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— Novel Ideas and Pilot Projects —

ASSESSMENT OF POLLEN ASSEMBLAGES FROM THE HIVES OF *TETRAGONULA CARBONARIA* FOR THE PRESENCE OF THE THREATENED SPECIES *GREVILLEA PARVIFLORA* SUBSP. *PARVIFLORA*

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Abstract—Pollen assemblages from managed hives of the Australian social stingless bee *Tetragonula carbonaria* were examined for the presence of the threatened species *Grevillea parviflora* subsp. *parviflora.* Managed hives of *Tetragonula carbonaria* were placed in bushland at Lake Macquarie, New South Wales, Australia, in four known populations of *Grevillea parviflora* subsp. *parviflora* through the main flowering period of 12 September to 2 November 2014. Samples of honey and propolis were collected from the hives at the end of this period and analysed using palynology techniques for the presence of *Grevillea parviflora* subsp. *parviflora* pollen. *Grevillea parviflora* subsp. *parviflora* pollen was detected in all propolis samples from the four study sites but was not found in the honey samples. Field observations identified that honeybees were the dominant insect visitor to *Grevillea parviflora* subsp. *parviflora* at all sites. There were no sightings of *Tetragonula carbonaria* foraging on *Grevillea parviflora* subsp. *parviflora* inflorescences during field observations. This study also demonstrates the application of managed hives of the Australian stingless bees *Tetragonula carbonaria* for monitoring floral diversity where propolis samples can be used to indicate plant species richness (biodiversity) and the presence of specific species relevant to conservation within their foraging area.

Keywords: Honey, palynology, pollen identification, propolis, stingless bees

INTRODUCTION

Grevillea parviflora R.Br. subspecies *parviflora* (Small-flower Grevillea) is a threatened plant species listed as vulnerable under state and federal legislation, and is a matter of national environmental significance. Significant populations of this Grevillea exist in the Lake Macquarie local government area (NSW, Australia) and are facing increasing pressure from land clearing, fire activities, weed invasion, and habitat fragmentation (SEWPaC 2013d). The interim Lake Macquarie *Grevillea parviflora* subsp. *parviflora* Planning and Management Guidelines (June 2013), summarises the current knowledge and status of this Grevillea in Lake Macquarie and identifies scientific research priorities. A key gap in the scientific knowledge is the lack of reproductive biology and ecology information of the species however it was assumed that bees would pollinate the species.

The approach to identifying potential pollinators of rare species typically involves undertaking a vegetation survey and, once rare species have been located, many hours of time consuming observations at several sites (sometimes in remote areas). Since bees forage through the flowering season, it may be useful to look at pollen in the products they produce, such as honey and propolis in hives, to determine which plant species bees are using and whether they are visiting a rare plant in particular. This approach could be particularly useful for creating a "pollen library" for a given area.

Palynological studies of the ecological links between native bees and plant groups have been conducted over the past thirty years in the Amazon region of South America (Santos de Novais & Absy 2013). Recent studies in 2013 from Spain and Brazil have shown that the pollen content in native bee honey is an effective predictor of vegetation in a geographical area around the hive (Gonzalez-Porto et al. 2013; Santos de Novais & Absy 2013). A melissopalynological analysis of honey from the Regional Park of Monti Lucretili identified that bees were visiting a rare and critically endangered species of plant (Canini et al. 2009). There are no published melissopalynology studies of Australian native stingless bees. Australian stingless bees are generalist flower visitors and have an important ecological role as pollinators of native vegetation and agricultural crops (Vit et al. 2013; Slaa et al. 2006; Heard 1999).

Tetragonula carbonaria (Apidae: Meliponini) are the most common species of stingless bees in Australia both in the wild and in managed hives (Halcroft et al. 2013). *Tetragonula carbonaria* are active throughout the year and nest in hollow trees or dead/fallen trees. They form large colonies of approximately 10,000 adult bees where at least 1,000 are foragers and are typically found along the sub-tropical east coast of Australia. *Tetragonula carbonaria* adapt

well to artificial hives and to disturbed environments (Heard 2016). Based on field observations, Grevillea plant species are listed in the top ten best plants for attracting Australian native bees (Dollin et al. 2007).

The objective of this study was to use palynology techniques to investigate the pollen assemblages in *Tetragonula carbonaria* hives placed in close proximity to known populations of the threatened species *Grevillea parviflora* subsp. *parviflora* to determine whether *Tetragonula carbonaria* includes this plant species in its foraging. Data were derived from analysis of pollen (% abundance) from the threatened species *Grevillea parviflora* subsp. *parviflora* in the honey and propolis from hives of the Australian social stingless bees *Tetragonula carbonaria.*

MATERIALS AND METHODS

Four managed hives of *Tetragonula carbonaria*, at the same age and size, and reared under the same conditions were located in four distinct populations of the threatened species *Grevillea parviflora* subsp. *parviflora* as identified by Lake Macquarie City Council's Sustainability Department (see Fig. 1). The information regarding the location of populations was supplied on a confidential basis. All sites are contained in the vegetation community classification 30f named Freemans Peppermint-Apple-Bloodwood Forest as described in the Lake Macquarie City Council Working Draft Composite Vegetation Community Map 2014 (Bell et al. 2014). Plant populations varied in stem counts between sites, with approximately 30 at Colliery, 40 at Ryhope, 100 at Hawkmount, and 140 at Becks. The plant patch size was similar at all sites with plants located within an area of 15 square metres. There was at least 5km between a site and the next nearest study site.

The foraging range of *Tetragonula carbonaria* is 500 m (Heard 2015). The hives were placed on site within 10m of the patches of *Grevillea parviflora* subsp. *parviflora* for the duration of the main flowering period, September and October 2014. In early November, 10 mL of honey and 1.0 g of propolis samples were collected from each of the hives prior to them being removed from the study sites.

Field observations to monitor *Grevillea parviflora* subsp. *parviflora* flowering, insect visitation and pollination/seedpod set, were conducted for 30 mins/site, between 10 am and 2 pm, at least twice a month from August 2014 to March 2015 at the Becks, Hawkmount and Ryhope sites (see Tab. 1). The Colliery site had restricted access and observations were made on only three occasions. Two night-time observations occurred in October and November at all sites.

Honey and propolis samples were processed and analysed in the Australian Palynology Laboratory located at the Australian National University. Honey samples were extracted from honey pots in three study beehives (Colliery,

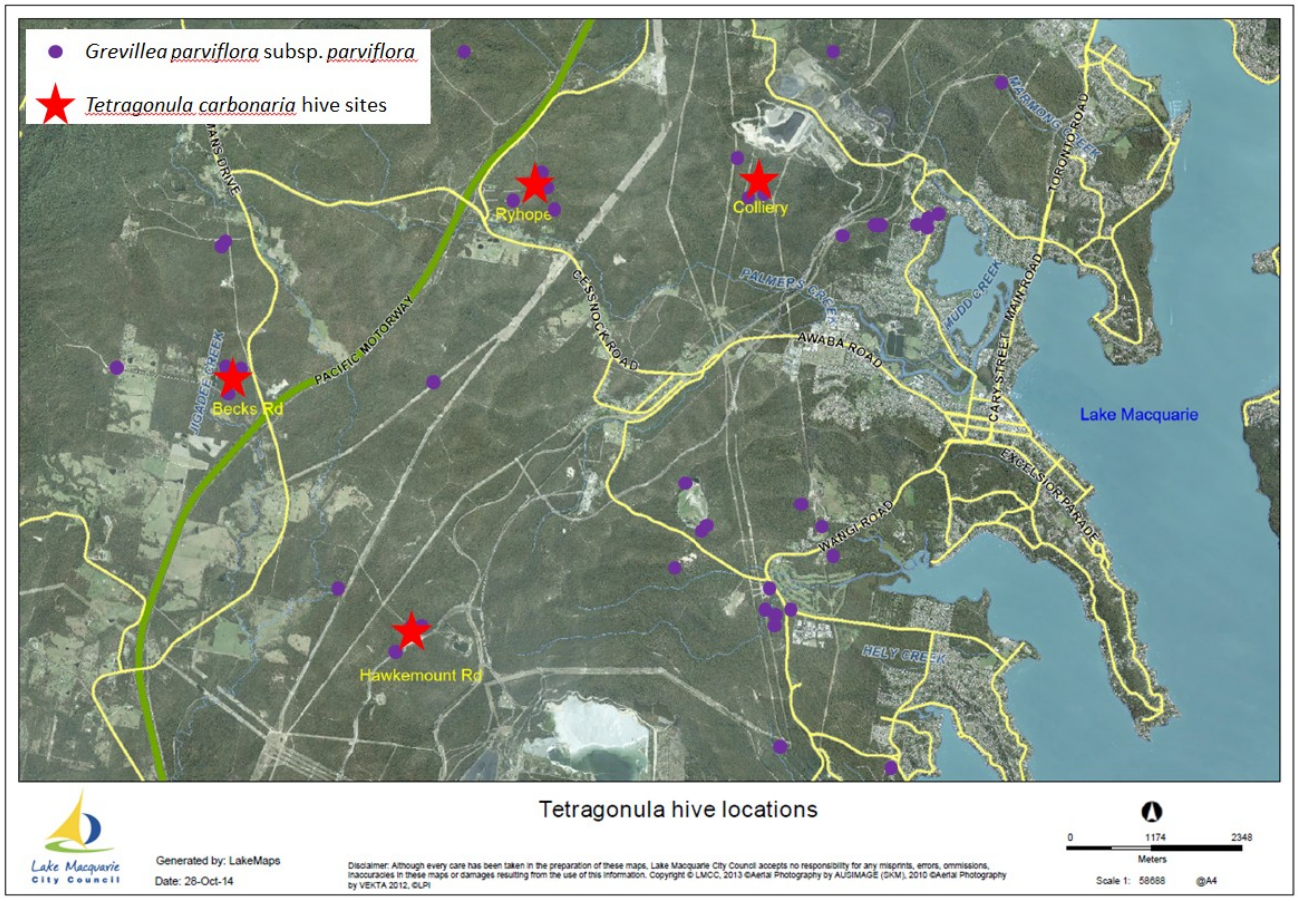

FIGURE 1. Site locations of the four *Tetragonula carbonaria* monitoring hives within patches of *Grevillea parviflora* subsp. *parviflora* in areas of western Lake Macquarie, NSW.

TABLE 1. Record of observational field trips (Hives present from the 12/9/2014 – 2/11/2014).

Site	Date	Time	Flowering	Honey Bees	Moths	Butterflies	Flies	Wasps	Native Bee
Ryhope	21/08/2014	am	Yes	Yes	Yes				
	29/08/2014	pm	Yes	Yes	Yes				
	12/9/2014 (hives installed)	pm	Yes	Yes					
	26/09/2014	am	Yes	Yes	Yes				
	1/10/2014	am	Yes	Yes	Yes				
	6/10/2014	am	Yes	Yes	Yes				
	14/10/2014	pm	Yes						
	23/10/2014	am	Yes	Yes	Yes				
	25/10/2014	pm	Night time visit - no animal activity on plants						
	1/11/2014	am	No						
	2/11/2014 (hives removed)	pm	No						
Hawkmount	21/08/2014	pm	No						
	29/08/2014	pm	No						
	12/9/2014 (hives installed)	am	Yes	Yes					
	26/09/2014	am	Yes	Yes					
	1/10/2014	am	Yes	Yes					
	6/10/2014	am	Yes	Yes			Yes		
	14/10/2014	pm	Yes						
	23/10/2014	am	Yes	Yes	Yes		Yes		
	25/10/2014	pm	Night time visit - no animal activity on plants						
	1/11/2014	am	Yes	Yes					
	2/11/2014 (hives removed)	pm							
Becks	21/08/2014	pm	Yes	Yes	Yes		Yes		Yes
	29/08/2014	pm	Yes	Yes	Yes	Yes		Yes	
	12/9/2014 (hives installed)	am	Yes	Yes					
	26/09/2014	pm	Yes	Yes	Yes				
	1/10/2014	am	Yes	Yes	Yes				
	6/10/2014	am	Yes	Yes	Yes		Yes		
	14/10/2014	pm	Yes						
	23/10/2014	am	Yes	Yes	Yes				
	25/10/2014	pm	Night time visit - no animal activity on plants						
	1/11/2014	am	Yes	Yes					
	2/11/2014 (hives removed)	pm	Yes						
Colliery	26/09/2014	pm	Yes	Yes					
	25/10/2014	pm	Night time visit - no animal activity on plants						
	2/11/2014 (hives removed)	pm	No						

Ryhope and Hawkmount), using a pipette for suction. The Becks beehive had insufficient stores of honey and no sample was collected. The honey was prepared for qualitative microscopic analysis based on the palynology acetolysis method described by Louveaux et al. (1978). Due to the small volume of native bee honey samples collected only 5 mL was used in the acetolysis procedure not 10 mL as described by Louveaux et al (1978).

Propolis

Propolis samples were scraped from the insides of all four study hives with a knife and kept in a fridge at 4°C for 4 weeks until processed for microscopic analysis. The preparation of the propolis for pollen analysis was based on the methods of Barth (1998). They included an initial overnight extraction using ethanol followed by a KOH and ultrasonic treatment before acetolysis.

The prepared microscope slides were analysed at The Australian National University for pollen counts and type using a light microscope at magnifications of 400× to 600×. The counts averaged between 200 and 300 pollen grains per slide. A reference slide of pure *Grevillea parviflora* subsp. *parviflora* pollen was prepared from dried plant specimens following the same process as the propolis samples. The reference slide was used for comparison of Grevillea type pollen found on the sample slides. The Australian Pollen and Spore Atlas (APSA) and The University of Newcastle's Hunter Region Pollen Collection were also accessed for pollen identification.

RESULTS AND DISCUSSION

Field observations for plant visitations by potential pollinators

Over 35 hours of field observations were carried out on 24 separate days between August 2014 and March 2015 across the study sites. Honeybees (*Apis mellifera*) were found to be the most common visitors to *Grevillea parviflora* subsp. *parviflora* inflorescences during the main flowering period of September to November 2014. Honeybees were observed foraging for nectar only, and had no pollen within their corbicula (pollen baskets on their hind legs). They have the potential to pollinate *Grevillea parviflora* subsp. *parviflora* as occasionally while harvesting nectar, the hairs on the heads and backs of honeybees received a dusting of pollen as they encountered the pollen presenter, which is also the stigma.

Other observed insect visitors to *Grevillea parviflora* subsp. *parviflora* included flies, moths, butterflies, wasps, and a *Megachile* species of native bee (see Fig. 2). The native stingless bees (*Tetragonula carbonaria)* were not observed visiting the *Grevillea parviflora* subsp. *parviflora* inflorescences. No insects were observed purposefully foraging for pollen on *Grevillea parviflora* subsp. *parviflora.* No birds or small mammals were observed visiting *Grevillea parviflora* subsp. *Parviflora;* and there was no animal night-time activity observed on *Grevillea parviflora* subsp. *parviflora.* During field observations of *Grevillea parviflora* subsp. *parviflora* the number/ presence of seedpods were

FIGURE 2: Insect visitors collecting nectar from *Grevillea parviflora* subsp. *parviflora* at the Becks Rd site, A) Fly type, B) Native bee *Megachile sp.* and C) Brown potter wasp *Delta sp.* (photos Graham Prichard). The wasp photo was taken in early March 2014 but this wasp species was observed at the Becks Rd site in August 2014 and February 2015.

FIGURE 3: Seedpod of *Grevillea parviflora* subsp. *parviflora* at the Becks Rd site

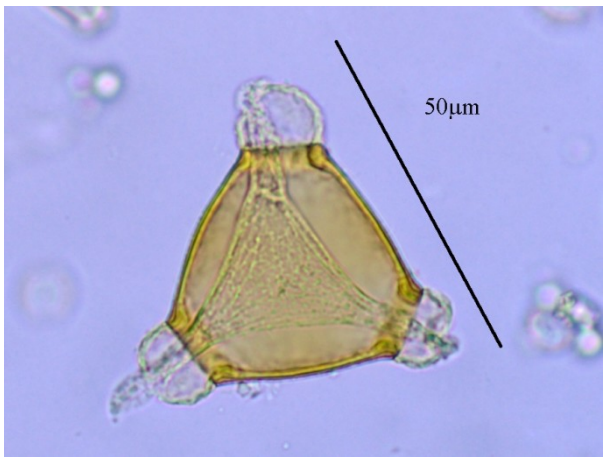

FIGURE 4. Image of Proteaceae pollen (most likely *Grevillea parviflora* subsp. *parviflora)* from the propolis samples from *Tetragonula carbonaria* beehives at 40X magnification.

recorded for each site. Becks produced 21 seedpods and was the only site to do so (see Fig. 3).

Pollen analysis

A total of 27 pollen types were identified from the pollen assemblages of 3 x honey samples from the Ryhope, Hawkmount and Colliery sites, and 4 x propolis samples from the Becks, Ryhope, Hawkmount, and Colliery sites. Propolis samples showed a greater species richness than honey samples. The family Myrtaceae (*Eucalyptus spp.*) was the dominant pollen type in all honey samples (50 – 79%). Cunonaceae was the dominant pollen in the Hawkmount propolis sample (45%), Sapindaceae (*Dodonea sp.*) was dominant in the Becks and Colliery propolis samples (73% and 45% respectively), and Polygonaceae (*Rumex sp.*) dominated the Ryhope propolis sample (50%).

Proteaceae (*Grevillea sp.*) pollen was present in the propolis samples from all four sites (see Fig. 4), but was not present in any of the honey samples. Figures 5 to 8 show the % abundance of species from pollen extracted from honey and propolis at all study sites. The data reflects the biodiversity of flowering plants and bee forage species in the study areas at the time.

Pollen analysis of propolis from the monitoring stingless bee hives in this study showed the presence of *Grevillea sp.* pollen. Comparison with a reference slide of local *Grevillea parviflora* subsp. *parviflora* pollen identifies this as the most likely species. There were other members of the Proteaceae family flowering within the 500 m foraging distance of the hives (including *Grevillea sericea* at the Hawkmount site and *Hakea sericea* at the Becks Rd site) which have similar triangular shaped pollen. Comparisons with reference slides in the Australian Pollen and Spore Atlas helped clarify identifications. Microscopic identification of pollen requires specialised taxonomic skill and there is always the potential for variations in pollen morphology between specimens of the same species. More sensitive techniques for pollen analysis in honey are being developed such as DNA metabarcoding and will be worth considering for future studies (Hawkins et al. 2015).

An interesting observation was the native solitary 'Brown Potter Wasp' feeding on nectar from the *Grevillea parviflora* subsp. *parviflora* flower. As it fed, its body size and shape was perfect for the back of its head to have contact with the pollen presenter/ stigma. This resulted in a dusting of pollen collecting over fine hairs on its head that it carried between flowers and plants (see Fig. 2C). This wasp species was only observed at the Becks Rd site, which was also the only site to record the presence of seedpods, therefore it is considered a most likely pollinator of this species.

The fact that Proteaceae (*Grevillea sp.*) pollen was present in the propolis samples from all four sites, but was not present in any of the honey samples, poses some interesting questions. Does pollen in honey reflect short-term foraging (season) versus pollen in propolis which might reflect long-term foraging (over several seasons)? Which one is best for monitoring biodiversity? Results from this study indicate that propolis is better as it detected *Grevillea parviflora* subsp. *parviflora*, even though field observations did not witness *Tetragonula carbonaria* visiting or foraging on the flowers. While some limitations are acknowledged, this study has shown the great potential for using honey/propolis to assess biodiversity and/or the presence/non-presence of threatened species utilised by these bees.

Conclusion

Pollen analysis of propolis from the monitoring stingless bee hives in this study showed the presence of Grevillea sp. pollen. Honey samples were unsuccessful in detecting the presence of *Grevillea* pollen. This is not surprising as the size of the stingless bees, compared to the distance between the nectaries and the pollen presenter on *Grevillea parviflora* subsp. *parviflora,* makes it virtually impossible for them to remove pollen while collecting nectar. The presence of Grevillea pollen in the propolis suggests the bees have actively gathered this pollen and have encountered the pollen presenter/stigma. *Tetragonula carbonaria* are known to forage pollen from Grevillea flowers (Dollin et al. 2007)

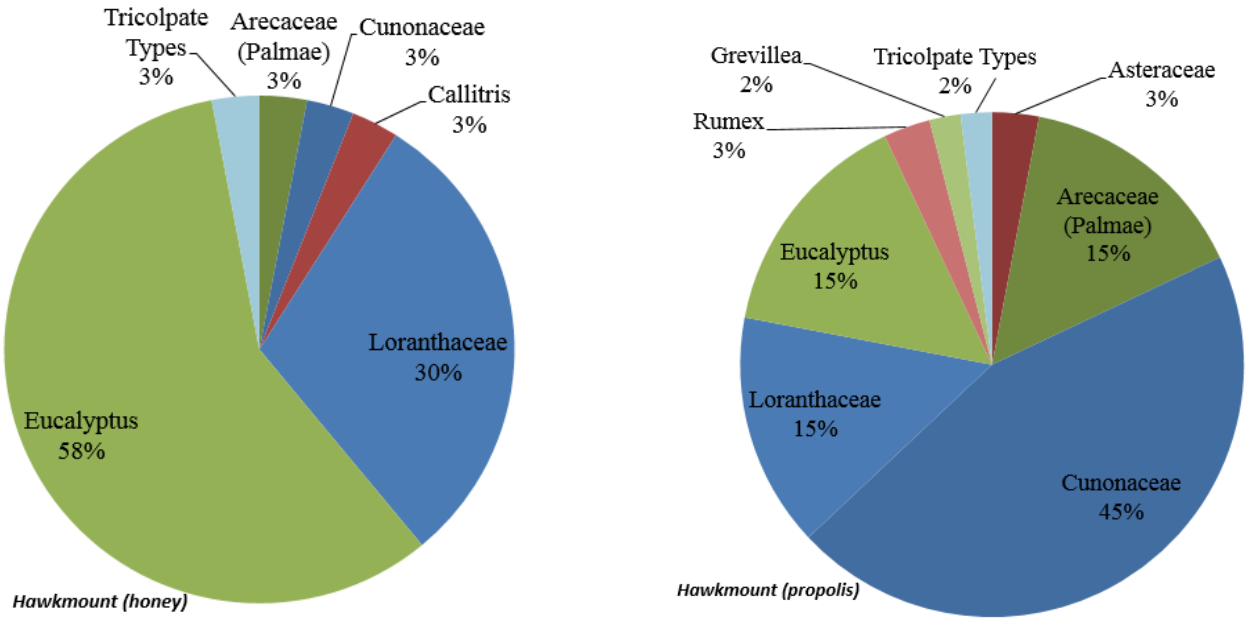

FIGURE 5. Pollen types in honey and propolis samples from *Tetragonula carbonaria* native stingless beehives (Hawkmount Rd)

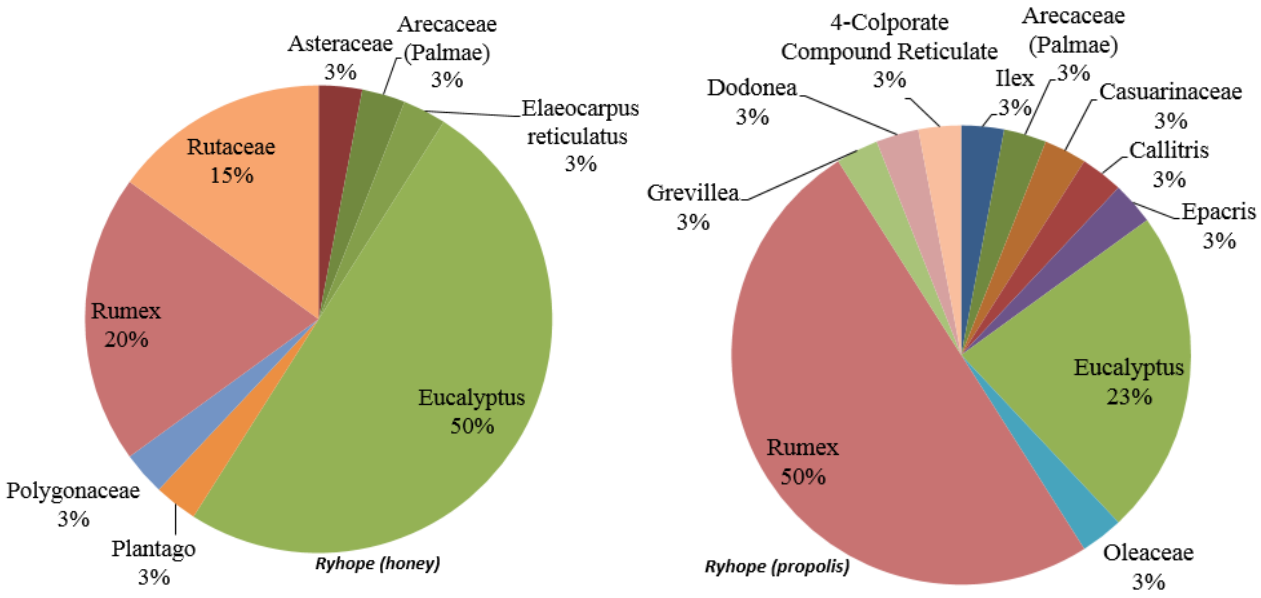

FIGURE 6. Pollen types in honey and propolis samples from *Tetragonula carbonaria* native stingless beehives (Ryhope)

however due to the lack of observed foraging activity of *Tetragonula carbonaria* on Grevillea *parviflora* subsp. *parviflora* in this study, the effect on pollination is undetermined.

This study provided a test case for the application of native stingless beehives and palynology in an innovative method of flora assessment for the detection of a threatened plant species. The study demonstrated the use of propolis samples to quantify floral diversity and monitor the presence of a species relevant to conservation within a foraging area. Studies are underway that utilise both honey bee and *Tetragonula carbonaria* hives to monitor biodiversity in mined land rehabilitation areas in the coalfields of the Hunter Valley (NSW) and all data will form the basis of a pollen library for the Hunter Valley.

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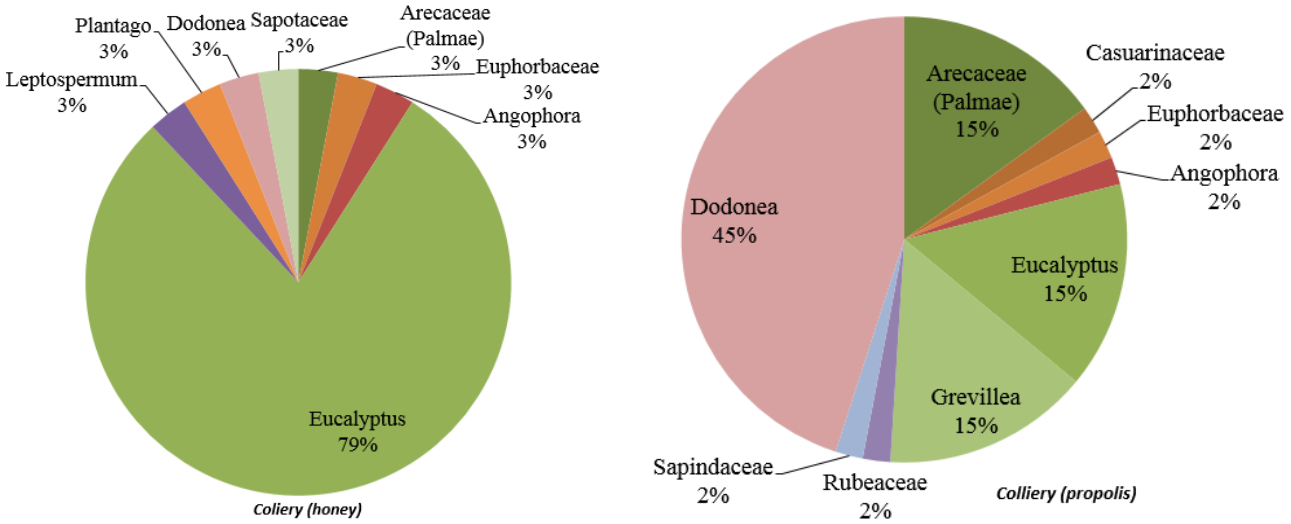

FIGURE 7. Pollen types in honey and propolis samples from *Tetragonula carbonaria* native stingless beehives (Colliery)

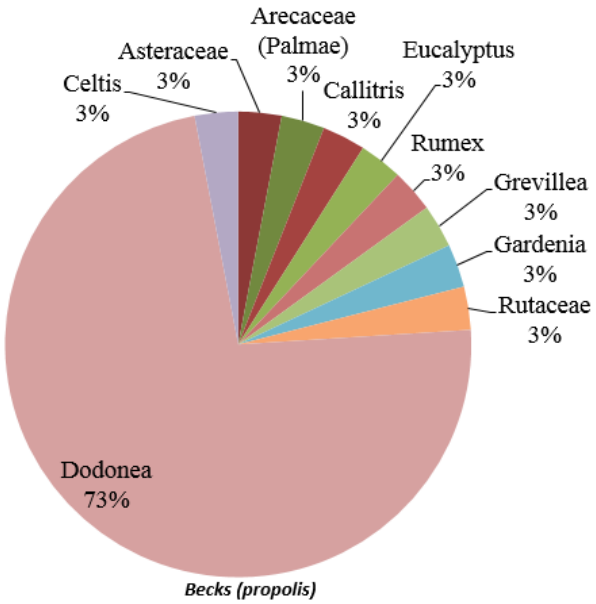

FIGURE 8. Pollen types in propolis samples from *Tetragonula carbonaria* native stingless beehives (Becks Rd) – Note: There was insufficient honey from the Becks Rd beehive to conduct pollen analysis.

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