Pollination of an endangered *Caladenia* (Orchidaceae) via nectar foraging behaviour of a widespread species of colletid bee

Noushka Reiter [1, 2*], Björn Bohman [2, 3, 4], Michael Batley [5], Ryan D. Phillips [2, 6, 7]

[1]Royal Botanic Gardens Victoria, Corner of Ballarto Road and Botanic Drive, Cranbourne, VIC 3977, Australia

[2]Ecology and Evolution, Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia

[3]School of Molecular Sciences, The University of Western Australia Crawley, WA 6009 Australia

[4]Research School of Chemistry, Australian National University Acton, ACT 2601, Australia

[5] Australian Museum, 1 William Street, Sydney, NSW 2010, Australia.

[6]Kings Park and Botanic Garden, The Botanic Garden and Parks Authority, West Perth, WA 6005, Australia

[7]Department of Ecology, Environment and Evolution, La Trobe University, Melbourne, Victoria 3086, Australia

*corresponding author: noushka.reiter@rbg.vic.gov.au

Abstract

The geographic range of pollinators is an important factor determining the distribution of plants with specialised plant pollinator interactions. Further, in specialised plants pollinator availability can be critical for the success of conservation translocations of threatened flora. Here, we investigated the pollination biology of the endangered orchid *Caladenia versicolor*, with the aim of improving both management of wild populations, and conservation translocations. Using portable groups of cultivated plants to attract pollinators, we found that at natural sites *Caladenia versicolor* is predominantly pollinated by food-foraging males of one species of bee, *Leioproctus platycephalus* (Colletidae), with only occasional visits from females or other bee species. Interestingly, this apparently high degree of specialisation occurred despite the presence of a co-occurring bee community of over 20 species. While previously thought to be nectarless, gas chromatography – mass spectrometry analysis of labellum secretions revealed that *C. versicolor* produces meagre quantities of sucrose on the upper surface of the labellum, on which some pollinators appeared to feed. Reproductive success was high in *C. versicolor* at both natural and translocated sites. While *C. versicolor* now has a restricted range, *L. platycephalus* is found across a broad area of southern Australia. While pollinator availability does not appear likely to have contributed to the rarity of *C. versicolor*, the apparent reliance on *L. platycephalus* means that the availability of this species needs to be taken into account for conservation management and translocations.

Introduction

Many plant species show some level of pollinator specialisation, often being primarily reliant on a particular functional group of pollinator (Fenster *et al*., 2004). Further, there is increasing recognition that many species of plants have highly specialised pollination strategies involving one or few pollinator species (Schiestl & Schlüter, 2009; Johnson 2010). In the event that pollinators have a narrow geographic range or occur at low abundance, this specialisation has the potential to limit the geographic range and abundance of the plant species (Duffy & Johnson, 2017). For example, in the highly specialised orchid genus *Drakaea*, the least abundant orchid species within a given region were those where the pollinator occupied the lowest proportion of suitable habitat patches (Phillips *et al*., 2014). This issue is likely to be exacerbated in anthropogenically modified landscapes, where populations of pollinators are likely to go extinct when they inhabit smaller, more isolated remnants (Biesmeijer *et al*., 2006; Pauw & Hawkins, 2011). The potential vulnerability of specialised pollination systems poses challenges for conservation, not only through maintaining populations of the pollinator to support populations of the rare plant (Phillips *et al*., 2015), but also when selecting sites for the establishment of new populations via conservation translocation (Reiter *et al*., 2017; see IUCN (2013) for definition).

Among plant families, the orchids are characterised by a high incidence of specialised pollination strategies (Tremblay, 1992; Johnson & Steiner, 2003; Schiestl & Schlüter, 2009),

and pollen-limited fruit set (Tremblay *et al*., 2005), making resolving the availability of pollinators an important issue for the conservation of many species. In orchids, specialisation can arise in part through the gullet-shaped flowers, where only pollinators of a particular size may be capable of transferring pollen (e.g. Li *et al*., 2008; Reiter *et al*., 2018). However, many orchid species are specialised at the attraction phase, where only one or few pollinator species respond to the orchid's floral signals (Nilsson, 1992). In these cases, the orchid often utilises a deceptive strategy, such as deception of food-foraging pollinators through the use of floral signals typically associated with a reward (Jersáková *et al*., 2012; Nilsson, 1992), sexual deception of mate-seeking male insects via mimicry of females (Coleman, 1927; Stoutamire, 1983; Schiestl *et al*., 1999), or attraction of female Diptera through the mimicry of brood sites (Martos *et al*., 2015). From a conservation perspective, differences between specialised strategies may have important consequences for the management of threatened orchids. For example, there is evidence in orchids that pollination strategies differ in levels of reproductive success (Neiland & Wilcock, 1998; Phillips *et al*., 2009b; Tremblay *et al*., 2005) and resilience to small population size (Jacquemyn *et al*., 2007; Johnson *et al*., 2009; Phillips *et al*., 2014). Further, the mechanism of attracting pollinators, and the group of animals involved, affects the capacity to survey for the availability of pollinators (Phillips *et al*., 2014; Van der Niet *et al*., 2015)

Caladenia R.Br. is a large genus of orchid with over 370 species (Backhouse *et al.*, 2016), most of which occur exclusively in Australia. Ninety-seven species of *Caladenia* are considered to be of conservation concern (Backhouse, 2007) and, without on-ground conservation efforts such as conservation translocation, many of these species may go extinct this century (Swarts & Dixon, 2009; Reiter *et al*., 2017. *Caladenia* is unusual in that a range of pollination strategies have been recorded, including species pollinated by sexual deception, nectar reward, food deception and self-pollination (Stoutamire 1983; Phillips *et al*., 2009b; Reiter *et al*., 2018). *Caladenia* pollinated by the sexual deception of thynnine wasps (Tiphiidae) typically have green/red flowers, which are often reduced in size and accompanied by a dense aggregation of calli on the labellum (Stoutamire, 1983; Phillips *et al*., 2017). Many other *Caladenia* are considered likely to be pollinated by food deception (Bower, 2006; Bower, 2008), based on bright colouration (pink, yellow, white) and a lack of visible nectar. Interestingly, there has been no detailed study of food-deception in *Caladenia*, and some putatively deceptive species have been shown to produce small quantities of nectar (Faast *et al*., 2009; Reiter *et al.*, 2018). Studies of the pollination of nectar producing *Caladenia* have revealed systems using nectar foraging thynnine wasps in *C. colorata* (Reiter *et al*., 2018), and a strategy involving the attraction of a suite of Diptera and solitary bees in *C. rigida* (Faast *et al*., 2009). Given the specialisation on one or few pollinator species in many *Caladenia*, and the low pollination rate exhibited by some species (Phillips *et al*.,

2009), management could potentially be improved through an understanding of the pollination strategy, and the biology of the animals involved.

Caladenia versicolor G.W.Carr. is listed nationally under the *Environment Protection and Biodiversity Act 1999* and is considered endangered using IUCN criteria (Backhouse & Cameron, 2005). Formerly distributed from central Victoria to near Adelaide in South Australia, *C. versicolor* has undergone a substantial range reduction and is now known from only four wild sites, all of them in Victoria. Prior to clearing, *C. versicolor* may have been widespread in areas of productive, low-lying soils (Duncan *et al.*, 2010) in western Victoria, a habitat that has been extensively cleared for agriculture. The current major issues for the persistence of this species are the small numbers of populations, small population size, and reduced rainfall during the growing and flowering season. In addition, parts of the Black Range west of the Grampians are now under threat from logging (VNPA, 2017), which may potentially threaten populations of *C. versicolor*. *Caladenia versicolor* is part of a major conservation translocation program to propagate and translocate this species back within its former range (Reiter *et al.*, 2016). An understanding of the pollination ecology of *C. versicolor* is likely to be important for the conservation and recovery of the species.

Despite intensive efforts, a previous attempt to identify the pollinators of *C. versicolor* was unsuccessful. Using the baiting method developed for sexually deceptive orchids, 163, 3-

minute trials using 3 flowers were undertaken within the natural range of *C. versicolor* (Bower, 2006). Due to the absence of a pollinator response to a small number of bait flowers, the absence of visible nectar, and the bright floral colouration (pink and white) and strong floral odour of *C. versicolor,* it was concluded that the species is likely to use food deception to attract pollinators rather than sexual deception (Bower, 2006). Given the concerns over the conservation of *C. versicolor*, and the lack of resolution of the pollination strategy used, we aimed to address the following questions: (I) What are the pollinators of *Caladenia versicolor*? (II) Are the pollinator species the same at different populations, including those initiated via conservation translocation? (III) Is the pollination system specialised relative to the available community of potential pollinators? (IV) Does *C. versicolor* produce a nectar reward? (V) Does fruit set vary between populations, including those initiated via conservation translocation? We also provide information on the diet and geographic range of the pollinator species, and discuss our findings in the context of the conservation of *C. versicolour*. Critically, the approach we use here will likely have important implications for other specialised orchids pollinated by food-foraging insects.

Methods

Study Species

Caladenia versicolor is an annually dormant geophyte, with a single leaf up to 10 cm long emerging in May. Flowering is in late September to early November (Jeanes & Backhouse,

2006). A solitary scape is produced per year, which is approximately 25 cm tall and produces one or rarely two flowers (Jeanes & Backhouse, 2006). Floral colours range from an off white through to a dusky pink with characteristic dark, densely glandular osmophores on the tips of all sepals and petals. The flowers of *C. versicolor* produce a distinct, strong musky odour, particularly on hot days. Observation of a large number of plants in shade house conditions has demonstrated that flowers of *C. versicolor* require a vector to achieve pollination. In the wild, almost all pollen deposition events in *C. versicolor* lead to fruit set, the rare exception being those years that are in drought which see plants wither before fruit set (N. Reiter, unpublished data).

Caladenia versicolor is now known from only four wild locations within Victoria, with one of these sites being discovered during the course of this study. Three of these locations contain populations that may be viable in the long term, while one in central Victoria contains only a single known extant plant. In addition to wild sites, two new sites have been initiated through conservation translocations.

Study Sites

We based our study at the three remaining wild sites of this species that have more than one plant (LF, BR1 and BR2; Figure 1), and the two large conservation translocation sites (CT1 and CT2; Figure 1) grown from seed from sites BR1 and LF. We also used one site for

pollinator observations (with cultivated plants) well outside the current range of this species, but within potentially suitable habitat (M, Table 1).

Propagation

We used trays of propagated plants placed in the field as the basis for pollinator observations. These plants were grown from seed collected from the BR and LF sites following the methods of Reiter *et al*., (2016) with minor modifications. Mycorrhizal fungi were isolated from the underground stem-collar by peloton rinsing (Rasmussen & Whigham, 1993) and grown on fungal isolating medium (Clements *et al*., 1986) with streptomycin at 0.05 g/L. Seed collected from across the wild populations (BR1 and LF) was surface sterilized using 0.05% NaOH for 3 minutes, drained through a vacuum filter, and rinsed in sterile water. Seed was then placed on filter paper on modified OMA (Clements and Ellyard, 1979) consisting of (2 g oats + 0.1 g yeast + 9 g agar)/L water. Two cubes of media containing fungi (approximately 1 cm square) were placed on either side of the seed, the petri dish was wrapped with parafilm, and incubated in the dark for one month. Seedlings were then grown under lights for a further 2 months and transferred to tubs containing a layer of OMA covered in 2 cm of sterile vermiculite mix (i.e. 2 g oats, 0.1 g yeast, 100 ml water per 1 L volume of vermiculite). Seedlings were then grown for a further 12 weeks before being deflasked in the nursery to 12 inch tubs containing Terrestrial Orchid

Conservation Mix (BioGro®). Seedlings were watered as required and grown for two years in the nursery, at which point they were mature enough to flower and be used as bait plants.

Pollinator Observations

Baiting using orchid flowers is an effective tool for attracting pollinators for many sexually deceptive orchids. This method involves picking flowers and moving them to a new part of the landscape, often leading to a rapid response by the pollinator species if present (Stoutamire, 1983; Peakall, 1990; Peakall & Beattie, 1996). However, baiting with three flower stems (Peakall & Beattie, 1996) with *C. versicolor* has previously proven unsuccessful (Bower, 2006). Here, we used a modification of this method (Reiter *et al*., 2018), which has been trialled on nectar foraging thynnine wasps. We used floral displays of between 15 and 20 propagated flowering plants in a tray to provide an increased stimulus to pollinators. Based on observations of thynnine wasp and bee activity at the study sites, baiting trials were only conducted between approximately 10am and 3:30pm on days that were dry, mostly clear, and above 18°C. When surveying sites for the presence of pollinators, bait flowers were exposed for a 10-minute period and, if there was no response, the bait flowers were moved to a new position approximately 20 m away. At the BR1 site, at baiting locations where pollinators continued to respond consistently beyond the 10-minute period, trays were left in the same position for 2-hour periods. We baited across five sites (LF, BR1, BR2, CT1 and M) over 2015 and 2016 with a total of 213 trials. Baiting in 2016

was restricted due to exceptionally long periods of cold and wet weather during the flowering of the orchid. Trials were conducted between the 1st and 29th of October 2015 and 2016.

Visits by potential pollinators to *C. versicolor* were scored in the following way: time of landing after the commencement of the trial; where on the flower the insect landed; behaviour of the insect i.e. if they exhibited copulatory or feeding behaviour; if the insect contacted the column, and if pollinia were removed or deposited. To investigate the time taken to respond to the flowers, 37 6-minute baiting trials were undertaken at a site of relatively high pollinator abundance (BR1). For each floral visitor, the time taken to land after the commencement of the trial was recorded. For quantifying behaviour, these observations were combined with those from the ten-minute trials. A selection of the floral visitors was caught with an insect sweep net and either pinned or stored in 80 % ethanol to allow identification. The plants used as a bait flowers are kept as part of the Royal Botanic Gardens Victoria's living plant collection as accession 150878. The seed that was sourced to grow these plants came from the LF and BR1 populations (Table 1).

Quantifying the pool of potential pollinators

Following evidence in the course of our study that *C. versicolor* is pollinated by bees, we surveyed the diversity of the bees in the community relative to those visiting the orchid. We

used two approaches to collect bees, sweep netting transects (Janzen, 1973) and pan traps (Gollan *et al*., 2011; Leong & Thorp, 1999). At the BR1, LF and CT1 sites 100m transects were walked through the most floriferous parts of the bushland over the course of a day, and bee species were collected with a sweep net. Bees were either stored in 80 % ethanol or pinned for later identification. At the CT1 and CT2 sites, fifteen white, yellow and blue plastic dishes filled with 3 cm of water with a drop of detergent were placed among the *C. versicolor* population for 5 days at the start of the flowering period. Each morning all insects captured were checked for orchid pollen then sorted into bees, wasps, flies and others, and stored in 80 % ethanol for further identification. All bees collected were identified using the keys of Batley & Houston (2012), Maynard (2013) and Walker (2015).

Distribution of C. versicolor *and its pollinators*

All museum records of the pollinator species on the Atlas of Living Australia database (ALA, 2018) (excluding photos and unconfirmed sightings), together with specimens from this study, were compiled. Herbarium records of *C. versicolor* from both extant and extinct sites were then overlaid into a distribution map. A record from well outside of the known range of *C. versicolor* (in northern Tasmania) was considered erroneous and removed from the dataset.

Forage plants of the pollinator species

To determine the food plants of the pollinator species, and test if there is any difference in foraging between males and females, we undertook sweep-netting at co-flowering plants at BR1, CT1 and LF. In addition, we extracted information from a database of museum collections established by MB with data on site of collection, food plant, and sex of the bee.

Fruit set

Fruit set for *C. versicolor* was scored between 2015 and 2017 at the two conservation translocation sites and across three wild populations over 2016 and 2017. However, not all sites were surveyed in each year (Table 4), in part due to one of the conservation translocations not yet being established in 2015. To quantify the reproductive success of *C. versicolor*, the average fruit set across years was calculated for each site, then these values were averaged to give an overall value for the species.

Does Caladenia versicolor *produce nectar?*

On three occasions in 2016 when the temperature was above 21° C, 33 flowers in total (cultivated plants in an insect proof shade house) were sampled for nectar by adding 5 µl of deionised MilliQ water onto the labellum of *C. versicolor* and collecting the aqueous extract for analysis using a 5 µl microcapillary tube. *Caladenia tentaculata,* a sexually deceptive species that produces no nectar (Peakall and Beattie, 1996), was used as a negative control. Aqueous extracts were transferred to glass vials (2 ml) with glass inserts (50 µL) and stored

at -20°C. The solvent was evaporated with a gentle stream of nitrogen. Methoxyamine-HCl (20 µl of 20 mg/ml solution in pyridine) was added and the sealed vials were heated for 2 hours in a heating block at 37°C. At the same temperature, the extracts were treated with MSTFA (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide, 35 µl) in sealed vials for 1 hour before GC-MS analysis.

Following this preliminary analysis with combined samples, a second analysis was performed in 2017 on three individual plants using the methods of Reiter *et al*., (2018), also using shade house plants. Here, for each of 3 flowers, 5 µL of an aqueous solution of ribitol (internal standard, 0.20 mg/ml) was added with a glass syringe on to the labellum. The aqueous extract was subsequently collected with microcapillary tubes (5 µL) and immediately transferred from microcapillaries to GC vials (2 mL) with inserts (50 µL). This process was undertaken three times for each flower on different parts of the labellum, with the extracts for each flower combined in the same vial.

For both sets of extracts, GC-MS analysis was performed on an Agilent 5973 mass selective detector connected to an Agilent 6890 GC equipped with a BPX5 column [(5 % phenyl polysilphenylene-siloxane), 30 m × 0.25 mm × 0.25 µm film thickness, SGE Australia], using helium as carrier gas. An Agilent 7683 autoinjector was used and injections (3 µL) were performed in split mode (1 to 10). The oven temperature started at 40°C and increased to 300°C at a ramp rate of 5°C/min and maintained for 15 min. Tentative identification of trimethylsilylated monosaccharides and sucrose was based on the comparison of retention

indices and mass spectra with data from a mass spectral library (NIST-11). All tentative identifications were confirmed by co-injections with synthetic standards. For the second analysis, quantification of glucose, fructose and sucrose was achieved by comparison of peak areas of total ion chromatograms (TIC) of nectar samples with the known amount of the internal standard ribitol. The response factors for the respective carbohydrates sampled and the internal standard were included in the calculation of the amounts of analytes (see Reiter *et al*., 2018).

Results

Pollinator Identification and Behaviour

At the BR1 sites (natural populations) we observed 105 visits of *Leioproctus (Leioproctus) platycephalus*, with all of the vouchered specimens being male. At BR2 (natural) we made a single observation of a male *L. platychephalus*. At LF (natural) we had three observations of male *L. platycephalus*, and one observation of a male *Lasioglossum (Chilalictus) globosum*. At CT1 (conservation translocation population) we observed visitation by four male *L. platycephalu*s, one female *L. platycephalus*, and one female *Trichocolletes venustus.* At M, outside of the extant range of *C. versicolor*, but within its historic range, we observed visitation by a single female *Lasioglossum (Chilalictus) littleri.* The number of bees visiting *C. versicolor* per hour varied greatly between sites, with response rates much higher at the BR1 site than all other sites (Supp Table 3).

For the primary pollinator species, *L. platycephalus*, from 213 baiting trials there were 113 observations of visits to *C. versicolor* bait flowers across the five sites. Thirty nine percent (*N* = 46) of these visits involved a close approach to the bait flowers without landing. Of those *L. platycephalus* that landed on the bait flower 80 % landed on the labellum (*N* = 57) and the remaining landed on the sepals or petals (*N* =14). Of the *L. platycephalus* that landed, 38 % came into contact with the column (*N* = 27), 4 % deposited pollinia (*N* = 3), and 18% removed pollinia (*N* =13) (Figure 2). *Leioproctus platycephalus* displayed food foraging behaviour (Figure 4) when on the labellum, typically moving with head down, along the surface of the labellum to the base of the column. From photos it could be clearly seen that in at least some individuals the mouthparts were extended onto the surface of the labellum. No courtship or copulatory behaviour was observed. The other bee species, which were all comparatively rare visitors, exhibited similar food foraging behaviour, but pollinia removal was only observed for female *Lasioglossum (Chilalictus) littleri*, while pollinia deposition was not witnessed. Syrphid flies were attracted at all sites but none removed or deposited pollinia. In 6-minute trials, 62 % of responses of *L. platycephalus* (*N* = 37) were in the first 3 minutes, but some individuals continued to respond for the full six minutes (Figure 3).

Quantifying the pool of potential pollinators

In addition to *L. platycephalus*, twenty-one other bee species were caught via sweep-netting and pan traps, including an undescribed species of *Leioproctus.* The majority (13) of species (Table 3) belonged to the genus *Lasioglossum*.

Distribution of the pollinator species

Caladenia versicolor was originally distributed from South Australia to Port Phillip Bay in Victoria, but is now known only from south-western and central Victoria (Figure 5). In contrast, *L. platycephalus* is widespread in eastern and southern Australia, with records from as far north as Queensland, and some records from semi-arid areas of south-western Western Australia (Figure 5).

Forage plants of the pollinator species

There were 48 co-flowering species across the three sites surveyed (Supplementary Table 1). Following targeted collections from co-flowering species at BR1, CT1 and LF, male *L. platycephalus* were collected on three occasions, twice on *Hibbertia sericea* (R.Br. ex DC.) Benth. and once on *Leptospermum myrsinoides* Schltdl. In addition, an individual male *L. platycephalus* was seen to remove pollen from *C. versicolor*, before being immediately recaptured after it landed and began to feed on *Calytrix tetragona* Labill.. No female *L. platycephalus* were collected in pan traps or sweep-netting transects. Based on a compilation

of collection records, *L. platycephalus* has mostly been found on a range of genera in the Fabaceae, and less frequently on *Acacia* (Mimosaceae), *Boronia* (Rutaceae) and *Trimenia* (Trimeniaceae) (Supplementary Table 2).

Fruit set of Caladenia versicolor

The average pollination rate across the five sites was 49.73 ± 7.98 % (SE). Across three natural populations of *C. versicolor* the pollination rate (Table 4) averaged 56.51 ± 12.35 % (S.E.). At the two translocated sites pollination rate averaged 39.55 ± 2.55 % (S.E.). Pollination at an individual site varied between years by between 16.2 % and 33.8 %.

Does Caladenia versicolor *produce nectar?*

The three samples of nectar from *Caladenia versicolor* where nectar extracts were pooled across individuals contained ca 3.6- 4.7 µg saccharides per flower, with >95 % sucrose and < 5 % monosaccharides. In comparison, the sexually deceptive *C. tentaculata* contained less than 0.01 ng saccharides per flower (trace amounts). The three individual samples using the ribitol standard revealed a large variation in sugar content per flower, with 1.1 - 114 µg saccharides per flower (95 % sucrose and < 5 % monosaccharides) with an average of 39.5 ± 37.42 (S.E.) µg.

Discussion

The few remaining natural populations of *Caladenia versicolor* are primarily pollinated by one species of bee, *Leioproctus platycephalus*. Interestingly, the majority of *L. platycephalus* visiting *C. versicolor* were males, with only one confirmed visit by a female. However, our evidence suggests that *C. versicolor* is capable of attracting males and females of other bee species in low numbers, which may occasionally act as pollinators. The bees attracted to *C. versicolor* displayed food foraging behaviour, and the labellum of the orchid was shown to produce meagre quantities of nectar. Other nectar-producing species of *Caladenia* are either pollinated by a suite of pollinators including Hymenoptera and Diptera (Faast *et al*., 2009), specific species of nectar-foraging thynnine wasps (Reiter *et al*., 2018), or specific species of sexually deceived thynnine wasps (Dixon & Tremblay, 2009; Phillips *et al*., 2017). As such, this is the first study to show a specialised bee pollination system in *Caladenia* and, given that nectar wasn't visible to human observers, raises the question of how many species of *Caladenia* predicted to be food-deceptive are actually nectar producing? Further, we show that our modified baiting technique based on using large numbers of potted plants (Reiter *et al*., 2018) may be effective for a range of food-foraging pollinator species.

Specialisation on one bee species

Pollination systems that specialise on one or few species of bee have not been previously observed in *Caladenia*. Specialisation in orchids can arise from the gullet shaped structure of the flowers limiting the number of pollinator species with appropriate morphology to remove

and deposit pollen (e.g. Li *et al*., 2008; Reiter *et al*., 2018). In these cases a range of floral visitors are observed, but only a limited subset actually achieve pollination. In contrast in *C. versicolor*, almost all visitors belonged to *L. platycephalus*, indicating filtering of pollinators at the attraction phase. Our result is particularly interesting given that there were 21 species of co-occurring bees at our surveyed sites. Further, there was an additional species of *Leioproctus* present at our study sites of similar size to the primary pollinator *L. platycephalus* that was not attracted to the orchid. Given the unexpectedly high levels of specialisation seen in *C. versicolor*, teasing out the roles of visual and odour cues in pollinator attraction would be an interesting avenue of future research.

Among the 113 observations of *L. platycephalus* attracted to *C. versicolor* there was only one female attracted, indicating a strong male bias in floral visitation. There are several possible explanations for the male bias including: males emerging prior to females, as seen in six related species of *Leioproctus* (Maynard, 2013); different dietary preferences between males and females (Ostevik *et al*., 2010), though based on dietary records this seems unlikely in *L. platycephalus* (Table 6); and long range sexual attraction of the male that then switches to food foraging behaviour on the flower (Bino *et al*., 1982; see Paulus & Gack 1990 for an example of sexual deception of a colletid bee). Further, it is possible that more than one mechanism may contribute towards pollinator attraction. For example, typical food foraging behaviour may be responsible for the attraction of the occasional female and males of other

species, with male *L. platycephalus* being attracted by a specific odour-based mechanism at long range, before switching to food foraging behaviour on the flower.

Does Caladenia versicolor *produce nectar?*

Nectar production in *C. versicolor* is generally very low, with GC-MS analysis suggesting that most individuals had less than 10 µg of sucrose on the upper surface of the labellum. However, one flower contained more than 0.1 mg, which is comparable to some nectar rewarding species. Such variation is in line with some other studies on nectar producing orchids, where not all flowers have been detected with nectar (Indsto *et al*., 2007; Faast *et al*., 2009), or nectar volume or concentration has varied extensively between individuals (Koopowitz & Marchant 1998; Johnson & Hobbhan 2010; Nunes *et al*., 2015). When the mean was taken of the three flowers that were individually quantified, they were shown to have between 1- 470 times less nectar available than 13 species quantified in the literature (Ackerman *et al*., 1994; Galetto *et al*., 1997; Johnson, 1996; Johnson, 2006; Johnson & Hobbhahn, 2010; Nunes *et al*., 2016; Pansarin *et al*., 2014; Stpiczyńska *et al*., 2005; Van der Niet *et al*., 2015). However, the majority of these studies were undertaken on pollination systems involving birds or relatively large bodied insects, meaning that it could be expected that these orchids will produce higher volumes of nectar than bee pollinated species such as *C. versicolor*. Nonetheless, the meagre quantity of nectar present on the labellum of many individuals of *C. versicolor* raises the question of whether sufficient nectar is always present

to function as a reward? Further, the available sucrose may be insufficient to induce pollinator fidelity, but sufficient to encourage the pollinator to linger on the flower and increase the chance of pollen removal and deposition. When pollinators were observed landing on the flower, the majority of visits were brief, while a small proportion involved prolonged attempts to feed. While experimental work is required, this observation is consistent with the idea that not all flowers produce equal amounts of sucrose, leading to variation in the behaviour of visiting bees.

Similarly to *Caladenia rigida* (Faast *et al*., 2009) and *C. colorata* (Reiter *et al*., 2018), sucrose rather than fructose or glucose is the predominant reward in *C. versicolor*. Pollination systems in *Caladenia* based on nectar foraging behaviour now encompass pollination by thynnine wasps (Reiter *et al*., 2018), multiple species of Diptera and Hymenoptera (Faast *et al*., 2009), and *Leioproctus* bees (the present study). The main reward in all three orchid species has been sucrose, meaning that thus far there appears to be no association in *Caladenia* between sugar composition and the pollinator group involved.

Reproductive success

Across all populations *Caladenia versicolor* has a high pollination rate of on average 49.73% ± 7.98 (N = 5), with variation between years at the same site as much as 28%. This overall pollination rate is greater than (i) the average of 37.1% fruit set in a review of rewarding

orchids (*N* = 84; Tremblay *et al*., 2005), (ii) *Caladenia* thought to be food deceptive (mean fruit set = 36%, *N* = 5; Phillips *et al*., 2009b) and (iii) other food rewarding *Caladenia* (Reiter *et al*., 2018; 33.2% natural sites and 50.7% translocated sites). The high reproductive success of *C. versicolor* suggests that its pollination strategy may confer a high level of pollinator fidelity relative to food-deceptive systems, either through the provision of a reward, or a chemical attractant that enhances repeat visitation. While demographic data is needed to establish which phase of the life cycle limits population growth rates (Tremblay *et al*., 2009), it appears likely that levels of fruit set do not limit the persistence of populations of *C. versicolor*. Further, the high levels of fruit set at the populations originating from conservation translocations suggests that pollinator availability will not place a limit on the success of these recently established populations.

Conservation implications for Caladenia versicolor

Both biotic and abiotic interactions influence the distribution of plants at local and regional scales (Wisz *et al*., 2013), with recent evidence suggesting that pollinators can limit the geographic range and abundance of plants with specialised pollination systems (Phillips *et al*., 2014; Duffy & Johnson, 2017; Reiter *et al*., 2017). Although *C. versicolor* appears highly specialised on *L. platycephalus*, this bee is widely distributed across southern and eastern Australia (Figure 5). Overlaying the extinct and extant sites of *C. versicolor* with the distribution of *L. platycephalus* (Figure 5), it is clear that the geographic range of *C.*

versicolor is not limited by that of its' pollinator species. While it remains unknown how common *L. platycephalus* is within patches of suitable habitat within its geographic range, and what its preferred habitats are, given its broad geographic distribution it seems likely to occur across a range of vegetation communities. Nonetheless, because of the apparent specialisation of *C. versicolor*, it will be important to determine presence of *L. platycephalus* prior to undertaking conservation translocations.

At present, we have limited data on the diet of *L. platycephalus*. Based on specimens in the ALA, across its geographic range both male and female *L. platycephalus* frequently feed on members of the Fabaceae (Table 5), though other forage plants have been recorded. In the present study, the low number of *L. platycephalus* encountered while conducting transects may have arisen from *L. platycephalus* feeding on the *Eucalyptus* trees that were flowering at our study sites, preventing collection due to the height of the canopy. We do not know how heavily *L. platycephalus* relies on common plants such as *Calytrix tetragona* for food in sites like BR where there are few Fabaceae. A dietary study focussed on *L. platycephalus* would shed light on what this species is feeding on when the abundance of Fabaceae is low, or if the high number of Fabaceae records in museum collections accurately reflects their true diet or merely the dominant flowers present at the time of their emergence. Given that many solitary bee species are known to have foraging distances from the nest of < 600m (Gathmann & Tscharntke, 2002) and a maximum distance of 1.9 km was recorded in feeder training

experiments (Greenleaf *et al*., 2007) caution should be exercised in altering the vegetation at these sites. A mark-recapture study would be useful in determining foraging distances for this species to determine how large an area is required for adequate protection.

Implications for the study of rare orchids

In orchids, many studies of pollination by nectar foraging pollinators are characterised by low visitation rates (e.g. Galetto et al. 1997; Johnson 2006; Faast *et al*., 2009; van der Niet *et al*., 2015), placing a constraint on the generation of both ecological and experimental data. However, our use of numerous potted plants and a baiting-based approach to attract numerous individuals of the pollinator species may have partly removed this limitation (as trialled for food foraging thynnids in Reiter *et al*., 2018). In our study we did not find any *L. platycephalus* in our pan traps, and had limited success with sweep netting vegetation, suggesting that baiting may also be a more reliable method for determining the presence of the pollinator species at any given site. Should our baiting-based approach be transferable outside of *Caladenia*, this technique could potentially be utilised for a wide taxonomic range of orchids. Further validation of this approach should be undertaken, including testing whether the number of flowers used affects which pollinator species are attracted to the orchid, and testing how well survey data using the flowers as bait corresponds to established survey methods.

Based on floral colouration and the absence of visible nectar, it is predicted that a large number of rare *Caladenia* are pollinated by the deception of food-foraging insects (Bower, 2006, 2008; Phillips *et al*., 2011). Given our findings in *C. versicolor*, and recently in *C. colorata* (Reiter *et al*., 2018), *Caladenia* previously assumed to be food-deceptive may have specialised pollination systems that use a nectar reward. These results challenge how many *Caladenia* are actually deceptive, and suggest that all *Caladenia* species previously thought to be food deceptive should be examined for the presence of nectar using quantitative approaches. Such work should be accompanied by experimental studies testing the quantity of sugar required to function as a reward for bees visiting *Caladenia*. More broadly, our findings raises the possibility that many orchid species that are assumed to be food-deceptive may actually produce a meagre nectar reward.

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Tables

Table 1: Study sites and approximate number of plants. * Number based on survey data of flowering plants.

Site	Accession	Number of Plants
CT1		274
CT2		338
LF Wild Site	MEL 2020254A	100-600*
BR1 Wild Site	MEL 2325802A	100*
BR2 Wild Site		50*
M	N/A No plants present	0

Table 2: Bee voucher species collected landing on the labellum of *Caladenia versicolor* at different sites. Natural populations of *C. versicolor* occur at BR1, BR2 and LF. A population of *C. versicolor* initiated through conservation translocation occurs at CT1. While suitable habitat, *C. versicolor* does not occur at M. PR indicates that pollen removal was observed, PD indicates that pollen deposition was observed.

Species	**Year**	**M**	**CT1**	**BR1**	**BR2**	**LF**	**PR**	**PD**
Lasioglossum (Chilalictus) littleri ♀	2015	(*N*=1) F					Yes	No
Trichocolletes venustus ♀	2015		(*N*=1)F				No	No
Lasioglossum (Chilalictus) globosum ♂	2015					(*N* =1)M	No	No
♂ *Leioproctus (Leioproctus) platycephalus*	2016			(*N* =3)M	(*N* =1)M		Yes	Yes
Leioproctus (Leioproctus) platycephalus	2015		(*N* =1)F (*N* =1)M	(*N* =11)M		(*N* =1)M	Yes	Yes

Table 3: Bee community collected through pan traps and sweep netting at LF BR and CT1 sites of *Caladenia versicolor*. 'net' denotes specimens collected via sweep netting.

Species	**CT 1**	**BR**	**LF**
Euhesma sp. M ♀		1	
Euhesma nitidifrons ♀	1 (net)		
Exoneura (Brevineura) sp. ♀		1	
Lasioglossum (Chilalictus) clelandi ♀	8		4
Lasioglossum (Chilalictus) convexum ♀		1	
Lasioglossum (Chilalictus) helichrysi ♀	3		
Lasioglossum (Chilalictus) hemichalceum ♀	1 (net)		
Lasioglossum (Chilalictus) lanarium ♀	1		
Lasioglossum (Chilalictus) lanarium ♂	2		
Lasioglossum (Chilalictus) littleri ♀	1		
Lasioglossum (Chilalictus) repraesentans ♀	2		
Lasioglossum (Chilalictus) sculpturatum ♀		2	
Lasioglossum (Chilalictus) victoriellum ♀		1	
Lasioglossum (Chilalictus) victoriellum ♀	1		
Lasioglossum (Parasphecodes) hiltacum ♀		1	
Lasioglossum (Parasphecodes) lacthium ♀			1
Leioproctus (Exleycolletes) sp. A ♂	1		
Leioproctus (Leioproctus) platycephalus ♂			1
Leioproctus (Leioproctus) sp. B ♂		2	
Lipotriches (Austronomia) flavoviridis species group ♀	1		

Megachile canifrons 2 ♂	2

Table 4: Natural fruit set at wild and reintroduced sites of *Caladenia versicolor*, *N* = number of flowering plants.

Site	2015	2016	2017	Average for site
BR1		70% (*N* =20)		70%
BR2		67.7% (*N* =31)		67.7%
CT1	42.6% (*N* =75)*	58.8% (*N* =153)	25.6% (*N* =82)	42.3%
CT2		28.5% (*N* =56)	45.5% (*N* =244)	36.9%
LF		21.7% (*N* =46)	42.0% (*N* =38)	31.8%

Figures

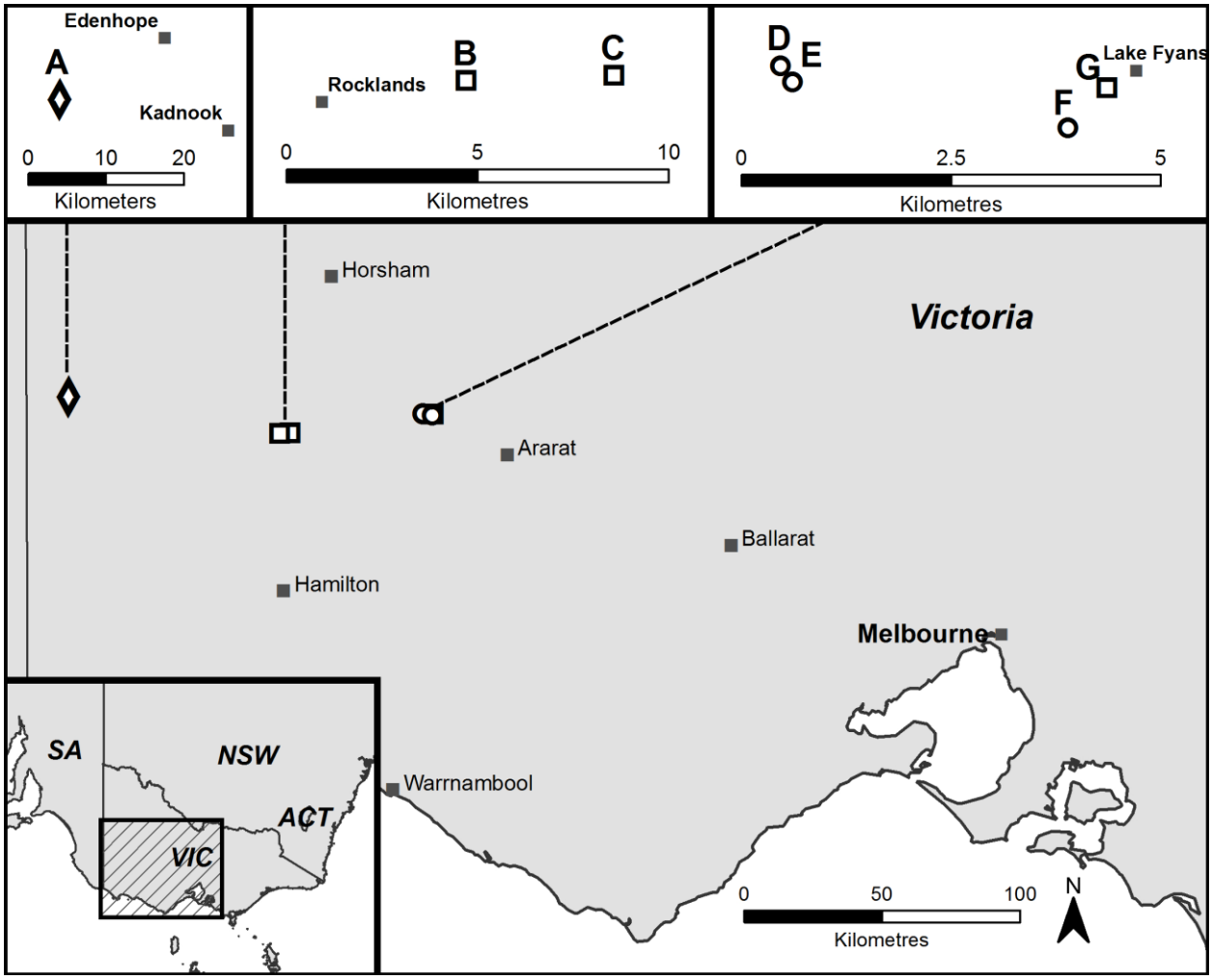

Figure 1: Pollinator study sites of *Caladenia versicolour*. wild sites = white squares; conservation translocation sites = white circle; baiting site outside of known range = white diamond.

Figure 2: ♂ *Leioproctus platycephalus* removing pollinia from *Caladenia versicolor*.

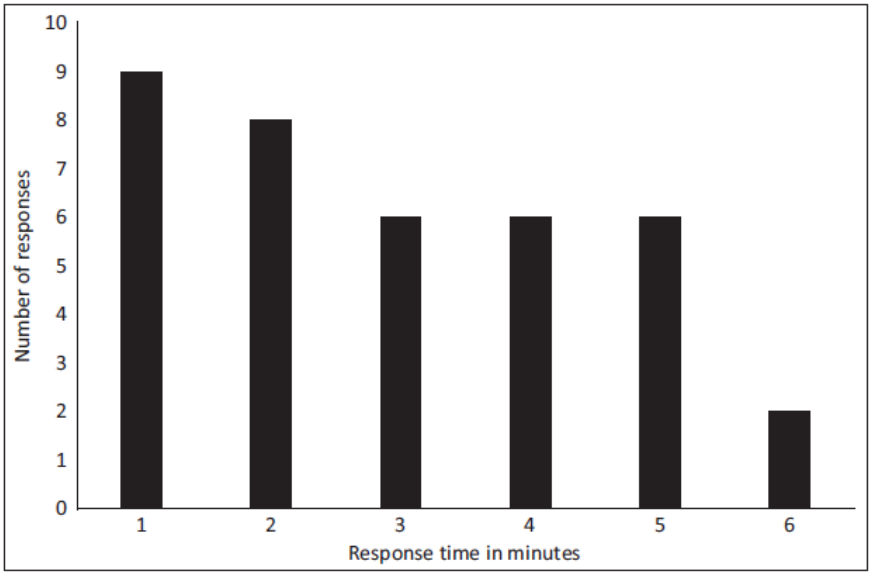

Figure 3: Graph of *Leioproctus platycephalus* ♂ responses during 6-minute trials. All trials were undertaken at the site BR1.

Figure 4: *Leioproctus platycephalus* ♂ food foraging behaviour on *Caladenia versicolor*.

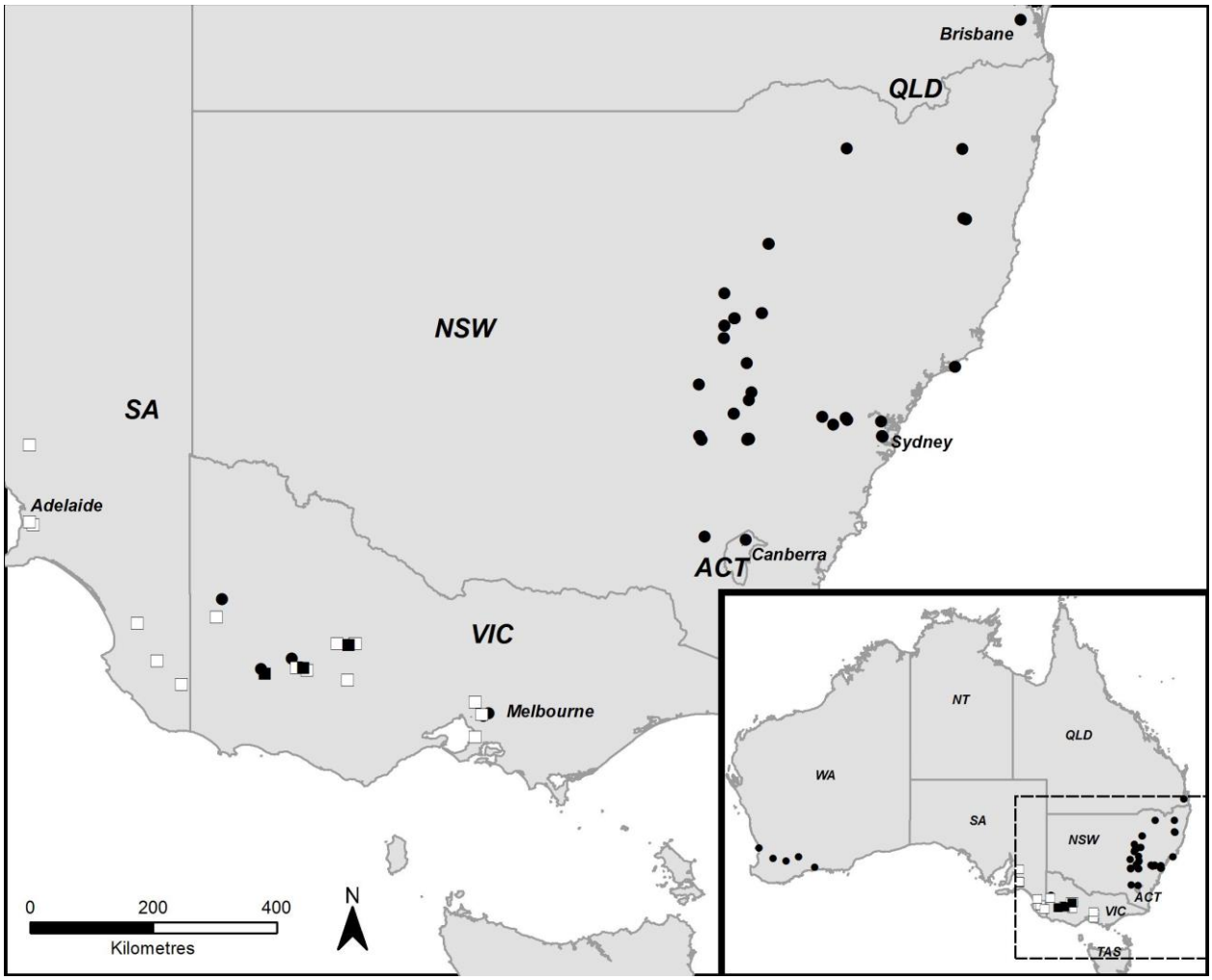

Figure 5: Distribution of *Caladenia versicolour* based on herbarium records, including extant (black squares) and extinct (white squares) populations, overlaid with *Leioproctus platycephalus* collections (black circles) vouchered in Museums.

1

Supplementary Table 1: Co-flowering species at the BR and LF wild and CT1Translocation sites.

Species	BR	CT1	LF
Acacia verniciflua A.Cunn.	*	*	
Acacia paradoxa DC.	*		*
Acacia pycnantha Benth.			*
Allittia cardiocarpa (F.Muell. ex Benth.) P.S.Short	*		
Arctotheca calendula (L.) K.Lewin		*	
Arthropodium fimbriatum R.Br.			*
Astroloma conostephioides (Sond.) F.Muell. ex Benth.	*		*
Brachyscome perpusilla (Steetz) J.M.Black	*		
Brachyloma daphnoides (Sm.) Benth.			*
Bulbine bulbosa (R.Br.) Haw.		*	*
Caladenia carnea R.Br.	*	*	*
Caladenia deformis R.Br.			*
Caladenia parva G.W.Carr	*	*	*
Caladenia venusta G.W.Carr	*		
Caladenia tentaculata Tate			*
Calytrix tetragona Labill.	*		*
Chamaescilla corymbosa (R.Br.) F.Muell. ex Benth.	*	*	*
Comesperma volubile Labill.	*		*
Craspedia variabilis J.Everett & Doust	*		*
Dillwynia hispida Lindl.			*
Diuris chryseopsis D.L.Jones		*	*
Diuris orientis D.L.Jones	*		*
Diuris pardina Lindl.	*		*
Drosera hookeri R.P.Gibson, B.J.Conn & Conran			*
Drosera peltata Thunb.	*	*	*
Eutaxia microphylla (R.Br.) C.H.Wright & Dewar	*		
Glossodia major R.Br.	*	*	*

Species	BR	CT1	LF
Grevillea aquifolium (Lindl.) Meisn.		*	
Grevillea alpina Lindl.		*	*
Hakea rostrata F.Muell.		*	
Hibbertia sericea (R.Br. ex DC.) Benth.	*	*	*
Hibbertia australis N.A.Wakef.	*		*
Hypoxis vaginata Schltdl.			*
Kunzea parvifolia Schauer		*	
Leptospermum myrsinoides Schltdl.	*	*	*
Leucopogon virgatus (Labill.) R.Br. var. virgatus			*
Millotia tenuifolia Cass.	*		
Microseris lanceolata (Walp.) Sch.Bip.			*
Pimelea humilis R.Br.	*	*	*
Ranunculus lappaceus Sm.	*		*
Senecio quadridentatus Labill.	*		
Senecio sp.	*		
Tetratheca ciliata Lindl.	*		*
Thelymitra antennifera (Lindl.) Hook.f.		*	*
Thysanotus patersonii R.Br.		*	*
Wurmbea dioica (R.Br.) F.Muell.	*	*	*
Wurmbea latifolia T.D.Macfarl.	*	*	*
Xanthorrhoea australis R.Br.		*	

Supplementary Table 2: Flower-visiting records for *Leioproctus platycephalus* specimens in the Australian Museum.

Collected from	Family	Sex	Location	State	Year	Month	Specimen unique identifier
Acacia decora Rchb.	Mimosaceae	3♀	Conimbla NP	NSW	2005	September	K.220760–62
Boronia ledifolia (Vent.) DC.	Rutaceae	1♂	Maroota	NSW	2001	Aug	
Boronia occidentalis Duretto	Rutaceae	2♀, 2♂	Goonoo SCA	NSW	2015	September	K.516449–52
Bossiaea lenticularis Sieber ex DC.	Fabaceae	1♂	Mount Banks	NSW	2003	September	K.182716
Daviesia leptophylla A.Cunn. ex G.Don	Fabaceae	2♀, 2♂	Pennsylvania SF	NSW	2012	October	K.345513, 38–40
		2♀, 4♂	Roseberg SF	NSW	2010	October	K.361757, 58, 65–68
Daviesia mimosoides R.Br.	Fabaceae	1♂	Black Mountain	ACT	2006	October	K.278254
		1♀	Clarence	NSW	2002	October	K.182715
Dillwynia sp.	Fabaceae	3♀	Dubbo	NSW	2012	October	K.345424, 25; K.345564
Diuris platichila Fitzg.	Orchidaceae	1♀	Dunedoo	NSW	2005	September	K.224662
Gastrolobium crassifolium Benth.	Fabaceae	1♂	Narrogin	WA	2009	October	K.359718

Gastrolobium sp.	Fabaceae	1♀	Lake King	WA	2004	September	K.360018
Gompholobium grandiflorum Andrews	Fabaceae	3♀	North Epping	NSW	1999	September	K.182709, 10
Goodia lotifolia Salisb.	Fabaceae	2♂	Gibraltar Range NP	NSW	2000	September	K.182713
Jacksonia sp.	Fabaceae	1♀	Ravensthorpe	WA	2009	October	K.359707
Phyllota phylicoides (Sieber ex DC.) Benth.	Fabaceae	1♂	Marramarra NP	NSW	2009	August	K.273247
Platylobium sp.	Fabaceae	1♂	Orange	NSW	2005	September	K.220751
Podolobium ilicifolium (Andrews) Crisp & P.H.Weston	Fabaceae	1♀, 1♂	Point Lookout	NSW	2007	October	K.361820, 21
Pultenaea flexilis Sm.	Fabaceae	1♂	Bilpin	NSW	1999	September	
Pultenaea foliolosa A.Cunn. ex Benth.	Fabaceae	2♀	Goonoo SCA	NSW	2012	October	K.345577; K.345667
Pultenaea microphylla Sieber ex DC.	Fabaceae	1♀, 2♂	Wongarbon	NSW	2000	September	K.182711, 17
		1♀	Dunedoo	NSW	2000	September	
		1♀, 3♂			2005	September	K.224658-61

		1♀, 3♂			2006	September	K.278242-45
		2♀, 1♂	Pilliga NR	NSW	2005	September	K.224663-65
		1♀	Burrendong Dam	NSW	2008	October	K.360799
		1♀	Goonoo SCA	NSW	2015	September	K.516392
Pultenaea procumbens A.Cunn.	Fabaceae	2♀, 3♂	Conimbla NP,	NSW	2012	October	K.345543-47
		1♀	Goobang NP	NSW			K.345606
		2♀, 1♂	Pennsylvania SF	NSW			K.345516-18
Pultenaea scabra R.Br.	Fabaceae	2♀	Bilpin	NSW	2005	September	K.220743
		1♀	Mount Banks	NSW	2003	September	
Pultenaea setulosa Benth.	Fabaceae	1♀	Gilgandra	NSW	2008	October	K.360820
Pultenaea sp.	Fabaceae	1♀, 2♂	Mount Canobolas	NSW	2008	October	K.360805-07
		2♀	Warialda	QLD	2000	September	K.182712
Pultenaea villosa Andrews	Fabaceae	1♀	Karawatha	QLD	2008	August	K.362527
Trimenia moorei (Oliv. ex Benth.) Philipson	Trimeniaceae	1♂	Cunnawarra NP	NSW	2002	October	K.182714

Supplementary Table 3: Number of pollinator responses of bees to *Caladenia versicolor* bait flowers per hour of baiting.

Site	Year	Number of trials	Number of responses	Response per Hour
BR1	2015	76	88	11.57895
BR1	2016	49	17	2.081633
BR2	2016	7	1	0.857143
LF	2015	38	3	0.473684
LF	2016	3	0	0
CT1	2015	22	4	1.090909
CT2	2016	16	2	0.75
M	2016	2	2	6