey-related products. Recommendations are tabulated in Box 1.

Box 1. Research opportunities for understanding the relationship between annual and perennial pasture legumes adapted to southern Australia and the Australian apiculture industry.

- Assess a range of Australian-adapted pasture species for nutritional provision for honey bees, including:
    - Nectar: sugars (primary analysis), secondary metabolites, amino acids, proteins, lipids, vitamins, terpenoids, metal ions, hormones, microbes.
    - Pollen: crude protein (primary analysis), water content, specific amino acids, starches, lipids, sterols, vitamins, toxins.
- Assess pasture species for attractiveness of honey bees to a range of Australian-adapted pasture species, including:
    - Flower size, shape, colour, scent.
    - Species preference in mixes.
- Assess how climate and management affect the reproductive response of pasture species for honey bee nutrition, including:
    - Plant genotype: cultivar, floral morphology, flower age, floret age in multiple flowers, plant age, time of day, phenology.
    - Plant management: planting density, soil structure, soil nutrition, plant nutrition, water availability, pest and disease presence, treatment effects.
    - Climate: diurnal temperatures, humidity, photoperiod.
    - Previous pollinator visitation.
- Assess the impact of incorporating Australian-adapted pasture species in a co-flowering pollination system, including:
    - Does a certain ratio of pasture species to pollination target species facilitate or detract from pollination services?
    - Does diversity of pasture species improve bee diet balance?
    - Do certain spatial scales of multispecies floral provision impact honey bee health?
- Assess the potential for Australian-adapted pasture species to provide new honey markets, including:
    - Organoleptic analysis of monofloral honey suited to the domestic and international market, and identified by geography and species or 'clover.'
    - Phytochemical analysis and bioactivity profiling of honey for promotion of honey as a functional food.
- Assess the value of increasing the commercial viability of using pasture legumes in sustainable farming systems through additional revenue.

Conclusion

Honey bees populations have rapidly declined in Europe and North America due to declines in the floral resources required for bee nutrition, pest and disease pressures, and an ageing commercial beekeeper population. Similar reductions in Australia necessitate ensuring adequate floral resources for honey bees for commercial and conservation purposes. Annual and perennial leguminous pasture species are adapted to southern Australian regions suited to apiculture. These species can offer floral resources that are highly attractive, accessible and nutritious to bees over a temporal span that is lengthy and predictable.

Using pasture species that are already integral to sustainable agricultural systems to support pollination services is crucial. Mutually beneficial outcomes for pasture seed producers and the apiculture industry are valuable and worthy of further investigation. Indeed, the production of honey and honey products with superior organoleptic and bioactive quality is likely from these species. A deeper exploration of leguminous pasture species outside the model clovers (*T. repens* and *T. pratense*) is recommended to exploit these opportunities to suit Australian conditions. An intentional re-population of floral resources for the apiculture industry in Australia must be considered to ensure the longevity of the beekeeping industry and food security in general.

Chapter 2. Field-based trials for the pasture-based and beekeeping industries that practically demonstrate how to produce premium-grade honey

Background

Based on the literature review, we established plots of bee-friendly pasture species in enclosed shade houses at The University of Western Australia (UWA) Shenton Park field station in Perth. We also used two commercial crops of bee-friendly pasture species at the property of our commercial partner (Bell Seeds) at Elgin, WA. We noted flowering time range and collected nectar and pollen from flowers.

Methodology

Plant establishment and maintenance at Shenton Park field station

Choice of pasture legume cultivar/selection

Thirteen legume pasture cultivars/selections were grown (Table 5). The choice of legume species was based on the literature review (Chapter 1). Most chosen species have physiology and attributes that mean they are adapted to many of the soil types and rainfall zones across southern Australia, and are safe to be grazed by ruminants. They are suitable to be grown in farming systems in such a way as to be mutually beneficial to both bees and pastures managed for ruminant livestock production. The only exceptions were the two patented *Trifolium pratense* cultivars; these were bred for non-forage purposes and are not intended to be grazed. Most species were annuals, but several were biennial or perennial. Commercially available cultivars represented most of the species.

Choice of growing conditions: use of enclosed shade houses

Thirteen cultivars or selections of nine species (including two *T. pratense* clover patented cultivars 'NSE' and 'NFE') were sown and maintained in enclosed shade house plots at the UWA Shenton Park field station (31° 56' 55.87" S, 115° 47' 44.35" E) in Shenton Park, WA (Table 5, Figure 2). The purpose of the enclosed shade house plots was to collect monofloral nectar, pollen and honey from a hive of bees foraging on each species. There were two sizes of bee enclosures, 10 m × 4 m and, for one enclosure only, 40 m × 10 m. The shade houses were adjacent to each other and were expected to be similar in soil types and climate. Under suitable conditions, when honey bees receive adequate food and are not subject to sudden fluctuations in temperature, they have been reported to forage in a greenhouse as they would when visiting a crop in the field (Free, 1993; see also Palmer-Jones et al., 1962). For most species, a single plot was maintained, and early and late-flowering species were co-located within a single shade house to use space efficiently and save costs (Figure 2). These pairs of species were selected based on the need for one species to finish flowering before the second species commenced flowering. However, if there was any crossover of flowering windows, insect-proof netting was placed over the later-flowering cultivar/selection. One species, *Hedysarum coronarium*, was established and maintained in the field (not within the enclosed shade houses).

Plant establishment

Prior to sowing, seeds were scarified using a brass cylinder lined with fine sandpaper driven by compressed air. The air compressor pressure was 30 kPa (200 psi) and ~100 seeds of each species were scarified for 10 seconds. On 15 May 2020, three seeds of each cultivar, except *T. glanduliferum*, were sown into hydrated Jiffy-9 peat pots (size 522, Jiffy Products Ltd, Norway). Each population was sown in a seedling tray containing 30 peat pots and covered lightly with potting mix. Three days after sowing, appropriate rhizobium inoculant was applied by watering can, by adding 40 mL of inoculum to 10 L of water (one watering can for seven trays). Peat pots were watered daily. Seedlings were transferred to outside benches for acclimatisation after four weeks in the glasshouse. Prior to transplanting, seedlings were thinned randomly to one per peat pot. On 1 July, the *H. coronarium* population was transplanted in the field into holes cut into plastic strips at 30 cm spacing. On 2 and 3 July, 100 seedlings of all remaining cultivars/selections were transplanted into their plots in the enclosed shade houses at 30 cm spacing. For *T. glanduliferum*, seed was inoculated with appropriate rhizobia and planted directly into the ground in a large, enclosed shade house on 3 July. Soluble fertiliser (Thrive® all purpose; 5 g per 10 L) was applied to all seedlings after transplanting and to the *T. glanduliferum* after sowing (1.5 L per m^2).

Irrigation was supplied as required by overheard watering until each cultivar flowered. Thereafter, water was supplied to each plant for 20 minutes three times a week through 19 mm premium black poly pipe (Holman) with micro jets (Pope half-circle Veri-flow). Plants were fertilised by hand, with single superphosphate with potash (6.8% P, 12.4% K and 8.3% S) applied prior to flowering.

Nucleus beehives

Each enclosed shade house had a nucleus beehive placed within it when a single cultivar appeared to have about half of all plants in flower. The design of the hives included a marked dry-drawn frame, foundation (× 2), brood (× 2) and a food frame (Figure 3). Unfortunately, there is little published literature on how to match the health requirements of bees and the productivity of pasture legume species to produce monofloral nectar and honey in small, closed production systems such as the enclosed shade house plots. As a consequence, the bee colony size maxima that could be sustained in the small shade house plots could only be estimated in advance of the bees being placed in the enclosures.

Table 5. The 13 cultivars/selections from 11 pasture legume species used in the study and their growth conditions. At the UWA Shenton Park field station, these were: SH, shade house (10 m x 2 m); SHL, shade house large (40 m x 10 m); and FP, field plot (50 m x 1 m). Two commercial crops (CC) were also sampled at Bell Seeds in Elgin, WA.

Scientific name	Life habit	Common name	Cultivar/selections	Growth conditions
Bituminaria bituminosa	Perennial	Tedera	Lanza	SH
Hedysarum coronarium	Biennial	Sulla	Flamenco	FP
Onobrychis viciifolia	Perennial	Sainfoin	Othello	SH
Trifolium glanduliferum	Annual	Gland clover	Prima	SHL
Trifolium incarnatum	Annual	Crimson clover	Caprera early selection	SH
Trifolium isthmocarpum	Annual	Moroccan clover	Selection	SH
Trifolium michelianum	Annual	Balansa clover	Bolta	SH
			Paradana	CC (24 ha)
Trifolium pratense	Perennial	Red clover	Patented NFE	SH
			Patented NSE	SHL
Trifolium purpureum	Annual	Purple clover	Electra	SH
Trifolium resupinatum	Annual	Persian clover	Kyambro	CC (54 ha)
Trifolium vesiculosum	Annual	Arrowleaf clover	Cefalu	SH

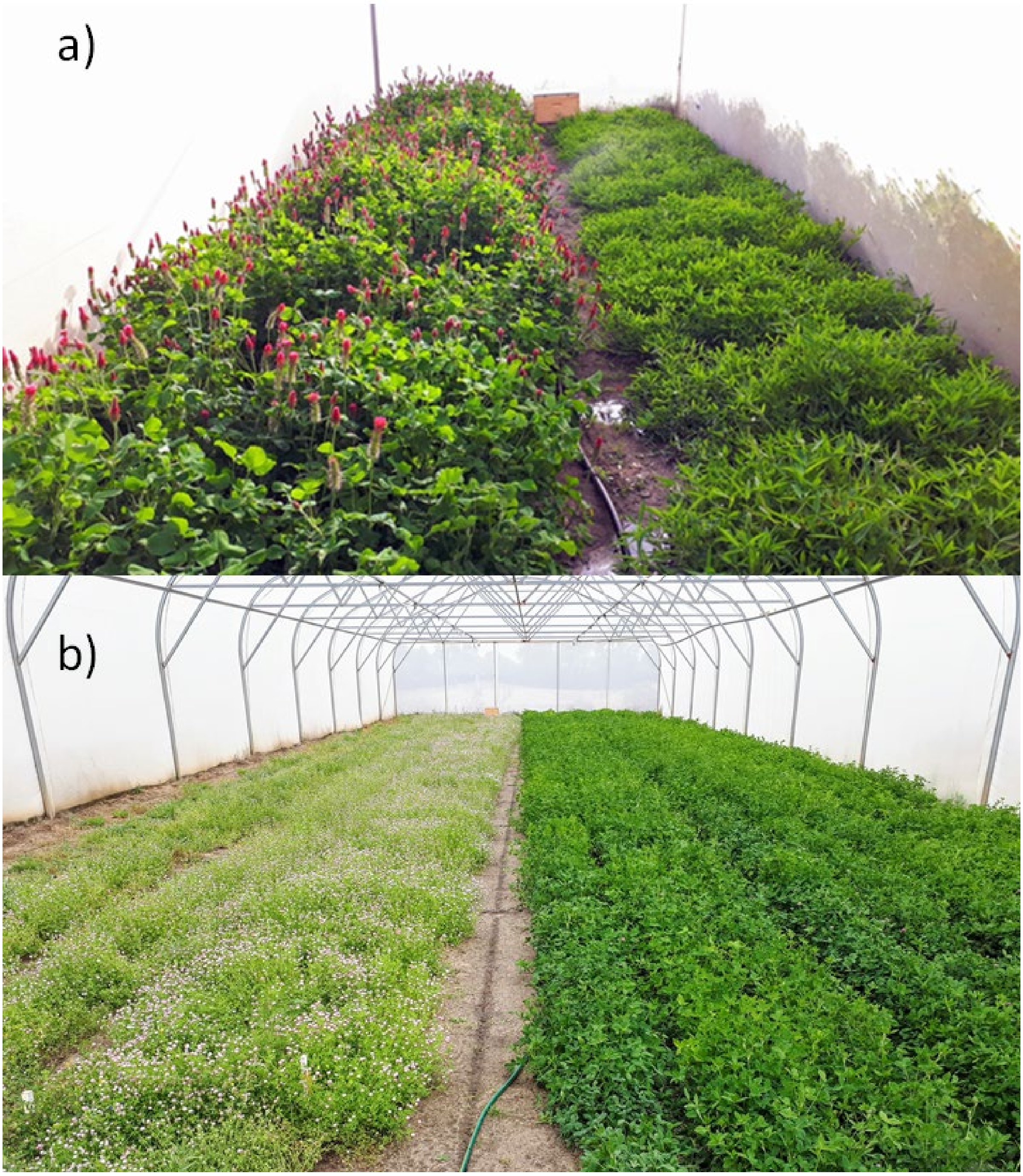

Figure 2. Example of a) 10 m × 4 m enclosed shade house plot with early flowering *T. incarnatum* 'Caprera' selection (left) and late-flowering *T. purpureum* (right); and b) 40 m × 10 m enclosed shade house plot with early flowering *T. glanduliferum* (left) and late-flowering *T. pratense* 'NSE' (right).

Figure 3. Example of nucleus beehive layout.

Plant establishment and maintenance of commercial crops

We collaborated with Bell Seeds in Elgin, WA, to sample honey from field-grown commercial crops (*T. resupinatum* and *T. michelianum*) (Figure 4). Crops were sown at 5 kg seed/ha in May 2020. Prior to sowing, glyphosate herbicide (1.5 L/ha) was applied for broad leaf weed control. A selective grass herbicide (Clethodim, 330 mL/ha) was applied in June and muriate of potash (75 kg/ha) fertiliser was applied in August. There are no guarantees that the honey from Bell Seeds was monofloral. However, we consider it likely, as there were very few other floral resources flowering in the district in 2020 (i.e. no eucalypts).

Figure 4. Beehives located at Bell Seeds, Elgin, WA, next to a late-flowering commercial *T. resupinatum* crop in December 2020.

Flowering time assessment

The Shenton Park site was visited at least weekly, and the presence of flowering recorded. The field site at Bell Seeds in Elgin was visited monthly.

Nectar collection and determination of sugars and volume

We collected nectar from individual florets within the flower heads of six legume species from enclosed shade house plots (*Bituminaria bituminosa*, *Onobrychis viciifolia, T. incarnatum, T. pratense* (NSE), *T. purpureum*, *T. vesiculosum)*, along with one species from the field plots *(H. coronarium)*. The florets of the other species were too small and required finer capillary tubes not available at the time.

Bees were excluded from flowers for 24 hours before sampling with nectar, then collected before 10am from dry flowers (i.e. flowers that had not experienced rain or irrigation prior to sampling for 12 hours). Nectar sampling then followed the capillary tube collection method of Corbet (2003), with samples collected using 2–10 µm microcapillaries tubes, slightly larger than the corolla tube. Samples were taken from 50 individual open florets, and the volume per floret was recorded. The number of open florets per flower head was recorded. Nectar was stored at –20 °C prior to high-performance liquid chromatography (HPLC) nectar sugar analysis as per Płachno et al. (2019). Ten replicates were analysed, with each containing nectar from five florets. The results were used to calculate the total weight of fructose, glucose and sucrose (in open florets) per flower head.

Pollen collection and determination of crude protein

Pollen traps could not be fitted to the nucleus beehives because they had been custom-built to accommodate the small population of bees suitable for the enclosed shade house plots. We were, therefore, unable to sample bee-collected pollen. As a proxy, pollen samples were taken from fresh individual florets of each legume (cultivars grown in the enclosed shade house plots and *H. coronarium* from the field) to analyse flower pollen for crude protein.

Pollen was sampled once only during the season for each cultivar, when about half the plants were flowering and flowers were in abundance. This occurred between 9am and 10am when the plants were not under heat or drought stress. The selected flowers were unopened to ensure that the pollen remained in the pollen sacs and had not dehisced. Pollen was removed from the anthers by hand from randomly selected young florets from the selected flowers within each cultivar. For each cultivar, pollen was collected from 20 flower heads. The pollen was bulked into a single sample, mixed, and three replicate samples were stored at –20 °C prior to analysis by HPLC for crude protein (%).

Honey collection from shade houses

The nucleus bee hives were checked regularly by the apiarist. The intention was to collect honey from the beehives in each shade house. However, unseasonal cold weather and high rainfall in November (more than double the monthly average) resulted in lower-than-expected floral resources, and too many bees relative to flowers. Hence, little or no honey or nectar was stored in the frames in some of the enclosed shade houses.

If there was sufficient nectar or capped honey in the frames, the frames were removed and replaced with clear frames. The removed frames were then transported to the laboratory. The honey was removed from the frame manually (i.e. without an extractor), using a clean knife to excise honey comb, and placed on a 3 mm brass sieve above a collection beaker. The honey was then allowed to collect at the bottom of the beaker. If nectar was present, it was removed from each cell of the frame using a disposable plastic pipette and stored in plastic vials. A single sample of honey and nectar was taken per frame. Both honey and nectar were stored in plastic containers in a 4 °C fridge until testing.

Honey collection from commercial field-grown pastures at Bell Seeds

Trifolium michelianum and *T. resupinatum* were grown in a commercial paddock at Bell Seeds in Elgin, WA. The hives were located next to each of the clovers when in full bloom. The hives were checked weekly for honey and nectar by the apiarist. Honey, nectar and pollen yields were calculated by how many days it took to fill the frames in each hive. Each box was weighed and processed individually. Full frames were removed and transported to the laboratory before honey was removed as detailed above.

Results

Flowering time

Flowering time varied between the cultivars/selections (Table 6). Notably, *T. incarnatum* 'Caprera' early selection commenced flowering in mid-winter (July). Several other cultivars commenced flowering in early spring (September), including *T. glanduliferum*, *T. isthmocarpum*, *T. michelianum* and *H. coronarium* cultivars. The remaining cultivars commenced flowering in October, November or December. *O. viciifolia* flowered for five months. Flowering extended well into summer for *B. bituminosa*, *O. viciifolia* and *T. pratense* 'NFE' and 'NSE'. Two cultivars of *T. michelianum* were grown in the enclosed shade house and the field; they both commenced flowering around the same time, but flowering in the shade house lasted about one month longer than in the field. Overall, flowering mainly occurred in a four-month window from September to December.

Table 6. Flowering range in enclosed shade house plots (SH), field plots (FP) and commercial crops (CC). Cultivars are indicated (x) where nectar and uncapped honey were taken for further analysis once sufficient nectar or honey was available.

Scientific name	Common name	Cultivar	Location	Flowering range	Date of sampling	Nectar	Honey
Bituminaria bituminosa	Tedera	Lanza	SH	Nov–Feb	–	–	–
Hedysarum coronarium	Sulla	Flamenco	FP	Sept–Nov	–	–	–
Onobrychis viciifolia	Sainfoin	Othello	SH	Oct–Feb	18 Nov	x	x
Trifolium glanduliferum	Gland clover	Prima	SH	Sep–Nov	16 Oct	x	–
Trifolium incarnatum	Crimson clover	Caprera early	SH	Jul–Oct	7 Oct	x	–
Trifolium isthmocarpum	Moroccan clover	Selection	SH	Sep–Nov	28 Oct	x	–
Trifolium michelianum	Balansa clover	Bolta	SH	Sep–Nov	–	–	–
		Paradana	CC	Sep–Oct	25 Oct	x	x
Trifolium pratense	Red clover	NFE	SH	Dec–Jan	28 Dec	–	x
		NSE	SH	Oct–Dec	7 Dec	x	x
Trifolium purpureum	Purple clover	Electra	SH	Oct–Dec	25 Nov	x	–
Trifolium resupinatum	Persian clover	Kyambro	CC	Oct–Dec	3 Dec	x	x
Trifolium vesiculosum	Arrowleaf clover	Cefalu	SH	Oct–Dec	23 Nov	x	–

Nectar sugars

Large differences among the cultivars occurred for the fructose, glucose, and sucrose weights in open florets per flower head (Figure 5). Sucrose always contributed the most to nectar sugars, followed by fructose and glucose with similar amounts. *H. coronarium*, *T. incarnatum* and *T. pratense* 'NSE' had the greatest sucrose weight and hence all sugars per flower head, and *B. bituminosa* had the lowest weight. *H. coronarium* and *T. incarnatum* had the highest fructose and glucose weights.

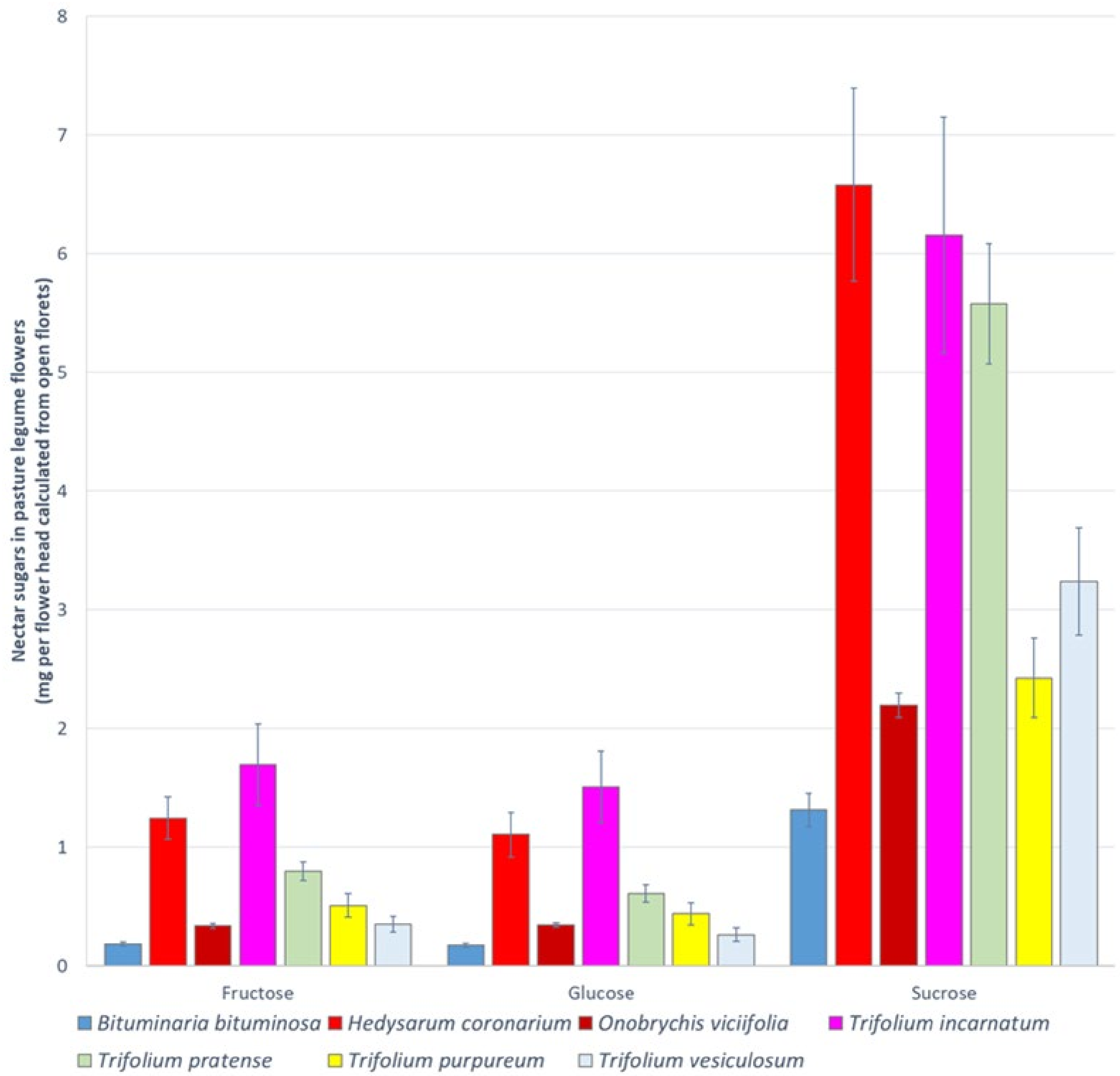

Figure 5. Weight of nectar sugars (fructose, glucose and sucrose) per flower (mean ± 1 standard error). All cultivars grown in enclosed shade houses other than *H. coronarium* (field plots). *Trifolium pratense* is the 'NSE' cultivar.

Nectar volume

Large differences among the cultivars also occurred for nectar volume per floret (Figure 6), which was highest for *H. coronarium* and lowest for *T. isthmocarpum* and *T. pratense* 'NFE'; all other cultivars had similar amounts. *Trifolium pratense* 'NSE' had significantly more nectar volume than *T. pratense* 'NFE'.

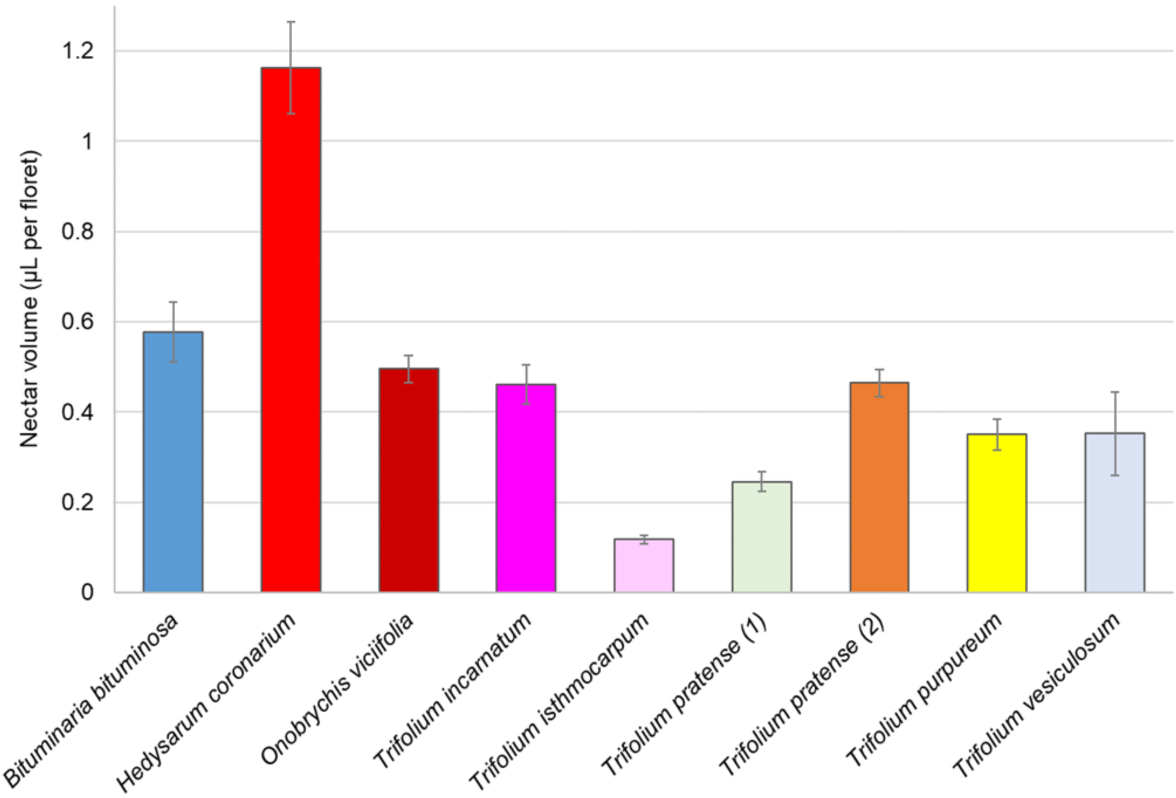

Figure 6. Nectar volume per floret (mean ± 1 standard error). All cultivars grown in enclosed shade houses other than *H. coronarium* (field plots). *Trifolium pratense* 1 is the 'NFE' cultivar and *Trifolium pratense* 2 is the 'NSE' cultivar.

Pollen crude protein

Crude protein of pollen collected directly from flowers ranged from 33.8–46.7% (Table 7). The highest crude protein was recorded for *H. coronarium*, followed by *B. bituminosa*. The *Trifolium* cultivars had similar crude protein, ranging from 33.8–39.3%.

Table 7. Crude protein in pollen (means of three replicates from a combined pollen sample from 20 flower heads). All cultivars grown in enclosed shade houses, other than *H. coronarium* (field plots).

Scientific name	Common name	Cultivar	Crude protein (%)	Std. error
Bituminaria bituminosa	Tedera	Lanza	41.4	0.29
Hedysarum coronarium	Sulla	Flamenco	46.7	0.81
Onobrychis viciifolia	Sainfoin	Othello	39.7	0.00
Trifolium glanduliferum	Gland clover	Prima	34.1	0.43
Trifolium incarnatum	Crimson clover	Caprera early	37.2	0.69
Trifolium isthmocarpum	Moroccan clover	Selection	36.3	2.07
Trifolium michelianum	Balansa clover	Bolta	33.8	0.13
Trifolium pratense	Red clover	NSE	34.6	0.30
Trifolium purpureum	Purple clover	Electra	34.0	1.39
Trifolium vesiculosum	Arrowleaf clover	Cefalu	39.3	0.39

Honey collection from commercial field-grown pastures at Bell Seeds

Trifolium michelianum and *T. resupinatum* each produced about 200 g of pollen per day (Rob Bell pers. comm.). *T. resupinatum* yielded 25–35 kg of capped honey per hive (based on yield per frame) over the eight-week flowering period from late October to early December. Similar yields were obtained for *T. michelianum*, which flowered from September to October.

Discussion

Methodology

The use of the enclosed shade houses enabled us to reasonably cheaply ensure that bees foraged on a monofloral pasture, as it removed the need to establish large areas well-separated from other pollen and nectar sources. The space- and cost-saving by growing paired cultivars that differed in flowering time worked well. However, we encountered problems collecting honey from the nucleus beehives due to cold weather in late spring and the high ratio of bees to flowers. To avoid this in the future, we suggest including one legume species per enclosed shade house of the dimensions we used and doubling the plant density, i.e. plants every 15 cm.

Flowering times

Flowering times differed among the cultivars. Flowering for *T. incarnatum* 'Caprera' early selection started in July, and continued into February for *B. bituminosa* and *O. viciifolia*. However, it should be noted that most of these cultivars, especially the annual cultivars, have indeterminate flowering and will continue to flower while seasonal conditions are suitable and there is sufficient rainfall. Hence, the flowering period can vary greatly between sites and among seasons. Also, there was a broad range of maturities among the species examined, designed to ensure maximum productivity in environments with different growing season lengths. For instance, maturity within the *T. michelianum* cultivars differed: 'Frontier' commenced flowering about 105 days after sowing, while 'Bolta' commenced flowering about 130 days after sowing. For the *T. resupinatum* cultivars, flowering time differed by about 40 days between the early-maturing 'Prolific' and later-maturing 'Maral'. Flowering time (early, medium, late) is often a goal of pasture legume breeding programs and could easily be manipulated for specific needs within a program designed to provide bee-friendly pastures that meet bee industry requirements. For instance, if winter floral resources were desirable, we could select from existing variation to develop an early-flowering cultivar; *T. incarnatum* 'Caprera' early selection is an example of the start of such a process.

In the future, producers could extend their flower resource availability – if there is no vernalisation requirement – by sowing separate crops of individual cultivars, as done by Bell Seeds, or by sowing mixtures in a single paddock. Sowing mixtures enable producers to hedge their bets on season length. The inclusion of cultivars with a range of flowering windows, combined with the indeterminate flowering nature of many legumes, could extend flowering, as long as soil moisture is available. Apiarists could then take full advantage of an extended season of diverse floral resources. Future work should focus on understanding the flowering patterns of annual clovers to define 'peak flowering' for maximum harvestable nectar and pollen yields of each legume cultivar. The impact of honey quality (see Chapter 3) also requires investigation.

Nectar sugar and volume and crude protein

Our nectar sugar and volume results were reasonably consistent with the, albeit variable, literature, including reports for *T. repens* and *T. pratense*. For instance, the sucrose:fructose:glucose ratio of most tested cultivars was ~6:1:1, while the literature reports values ranging from 1.5:1.0:0.7 to 6:2:2 to 20:2:1 (Table 4). Our values for nectar volume per floret (mostly ~0.1–0.6 µl per floret) are consistent with those reported in the literature for *T. repens* (0.1–0.75 µl per floret) (Table 4), with *H. coronarium* a standout at ~1.15 µl per floret. However, our values for total nectar sugars per flower (open florets only) were as high as 8–9 mg per flower head (*H. coronarium* and *T. incarnatum*) and ~6.5 mg per flower head for *T. pratense*, all well above the highest value reported in the literature (3.5 mg per flower head for *T. pratense*) (Table 4). While encouraging, we note that the growth of these cultivars in the enclosed shade houses under adequate fertiliser and well-watered conditions likely contributed to these high values. Many environmental conditions in the field could impact nectar quality and quantity; this warrants further investigation if bee-friendly pastures are to be developed further. It is worth noting that the flowers of all tested cultivars were very conspicuous and easily accessible in the upper canopy (see Figure 2); thus, the bees receive a high reward in terms of nectar for relatively little time and energy spent moving between florets and flowers.

All tested cultivars had pollen crude protein over the desired 25% (Somerville, 2005). However, it is hard to compare these values to the literature (Table 4), as pollen crude protein is usually collected on bee-collected pollen from pollen traps. Flower pollen likely provides a more accurate means of comparing cultivars as it avoids variability and contamination induced by the bees.

Honey production

We were unable to collect capped honey from nucleus hives in the enclosed shade houses. However, for all cultivars, when flowering, the bees in the shade house stored nectar for much of the time. The high honey yields in the commercial crops at Bell Seeds for *T. michelianum*, which was also grown in a shade house, indicates that the plant density was insufficient to meet the floral demands of the hive. The low plant density, combined with the unusually cold, wet spring that limited bee foraging, were the main factors responsible for limiting honey production in some enclosed shade houses. We are therefore confident that pasture legumes merit further investigation as sources of floral resources for apiarists.

Conclusion

Our methodology using enclosed shade houses requires further refinement but shows promise for further work. However, with a sufficient budget, commercial-scale field plots may be a superior option. The legume cultivars significantly differed for flowering time, nectar sugars and crude protein, and were similar or superior to other *Trifolium* species reported in the literature (see Chapter 1). Selecting a mixture of legume species (both annual and perennial) and cultivars within these species with diverse flowering periods could ensure floral continuity under variable conditions for apiarists.

Chapter 3. Honey quality produced from commercial legume cultivars: bioactivity and medicinal characteristics

Background

Honey consists of at least 200 phytochemicals, with its composition strongly dependent on geographical origin and the floral species the bees visit. Honey also contains a wide range of phenolic acids and flavonoids that exhibit antioxidant activity. The taste, colour, other physical properties and overall quality of honey are influenced by non-volatile compounds that include sugars and phenolic compounds. Honey has many therapeutically useful properties *in vitro*, including antioxidant, anti-inflammatory, antibacterial, antidiabetic, antiulcer, anti-atherogenic, antiviral and antimicrobial activity (Almasaudi et al., 2016). Based on their bioactivity profile, there is a strong impetus to develop some honeys into medicinal honeys (Oryan et al., 2016).

The *Trifolium* spp. or clover honeys are popular examples of pasture honeys. They are mainly appreciated for their distinct and complex flavour profiles; however, their bioactivity is under-explored. In this chapter, we report on the minimum inhibitory concentrations of honey and/or nectar from seven *Trifolium* cultivars against four common bacterial pathogens, and the antioxidant activity and pH, BRIX (g sugar per 100 g honey) and 100-BRIX (water content) of honey and/or nectar from 10 *Trifolium* cultivars and *O. viciifolia*.

Methodology

Antibacterial and antioxidant activities were measured on a range of nectars and honeys, produced as described in Chapter 2. Due to limited sample sizes, only nectar or honey from *T. isthmocarpum*, *T. glanduliferum*, *T. michelianum*, *T. pratense* 'NSE' and 'NFE' red clover, *T. purpureum* and *T. resupinatum* was tested (see Table 4).

Nectar and honey antibacterial activity

Minimum inhibitory concentrations (MICs) are the lowest percentage of honey or nectar that completely inhibits bacterial growth. MIC determination was based on a well-established broth microdilution method (CLSI, 2018; Green et al., 2020). Honey was prepared and tested for activity as described previously (Green et al., 2020), using a range of concentrations in 2% increments (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30%). Four common bacterial pathogens were examined: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853. MICs were determined visually after incubation at 37 °C for 18–22 hours. There was one sample from each cultivar/selection, which was tested twice on different days. If the results differed by more than 4% honey, the test was repeated a third time. The mode was then selected as the final MIC value.

Nectar and honey antioxidant activity

Total phenolic content was determined using the Folin–Ciocalteu method (Singleton et al., 1999) with some modifications (i.e. pH adjustment to eliminate sugar interference) by recording the absorbance of the samples at 760 nm (UV-Vis Carry 50 Bio UV-Visible Spectrophotometer). Total antioxidant activity was measured using the 2, 2-diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant capacity (FRAP) assays. To determine radical scavenging activity, the decay in the purple colour of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals in the presence of antioxidant compounds was measured, with the change in absorbance captured in a colorimetric assay at 517 nm (UV-Vis Carry

50 Bio UV-Visible Spectrophotometer). For the ferric reducing power (FRAP) antioxidant assay, antioxidant activity was recorded by spectrophotometric analysis (BMG Labtech POLARstar Optima Microplate Reader) as the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe (III)-TPTZ] to the corresponding ferrous complex at low pH. All tests were carried out in triplicate, and the mean reported.

Nectar and honey physicochemical characteristics

The pH of honey and nectar samples was measured in carbon dioxide-free water at room temperature using a calibrated pH meter (Eutech PC 2700, Eutech Instruments). The BRIX value (dissolved solids content) was measured with a Refractometer (HI96801, Hanna Instruments, Rhode Island, USA): it reflects a sample's total sugar content (expressed in g per 100 g of honey) and allows water content to be determined (100-BRIX). There was one sample from each cultivar/selection, which was tested twice on different days. The mean of the two values was then determined.

Results

Nectar and honey antibacterial activity

Low MICs are desirable as they indicate that the honey or nectar inhibits the pathogen at a low concentration. For antibacterial activity against all pathogens, the best results were obtained for *T. resupinatum* nectar, *T. michelianum* nectar and *T. resupinatum* honey. *T. glanduliferum*, *T. isthmocarpum*, and *T. pratense* 'NFE' had little antibacterial activity other than against *P. aeruginosa* (Table 8). For *T. michelianum* and *T. resupinatum*, nectar had a greater inhibitory effect than honey on all four pathogens. MICs varied greatly among *S. aureus*, *E. coli* and *E. faecalis*, with less variation evident for *P. aeruginosa*.

Table 8. Minimum inhibitory concentrations (MICs) (% w/v) of nectar and honey tested against four common pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (mode of 2–3 replicates of a single sample). All cultivars grown in shade houses other than *T. michelianum* and *T. resupinatum* (commercial crops).

Scientific name	Common name	Cultivar	Sample	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	*E. faecalis* ATCC 29212	*P. aeruginosa* ATCC 27853
Trifolium glanduliferum	Gland clover	Prima	Nectar	>30	>30	>30	24
Trifolium isthmocarpum	Moroccan clover	Selection	Nectar	25	30	>30	19
Trifolium michelianum	Balansa clover	Paradana	Nectar	4	14	15	10
			Honey	11	22	30	16
Trifolium pratense	Red clover	NSE	Nectar	15	28	28	14
	Red clover	NFE	Honey	28	29	29	14
Trifolium purpureum	Purple clover	Electra	Nectar	18	28	29	15
Trifolium resupinatum	Persian clover	Kyambro	Nectar	4	11	11	8
			Honey	4	18	20	11

Nectar and honey antioxidant activity

All three measures of antioxidant activity varied greatly among cultivars and samples (Table 9). Total phenolics content (TPC) and the FRAP assay varied ~three-fold, while the DPPH assay varied ~10-fold. The nectar from *O. viciifolia* had the highest values (desirable) for all three measures, particularly for the DPPH assay. Notable high values were also recorded for the TPC for *T. vesiculosum* nectar and the FRAP assay for *T. resupinatum* nectar. Low values for all three measures occurred for *T. michelianum* nectar and honey (commercial crop) and *T. pratense* 'NSE' honey. For *T. pratense*, it was notable that 'NSE' honey recorded values ~1.5–3 times higher than 'NFE' honey.

The three cultivars where both nectar and honey was measured had similar values for all three measures of antioxidant activity.

Table 9. Antioxidant activity indicators for nectar and honey: total phenolics content (TPC), ferric reducing antioxidant capacity assay (FRAP) and 2, 2-diphenylpicrylhydrazyl (DPPH) assay (means of triplicate analyses of a single sample). All cultivars grown in shade houses other than *T. michelianum* and *T. resupinatum* (commercial crops).

Scientific name	Common name	Cultivar	Sample	TPC (GAE eq.mg/100 g)	FRAP (mmol Fe/kg)	DPPH (µmol Trolox/kg)
Onobrychis viciifolia	Sainfoin	Othello	Honey	28.6 ± 0.2	5.88 ± 0.28	2288 ± 175
			Nectar	39.8 ± 0.9	7.55 ± 0.26	3543 ± 25
Trifolium glanduliferum	Gland clover	Prima	Nectar	27.0 ± 0.8	5.50 ± 0.32	1691 ± 42
Trifolium incarnatum	Crimson	Caprera early	Nectar	22.2 ± 0.3	3.55 ± 0.06	1407 ± 76
Trifolium isthmocarpum	Moroccan clover	Selection	Nectar	20.8 ± 0.4	3.35 ± 0.07	1188 ± 91
Trifolium michelianum	Balansa clover	Paradana	Nectar	13.2 ± 0.5	2.13 ± 0.15	341 ± 57
			Honey	14.8 ± 0.3	2.24 ± 0.17	552 ± 15
Trifolium pratense	Red clover	NSE	Nectar	25.7 ± 2.1	5.32 ± 0.44	205 ± 23
		NSE	Honey	29.9 ± 0.9	6.04 ± 0.44	2410 ± 168
		NFE	Honey	19.8 ± 0.5	2.58 ± 0.08	794 ± 66
Trifolium purpureum	Purple clover	Electra	Nectar	30.6 ± 1.7	6.58 ± 0.54	2417 ± 61
Trifolium resupinatum	Persian clover	Kyambro	Nectar	30.2 ± 0.5	7.17 ± 0.22	2429 ± 72
			Honey	25.7 ± 0.9	6.17 ± 0.24	2030 ± 180
Trifolium vesiculosum	Arrowleaf clover	Cefalu	Nectar	39.7 ± 3.0	6.21 ± 0.34	2270 ± 226

Nectar and honey physicochemical characteristics

The pH of five honey and nectar samples was measured and ranged from 3.92–4.27 (Table 10). The BRIX values varied little, from 77.97 for *T. incarnatum* to 82.33 for *T. michelianum*, translating into water contents of 22.03 and 17.67%. For two of the three samples (*T. michelianum* and *T. pratense* 'NSE'), the nectar had a higher water content than the corresponding honey, while the reverse was true for *T. resupinatum*.

Table 10. pH and BRIX (g sugar per 100 g honey) for honey and nectar samples. All cultivars grown in shade houses other than *T. michelianum* and *T. resupinatum* (commercial crops).

Scientific name	Common name	Cultivar	Sample	pH	BRIX (%)
Onobrychis viciifolia	Sainfoin	Othello	Honey	–	81.40 ± 0.20
			Nectar	–	78.00 ± 0.69
Trifolium glanduliferum	Gland clover	Prima	Nectar	4.27	78.33 ± 0.15
Trifolium incarnatum	Crimson	Caprera early	Nectar	–	77.97 ± 0.06
Trifolium isthmocarpum	Moroccan clover	Selection	Nectar	4.21	78.10 ± 1.04
Trifolium michelianum	Balansa clover	Paradana	Nectar	3.92	80.83 ± 0.21
			Honey	3.97	82.33 ± 0.57
Trifolium pratense	Red clover	NSE	Nectar	4.20	80.40 ± 0.10
		NSE	Honey	–	81.00 ± 0.26
		NFE	Honey	–	80.53 ± 0.21
Trifolium purpureum	Purple clover	Electra	Nectar	–	78.47 ± 0.16
Trifolium resupinatum	Persian clover	Kyambro	Nectar	–	81.13 ± 0.06
			Honey	–	80.27 ± 0.21
Trifolium vesiculosum	Arrowleaf clover	Cefalu	Nectar	–	78.47 ± 0.23

Discussion

Our results were compared with those from a large CRC for Honey Bee Products (CRC HBP) study using the same methodology (the results are currently being written up in a series of papers and will be published in due course). The CRC HBP study examined a wide range of Western Australian honeys and included iconic and commercially well-established honeys, such as jarrah (*Eucalyptus marginata*) and marri (*Corymbia calophylla*), as well as niche honeys, such as that of parrot bush (*Banksia sessilis*) and coastal peppermint (*Agonis flexuosa*).

Most of the analysed clover honeys and nectars in our study had higher antioxidant activities than the mean of 4.17 mm Fe/kg (FRAP) recorded for all honeys analysed as part of the CRC HBP study. Only *T. incarnatum* and *T. isthmocarpum* nectar, *T. michelianum* nectar and honey, and *T. pratense* 'NFE' honey were below the overall mean. Most of the analysed honeys and nectars from our study also had higher DPPH assay results than the mean recorded in the CRC HBP study (1613.28 µmol Trolox/kg) with the exception of *T. glanduliferum* nectar, which was slightly lower. Although not formally assessed due to limited sample availability, *O. viciifolia* and *T. vesiculosum* had the darkest nectar and the highest total phenolics contents (39.7 GAE mg/100 g). This is not surprising as colour can be used as a quick indicator of phenolics content (Otmani et al., 2019; Molaveisi et al., 2019).

Interestingly, antibacterial and antioxidant activities are not necessarily correlated. *T. michelianum* honey and nectar, for example, had the highest antibacterial activity for all four test organisms but low antioxidant activity compared to the other cultivars and the much larger honey dataset analysed as part of the CRC Honey Bee Product study. Most likely, the antibacterial activity of these samples is not associated with any phenolic compounds present, but further research is needed to confirm this assumption. This observation is not unusual, certainly not in the context of *Eucalyptus* honeys, which are known to produce enzymatically derived hydrogen peroxide as a main antibacterial compound. Similarly, the main antibacterial activity associated with Manuka honeys (obtained from a range of *Leptospermum* species) is the non-phenolic compound methylglyoxal. Thus, the phenolic fraction of many honeys might be associated more with antioxidant effects rather than antibacterial effects.

The physiochemical analysis of the various samples revealed that, as expected, nectar samples had higher water content than the respective honeys. The maturation of nectar into honey within the beehive involves the evaporation of water from the nectar via hive temperature, trophallaxis and the fanning of bee wings. According to the Codex Alimentarius, honey should not contain more than 20% water; all investigated honeys and three nectars (*T. michelianum*, *T. pratense* 'NSE' and *T. resupinatum*) met this requirement. As antioxidant activity is expressed in relation to the overall weight of the sample, it can be assumed that a lower water content in honey than in nectar will result in higher antioxidant activity. However, this anticipated trend was not seen across all samples where honey and nectar were collected. Further investigations are needed to assess whether this speculative correlation between water content and antioxidant activity holds. All pH values were similar, ranging from 3.92 for *T. michelianum* nectar to 4.27 for *T. glanduliferum* nectar, and within the typical pH range of honey (3.2–4.5).

Overall, the findings of the phytochemical investigation and bioactivity assessments confirmed that not all honeys and nectars are of the same quality, and can differ significantly in their chemical composition, antioxidant activity and antibacterial activity. These differences can be related to the floral source of the honey and nectar, meaning that honeys from different clover cultivars should be considered to obtain a broad range of bioactivities for potential future use as medicinal honeys.

Conclusion

The honeys and nectars tested exhibited a range of bioactivity profiles (antibacterial and antioxidant). Several had higher antibacterial and antioxidant activities than many of the honeys analysed as part of a large CRC for Honey Bee Product study. Based on their bioactivity profile, there may be an opportunity to develop some bee-friendly pasture honeys or nectars into valuable medicinal products.

Implications

There are five key messages from this report: (1) the literature review revealed that pasture legume species are well-suited to providing nutritious and melliferous floral resources for the beekeeping industry across southern Australia; (2) enclosed shade houses can be used as experimental plots for the collection and analysis of monofloral pollen, nectar and honey, as long as the ratio of bees to floral resources is suitable; (3) flowering times varied among the tested cultivars/selections, with flowering commencing in July for *T. incarnatum* 'Caprera' early selection, and continuing into February for *B. bituminosa* and *O. viciifolia*; (4) nectar sugar and pollen crude protein data indicate the suitability of pasture legumes as a floral resource for bees, consistent with the literature for *T. repens* and *T. pratense*; (5) all cultivars/selections had similar quality of honey and nectar but differed significantly in chemical composition, antioxidant activity and antibacterial activity – some had particularly favourable qualities, such as high antioxidant activity indicators.

(1) The literature review revealed that pasture legume species are well-suited to providing nutritious and melliferous floral resources for the beekeeping industry across southern Australia

The literature focuses on *T. pratense* and *T. repens*, traditionally grown in high-rainfall regions of Europe and North America. However, we found many other pasture legume species adapted to southern Australia that can match the needs of the apicultural industry. The aerially seeding clovers bred for Australian agriculture (from Mediterranean origins) have conspicuous flowers that exhibit morphology accessible to bees. The reviewed pasture legumes are adapted to a range of soil types and rainfall zones in southern Australia. *T. michelianum*, for instance, is already being used for grazing, hay, silage or green manure on >1.5 Mha in southern Australia (Craig and Ballard, 2000). Thus, further evaluation of these pasture legumes as floral resources for the beekeeping industry is merited.

(2) Enclosed shade houses can be used as experimental plots for the collection and analysis of monofloral pollen, nectar and honey, as long as the ratio of bees to floral resources is suitable

Further methodology development using enclosed shade houses is needed to gather sufficient quantities of monofloral honey for medicinal analysis. With some further development, they will provide a relatively cheap means for undertaking replicated work on a large number of species. However, if adequate budget is available, future work should consider the use of large-scale commercial field plots for commercial relevance.

(3) The cultivars/selections varied for flowering time, commencing with T. incarnatum 'Caprera' early selection in July, and continuing into February for B. bituminosa and O. viciifolia.

The time of flowering is generally predictable for the current pasture legume cultivars. However, selecting a mixture of legume species (both annual and perennial) and cultivars within these species with variable flowering periods could ensure floral continuity in variable conditions for apiarists. Further selections within current cultivars of other genetic material could extend flowering windows to early winter.

(4) Nectar sugar and pollen crude protein data indicate the suitability of pasture legumes as a floral resource for bees, consistent with the literature on T. repens and T. pratense.

The nutrition provided in nectar and pollen suggests that these pasture legumes are quality resources for the maintenance of bee colony health, with development potential as alternative floral resources for the beekeeping industry.

(5) All cultivars/selections had similar quality of honey and nectar but differed significantly in chemical composition, antioxidant activity and antibacterial activity – some had particularly favourable qualities, such as high antioxidant activity indicators.

There may be potential to develop new and unique honey types from these novel floral sources that have higher market value. There could be an opportunity to develop honey or nectar products for medicinal properties or pollen production specifically for health foods or supplementary feeding.

Recommendations

1. Extend the current work to a multi-year project to investigate the potential for production of high-quality honey from annual and perennial pasture legumes at the commercial paddock scale.

2. Investigate the utility of strategic mixes of pasture legume cultivars to extend the flowering window and produce nectar and honey with desirable characteristics.

3. Identify the best management practices for bees in pasture production systems currently used for ruminant livestock production or commercial seed production.

4. Examine the impact of farming practices (such as crop nutrition) on pollen, nectar and honey quality and quantity.

5. Commence preliminary investigations into other commercially available pasture legume species with existing cultivars and promising floral characters, to broaden the diversity and adaptation range of bee-friendly pasture options for apiarists.

6. Investigate the potential of bee-friendly pasture legume cultivars for developing new and unique honey types with medical or pharmaceutical significance.

7. Produce a 'bee-friendly pasture picker' tool and ute guide to help farmers choose the appropriate bee-friendly pasture mixes for soil and rainfall zones across southern Australia, and an agronomic package for farmers outlining how to best manage bee-friendly pastures for pasture and honey bee benefit.

8. Facilitate further discussions between the pasture seed industry and the beekeeping industry to identify common goals.

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Appendices

Appendix 1. Project steering committee

Table A1. Members of the project steering committee.

Name	Position	Contribution to project
Kevin Foster	Senior Research Officer	Principal investigator
Megan Ryan	Senior Lecturer – Pastures and Livestock	Project and budget management
Joanne Wisdom	Research Associate	Plant physiology and management, sample collection and analysis, method development, literature review
Daniel Kidd	Research Officer	Experimental design, plant physiology and management, sample collection and analysis, method development
Liz Barbour	CEO of CRC for Honey Bee Products	Provides in-kind support to the project from the CRC HBP
Tiffane Bates	Apiary manager	Hive management for field work
Cornelia Locher	Senior lecturer – Medicinal and Pharmaceutical Chemistry	Phytochemical and bioactivity analysis of honey
Kate Hammer	Senior Lecturer – Infection and Immunity	Antibacterial analysis of honey

Project industry advisory panel

The project industry advisory panel was carefully selected to comprise members who had the necessary expertise and passion for the Australian beekeeping industry (Table A2). The panel represented beekeepers, research scientists, commercial honey producers, marketing professionals, seed producers and those in agribusiness. All members are strong advocates for their industry.

Table A2. Members of the industry advisory panel.

Name	Company	Region	Position	Reason for appointment
Peter Cooke	Agknowledge	WA	Director	Strategic planning and agribusiness knowledge. Involved in developing current BICWA strategic plan
Thomas Holliday	MediBee Apiaries	Evans Head, NSW		Associated with the CRC HBP
Mike Bellman	Capilano Honey Ltd and Hive & Wellness Australia	Perth, WA	Branch manager	Strong supporter of the Australian beekeeping industry with commercial marketing experience with honey products
Cormac Farrell	UmWelt Pty Ltd	Canberra, ACT	Environmental consultant	Head beekeeper at Australian parliament with experience in bush and forest management
Lindsey Bourke	Australian Honey Products	Sheffield, Tas	Owner	Extensive experience in the beekeeping industry with knowledge of clover honey. Sits on several bee industry boards and government committees
Paul Costa	Gustare Honey	Kempsey, NSW	Curator and premium honey producer	Experienced honey curator with experience in the production and marketing of unique monofloral honeys with health benefits
Robert Manning	Ex WA government employee (DPIRD)	Rockingham, WA	Private consultant	Former beekeeping research scientist and current member of the AgriFutures Honey Bee and Pollination advisory panel (Note: only one meeting with Dr Manning)
Tiffane Bates	UWA CRC for Honey Bee Products	Perth, WA	Apiary Manager	Apiarist for the research project with previous experience on the AgriFutures Honey Bee & Pollination advisory panel
Rob Bell	Bell Seeds	Elgin, WA	Director	Pasture seeds producer and beekeeping enthusiast

Appendix 2. Extension activities and social media

COVID-19 prevented us from running a formal workshop to meet apiarists and producers face to face. Kevin Foster was the guest speaker at Western Australian Apiarists' Society (WAAS) in April 2021 during their monthly discussion group; he presented the bee-friendly pastures project and discussed rolling out bee-friendly pastures in a Q&A session. Beekeepers who attended agreed that a combined farmer/apiarist field day would be an excellent initiative, as well as a beekeeper's survey on bee-friendly pastures. There was support for the farmer–apiarist model of sharing the costs of establishing bee-friendly pastures, so both groups are invested in the relationship. The talk was streamed live and uploaded to the WAAS website, and is available to its 1000 members. We also presented the bee-friendly pastures project to the Bee Industry Council of Western Australia's (BICWA) executive board meeting in April 2021 to canvas members' opinions on the proposed farmer–apiarist collaboration model, and again received positive feedback. We also presented the project at the NSW Apiarists' Association conference at Tamworth in 2021 (via Zoom) to 150 apiarists and industry representatives. This annual conference is a major industry event on the NSW calendar; again, we received excellent feedback.

We presented our pilot project to BICWA members on 16 September 2020 in Bunbury to seek support for possible trial work in the region. We received excellent feedback and support from their members. We submitted an article to the BICWA in January 2021 for publication in its 'Smoke Signals' newsletter on our current project activities. We continued to engage with the beekeeping industry, farmers and the seed industry directly and via email, phone and social media. One of our tweets reached an audience of 35,000 followers (Perth Today) and was retweeted by ABC Rural (45,000 followers). We wrote a project update for the UWA Institute of Agriculture (IOA) annual research report, which has a mailing list of about 5800 people internationally, and 100 hard copy versions were distributed to industry and research leaders. It is available on the IOA website.

The project industry advisory panel met in April 2021 and we presented a project update, directions for a second phase and plans for longer-term industry goals. Kevin Foster has been appointed to the scientific steering committee for a Wheen Bee Foundation project with an enthusiastic and diverse group of industry representatives from across Australia. The Wheen Bee Foundation supports research projects and activities aimed at keeping bees healthy.

In summary: This project has increased awareness among farmers, agronomists and apiarists of the benefits of bee-friendly pastures.

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new opportunities for the production
of premium and medicinal honey

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