**Pollination by nectar-foraging thynnine wasps in the endangered *Caladenia arenaria*
and *Caladenia concolor* (Orchidaceae)**

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For translocations of plants with specialised pollination systems it is critical to identify the pollinating species and determine their presence at recipient sites. The endangered species *Caladenia concolor* and *Caladenia arenaria* are shown to be primarily pollinated by nectar-foraging thynnine wasps. For *C. concolor* the specialisation on one pollinator species means that pollinator availability needs to be taken into account prior to conservation translocations, while for *C. arenaria*, sharing of pollinators with related species means sites should be selected to avoid hybridisation.

Abstract

Prior to undertaking conservation translocations of plants with specialised pollination systems it is important to ensure the presence of pollinators at recipient sites. Here, for two threatened species, *Caladenia concolor* Fitzg. and *C. arenaria* Fitzg. (Orchidaceae) we determine (i) the pollination strategy used (ii), which floral visitors are involved in pollination, and (iii) if the pollinator species are present at potential translocation sites. For both orchid species, pollination was primarily achieved by nectar-foraging thynnine wasps, with a single species responsible for pollination in *Caladenia concolor*, whereas *Caladenia arenaria* utilised at least two species to achieve pollination. Both orchid species secreted meagre quantities of sucrose on the upper surface of the labellum. Visits to *C. concolor* occurred primarily in the late afternoon, with some wasps perching on the flowers overnight. Surveys revealed that pollinators were present at all extant populations and most potential translocation sites for both orchids. The specialisation on one pollinator species in *C. concolor* means that the distribution of the pollinator needs to be considered for conservation translocations. With *C. arenaria*, the risk of hybridisation with other *Caladenia* that are known to share one of its pollinator species needs to be taken into account when selecting translocation sites.

Introduction

The Orchidaceae is renowned for ecologically specialised pollination strategies (*sensu* Armbruster 2017), with many orchids being reliant on just one or a few pollinator species (Tremblay 1992; Schiestl and Schlüter 2009). In this circumstance, the geographic range of the orchid could be restricted by the distribution of pollinator species (Duffy and Johnson 2017). Further, natural scarcity of the pollinator within its geographic range could limit the area of suitable habitat for the orchid (Phillips *et al.* 2014). Specialising on a limited number of pollinators may increase the risk of extinction for plants, particularly as they are vulnerable to the loss of any given pollinator species following landscape modification (Mortensen *et al.* 2008; Anderson *et al.* 2011; Pauw and Bond 2011; Phillips *et al.* 2015).

Conservation translocations (for translocation terminology see IUCN 2013) have been widely used around the globe as a tool for bolstering small populations and increasing the number of populations of threatened flora, though in many species with limited success (Dalrymple *et al.* 2012, Godefroid *et al.* 2011; Menges 2008; Silcock *et al.* 2019). A common thread evident in unsuccessful conservation translocations is a lack of understanding of the ecology of the species (Reiter *et al.* 2016; Silcock *et al.* 2019). In particular, pollinator(s) availability needs to be considered when selecting sites, as the presence of pollen vectors will be critical for the long-term persistence of the translocation populations (Reiter *et al.* 2016). As such, threatened orchids reliant on specific species of pollinators may be limited in their potential translocation sites due to rarity of the pollinator species within otherwise suitable orchid habitat (Reiter *et al.* 2017).

Australia is home to over 1800 orchid species (Backhouse *et al.* 2016), the main diversity of which are terrestrial species concentrated in the south east and far south west of the country (ALA 2018; Gaskett and Gallagher 2018). *Caladenia* is the largest, primarily Australian genus of orchid, and is of major conservation concern with 68 (Department of Environment and Energy 2018) of the 370 known species listed under the *Environment Protection Biodiversity and Conservation Act 1999*. Preliminary work has shown that pollinator availability can severely limit candidate conservation translocation sites in some species of *Caladenia* (Reiter *et al.* 2017). However, different methods are required for detecting and surveying for pollinators depending on the pollination strategy used (e.g. Reiter *et al.* 2017, 2018, 2019). Therefore, understanding the pollination strategy is important to both optimise survey techniques for the pollinators of *Caladenia* and test for the presence of pollinators at candidate translocation sites, with the ultimate aim of increasing the likelihood of successful conservation translocations.

Caladenia is unusual among orchids in that it contains both species pollinated by food foraging insects, and species pollinated by sexual deception (Stoutamire 1975; 1983; Phillips *et al.* 2009b). Sexually deceptive *Caladenia* attract pollinators via mimicry of female sex pheromones of thynnine wasps (Bohman *et al.* 2016, 2018; Xu *et al.* 2017), with typically only a single wasp species pollinating any given orchid (Phillips *et al.* 2017). Like most other genera of sexually deceptive orchid, sexually deceptive *Caladenia* usually have red or green flowers, sometimes accompanied by small tepals, prominent calli and an insectiform labellum (Stoutamire 1983; Phillips *et al.* 2009, 2017). While a few species of sexually deceptive *Caladenia* have bright colouration and prominent floral displays (Phillips *et al.* 2017; Phillips and Peakall 2018), it is predicted that *Caladenia* species with colourful flowers are typically pollinated by food foraging insects (Phillips *et al.* 2009; 2011). However, at present there are

only three detailed published studies of pollination by food foraging insects in *Caladenia*: *Caladenia rigida* R. S. Rogers is visited by a range of species of solitary bees and families of Diptera (Faast *et al.* 2009), *C. colorata* D.L.Jones is pollinated by one species of food foraging thynnine wasp (*Phymatothynnus pygidialis*; Reiter *et al.* 2018a), while *C. versicolor* G.W.Carr is pollinated exclusively by bees, but predominantly by males of the colletid bee *Leioproctus platycephalus* (Reiter *et al.* 2019). Most *Caladenia* with white, pink or yellow colouration have been reported to lack nectar secretion (Stoutamire 1983; Jones 2006; Phillips *et al.* 2009; though see Hopper and Brown 2001), suggestive of pollination by food deception (Stoutamire 1983). Interestingly, although not visible to the naked eye, nectar has been detected by chemical analysis on the surface of the labellum in all three of these species (Faast *et al.* 2009; Reiter *et al.*, 2018, 2019). The lack of clearly visible nectar droplets in these nectar-secreting species means that the extent of nectar secretion in *Caladenia* could be underestimated. As such, for the large number of *Caladenia* thought to be pollinated by food foraging insects, little is known about which pollinator groups are involved, how specialised the pollination systems are, and the reward status of the flower.

Here we study the pollination biology of *Caladenia concolor* and *Caladenia arenaria*, two nationally threatened species that belong to the subgenus *Calonema*. While both species are characterised by a sweet scent and are similar in size, they contrast strongly in colouration. The flowers of *C. concolor* are typically uniformly dark red, a colour pattern seen in relatively few species of *Caladenia*, none of which have had their pollination strategy resolved. In contrast, *C. arenaria* has a colourful floral display, which is often associated with pollination by food-foraging insects, though some individuals have a dark-tipped labellum typical of species pollinated by sexual deception of thynnine wasps (Stoutamire 1983; Phillips *et al.* 2009; Phillips *et al.* 2017). Both species are part of a ten-year program of

conservation translocation (Waudby *et al.* 2018), thus identifying the pollinators, and the presence of these pollinators in suitable areas for conservation translocation is an important first step for this conservation program (e.g. Reiter *et al.* 2017). Specifically, for *C. concolor* and *C. arenaria* we address the following questions: (I) which insect species are attracted and which achieve pollination? (II) do the flowers secrete nectar? (III) are the pollinators present at the remaining wild sites and (IV) are the pollinators present at potential conservation translocation sites?

Methods

Study Species

Caladenia concolor is listed as vulnerable under the *Environment Protection Biodiversity and Conservation Act 1999*. *Caladenia concolor* is currently known from a total six extant populations in Victoria and New South Wales, Australia, though only three of these sites contain more than 100 individuals. Two of the sites with more than 100 individuals were discovered while we were surveying for pollinators as part of this study in 2017. In Victoria, the vegetation communities that *C. concolor* inhabits are Box-Ironbark forests with the dominant trees including *Eucalyptus goniacalyx* F. Muell, *E. sideroxylon* A. cunn. Ex Woolls, *E. macroryncha* F. Muell. ex Benth. and *E. polyanthemos* Schauer (N. Reiter pers obs). In New South Wales the community *C. concolor* inhabits tends to be open heathy Box-woodland on granite slopes and ridges dominated by *Eucalyptus macroryncha*, *E. polyanthemos* and *E. albens* Miq (N. Reiter pers obs). These habitats have been extensively cleared for agriculture (Kelly and Mercer 2005).

Flowering of *C. concolor* occurs during late August to September, with typically one flower produced on a solitary scape (Walsh and Entwisle 1996). Flower colour is a dark, dull red

with most individuals being either entirely red, or red with a slight green tinge to their sepals and petals. The osmophores on the sepals and petals appear dark and have densely clustered glands. Examination of flowers during warm conditions revealed a smell akin to mandarin to the human nose (N. Reiter pers. obs.). *Caladenia concolor* is reliant on pollinators to achieve pollination, though on rare occasions in nursery conditions will clonally reproduce (N. Reiter pers. obs.).

Caladenia arenaria is listed as endangered under the *Environment Protection Biodiversity and Conservation Act, 1999*. *Caladenia arenaria* is endemic to the southern slopes and Riverina of New South Wales and is known from five wild populations (Waudby *et al.* 2018). While *C. arenaria* appears to have a smaller geographic range than *C. concolor*, natural vegetation in this area has also been cleared extensively for agriculture (Thompson and Eldridge 2005), suggesting that habitat destruction played a key role in the current rarity of *C. arenaria*. The vegetation communities that *C. arenaria* inhabits are White Cypress Pine (*Callitris glaucophylla* Joy Thomps. & L.A.S. Johnson) woodlands growing on sandy soil, and Western Grey Box (*Eucalyptus macrocarpa* Hook.) woodlands on loamy soil with a grassy understorey (Waudby *et al.* 2018). *Caladenia arenaria* flowers in early September to mid-October, with typically one to two flowers per scape, though on rare occasions as many as four (N. Reiter pers. obs.). Flowers are a pale creamy yellow colour, though some flowers have a distinct dark red tip to their labellum. The osmophores are light brown and have sparsely distributed glands along the sepals and petals. Examination of flowers in warm weather reveals a subtle sweet floral scent to the human nose (N. Reiter pers. obs.). Like *Caladenia arenaria*, *C. concolor* is reliant on a vector to achieve pollination, but is not known to reproduce clonally in cultivation.

Study Sites

For *Caladenia concolor*, pollinator observations were made at four wild sites (C, NCH, B1 and B2, indicated by white circles in Figure 1) and five potential translocations sites (three within B and two within W, indicated by white triangles in Figure 1). Site B contained two separate populations of *C. concolor* separated by approximately 1 km but within the same large piece of reserve. In addition, for *Caladenia concolor* morning observations were taken at a conservation translocation site on NCH separated by about 1 km from the wild population. For *C. arenaria* pollinator observations were made at five wild sites (LP, C, Y, B, and N indicated by white circles in Figure 2) and five potential translocation sites (M, G, L, Me and A indicated by white triangles in Figure 2). Study site names have been masked due to the threatened status of these species (Lindenmayer and Scheele 2017).

General pollinator observation methods

Within each population or potential conservation translocation site, pollinator observations were undertaken at numerous locations using baiting trials (Figure 1; 2). Baiting trials were undertaken using the baiting method of Reiter *et al.* (2018), a modification of those used for sexually deceptive orchids (Stoutamire 1974; Peakall 1990). When baiting trials are used for sexually deceptive orchids, a small number of picked orchids are moved to a new location in the landscape, leading to a rapid response from sexually deceived male pollinators (Peakall 1990). However, pilot studies with *Caladenia* putatively relying on food foraging insects have proven largely unsuccessful using this technique (Bower 2006; Bower 2008; R. Phillips pers obs.). Here we used baiting with potted plants grown from seed from wild populations of each of our study species (20-30 flowers). This approach greatly increases the stimulus perceived by pollinators. Flowers used for pollinator observations were propagated using the seed and mycorrhizal fungi collected from NCH for *C. concolor* and the LP and C sites for *C.*

arenaria following the methods of Reiter *et al.* (2018b), modified from those of Clements and Ellyard (1979). Pollinator observations were made during the flowering period of wild populations of the orchid. Representatives of insects visiting or pollinating the bait flowers were collected for later identification. A selection of thynnine wasps from each site were identified using a series of unpublished keys to the Australian thynnine wasp fauna (G. Brown unpublished data). Michael Batley at The Australian Museum provided identification of bees using the key of Maynard (2013).

The behaviour of insects at the flower was recorded as follows: approach only (no landing), landing on flower (noting location of landing: labellum, sepals, petals), pollinia removal, and pollinia deposition or contact with the column. As many species of *Caladenia* are pollinated by sexual deception of thynnine wasps (Stoutamire 1983; Phillips *et al.* 2009; Phillips *et al.* 2017), any attempted mating behaviour with the flower was recorded. As the related *Caladenia colorata* is pollinated through nectar-foraging behaviour (Reiter *et al.* 2018a), we also recorded any evidence of food foraging behaviour.

Pollinator observations with Caladenia concolor

Pollinator baiting trials were conducted in 2016 and 2017 between late August and September for *C. concolor* (voucher for bait flowers: MEL 2423867A, MEL 2424868A, MEL 2324701A). For *Caladenia concolor*, initial baiting attempts over 3 days at sites NCH (25 trials) and C (25 trials) were unsuccessful, thus we revised the pollinator survey method to more intensively focus on the area around the orchid population or proposed translocation site, thereby reducing the risk of false absences. We surveyed for pollinators using 6-minute trials in an approximately 200 to 250 m grid (as the landscape and vegetation allowed), with each baiting position approximately 40- 50 metres apart. This approach was used once at sites

within B, and twice at sites NCH and C. In total, 273 baiting trials were conducted for *C. concolor* using the grid formation. For *C. concolor*, baiting was conducted in weather from 13 °C to 27 °C from 10 am to 5.30 pm. In other pollination systems using thynnines, including *C. arenaria*, activity generally ceases or is greatly reduced after about 3 p.m. (e.g. Peakall 1990; Phillips *et al.* 2017; Reiter *et al.* 2018a), but preliminary observations revealed that in *C. concolor* pollinators kept responding until almost dusk. To determine the optimal time of day and temperature to detect pollinators, A *G*-test with Williams's correction was performed using GenAlEx 6.5 (Peakall & Smouse, 2012) to test for a significant difference in the number of responses between the proportion of trials where a pollinator was detected, above and below 16 °C, above and below 18 °C, and above 18°C before and after 3 pm. Temperature was taken from the closest weather station. Pollinator observations for *C. concolor* were undertaken across four days in 2016 and 11 days in 2017.

Due to observations of thynnines arriving at plants in the late afternoon, in the mornings we embarked on inspections of flowers at wild sites to test if pollinators were likely to have remained on the flowers overnight. We conducted these surveys between 5.30 am and 7.30 am on four days across both the B1 and B2 wild populations ($N = 200$ flowering plants in total across both sites) and on one day at each of the C ($N = 6$ flowering plants) wild population and NCH ($N = 20$ flowering plants) plants that had been part of a conservation translocation two years earlier. We conducted these surveys on days that the previous day had reached 18 °C and thus were suitable for pollinator activity.

Pollinator observations with Caladenia arenaria

Observations were made across six days in both 2016 and 2017 for *C. arenaria*.

Baiting was conducted between September and October (voucher of wild plants: CANB 880803.1), between 10 am and 3 pm on days greater than 18 °C. Baiting was conducted for 6-minute periods, after which the bait plants were moved to a new location within the wild population or potential translocation site. A total of 105 baiting trials were undertaken for *C. arenaria*.

Nectar derivatisation and GC-MS analysis

We sampled nectar from ten flowers of each of *C. concolor* and *C. arenaria* from the ex-situ living collection plants grown from seed for conservation translocation at the Royal Botanic Gardens Victoria, Cranbourne site. Plants were kept in a glasshouse at 20°C for approximately 3 hours prior to sampling. We used the sampling procedure and methodology for nectar derivatisation as per Reiter *et al.* (2018, 2019). For each flower, an aqueous solution (5 µL) of ribitol (internal standard, 0.20 mg/ml) was added with a glass syringe on to the labellum. This quantity of solution only covered a portion of the labellum, but was positioned to cover some of the central calli (raised tissue on the upper surface of the labellum) that were thought to be the source of nectar secretions based on their glistening appearance and the localised feeding behaviour of pollinators. The aqueous extract was subsequently collected with microcapillary tubes (5 µL) and immediately transferred 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. Extracts were stored in a -20 °C freezer until analysis. As we followed the same methodology as Reiter *et al.* (2018, 2019), we were able to compare our results with those of the nectar secreting *Caladenia colorata* and *Caladenia versicolor*, and the nectarless *Caladenia tentaculata*.

As per the methods of Reiter *et al.* (2018), for each of the extracts, the solvent was evaporated to dryness with a stream of nitrogen. Methoxyamine-HCl (20 μ L of 20 mg/mL solution in pyridine, Sigma-Aldrich, St Louis, USA) 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 *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, 35 μ L, Sigma-Aldrich, St Louis, USA) in the same sealed vials for 1 hour before GC-MS analysis (Lisec *et al.* 2006). 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 $\times$ 0.25 mm $\times$ 0.25 μ m film thickness, SGE Australia], using helium as carrier gas. An Agilent 7683 autoinjector was used and injections (3 μ L) were performed in split mode (1 to 10). Tentative identification of trimethylsilylated monosaccharides and sucrose was based on the comparison of retention index and mass spectra with data from a mass spectral library (NIST-11). All tentative identifications were confirmed by co-injections with synthetic standards. 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 respective carbohydrate sampled and the internal standard were measured and included in the calculation of the amounts of analytes (Reiter *et al.* 2018a).

Pollination success

Reproductive success for *C. concolor* was scored in 2017 at two natural populations within Site B approximately 1 km apart. Other populations of this species were considered too small (only a handful of individuals flowering) for an accurate assessment of pollination success. Pollen removal and deposition was scored for each flower at the two populations at Site B. Female fitness was estimated as the proportion of flowers where pollen deposition occurred,

while male fitness was estimated as the proportion of pollen removal events that lead to deposition (pollen transfer efficiency; see Scopece *et al.* 2010). Reproductive success for *C. arenaria* could not be scored in 2017 or 2018, due to insufficient plants flowering in the prevailing drought in southern New South Wales.

Results

Pollinator identification and observations - Caladenia concolor

The main floral visitors were males of the thynnine wasp (Tiphidae) *Chilothynnus trochanterinus* ($N = 80$). In thynnine wasps only the male has wings, and carries the wingless female *in copula*, either feeding her directly or taking her to a food source (Alcock 1981). Of the male *C. trochanterinus* visiting the flower (Figure 3), 76 were solitary males and four were males carrying a female *in copula*. Three individual male *C. trochanterinus* attempted to feed from the upper surface of the labellum. An unidentified weevil, a flying ant, *Eirone* sp. (Thynnidae) and an unidentified fly were the only other visitors to *C. concolor*. While the *Eirone* sp. and fly attempted to feed from the upper surface of the labellum, none were carrying pollen or came in contact with the column. *Chilothynnus trochanterinus* typically landed on the sepals (64 % of responding individuals), and crawled over the surface of the flowers before flying away. A proportion landed on the labellum (36 %), then gradually moved forwards to the base of the column. Pollen was removed from the orchid flower when a *C. trochanterinus* that had fed ($N=3$) at the base of the labellum attempted to leave the flower. Pollen was deposited on the dorsal side of the thorax of the male wasp ($N=3$). Of the 80 *C. trochanterinus* attracted, three removed pollen and two deposited pollen. None of the insects attracted to the bait flowers showed any signs of attempted copulatory behaviour with the flowers. Representative specimens of the pollinator from site B1, B2, W, NCH,

translocated site at NCH and C were lodged at the Museum and Art Gallery of the Northern Territory NTM1008677-NTM1008686.

Pollinator presence at extant populations and potential conservation translocation sites

- Caladenia concolor

Chilothynnus trochanterinus was found at the four wild populations of *C. concolor* surveyed (NCH, C and both wild populations in B), the translocation site near NCH and the five potential conservation translocation sites (three from B and two in W). At the wild sites, wasps were attracted in 9 % ($N=13$ of 143 trials) of these trials, while at the potential translocation sites, the wasps were detected in 22 % ($N=26$ of 114) of trials.

Revising survey methods for Caladenia concolor

Responses of *C. trochanterinus* to the bait flowers were observed at temperatures between 13° C and 29° C (full range of temperatures = 9-29 °C). *Chilothynnus trochanterinus* were observed significantly more frequently at temperatures above 16 °C, than below 16 °C ($G=6.510$, $df=1$, $N=332$, $P=0.011$). Detection of *C. trochanterinus* was also significantly greater above 18° C, than below 18° C ($G=26.514$, $df=1$, $N=332$, $P=0.000$). The ability to detect the presence of *C. trochanterinus* was further significantly increased above 18 °C and after 3pm than above 18 °C and before 3pm ($G=21.765$, $df=1$, $N=61$, $P=0.000$).

The morning inspections of orchid flowers at sites B1 and B2 ($N=4$) revealed motionless wasps perched on the flowers on two of the four mornings at both sites that were surveyed. The two mornings where wasps were not detected on flowers were warmer than 12-13 °C (and the wasps had likely moved on for the day). Conversely, on both mornings when pollinators were detected on flowers at both site B1 and B2, the temperature was around 9-10

°C. Orchid flowers were surveyed for wasps once at site C and once at site NCH. On both these occasions, the temperature was 8-9 °C and both sites had motionless wasps present on the orchids.

The proportion of flowers where wasps were detected in morning surveys was generally low. At NCH, 3 of 16 flowers had motionless wasps (18 %), at population B over both wild sites, 8 of the 200 flowering plants surveyed over each of two mornings had motionless wasps (4%) (Figure 6). However, at population C one of six flowers (16 %) had wasps on the one morning that it was surveyed. Wasps were observed perched motionless on sepals, petals and labellum. Pollinia removal and deposition was not observed, though one wasp present early in the morning had pollinia on its thorax, which it did not deposit before flying off.

Pollinator identification and observations – Caladenia arenaria

The most common floral visitors were males of various species of thynnine wasps ($N = 112$). Of the 112 thynnines attracted, 83 landed, 4 removed pollen and two deposited pollen (Figure 3). *Tachynomyia* sp. nr *volatilis* removed and deposited pollen ($N=2$, $N=1$ respectively) and *Aelothygnus westwoodii* both removed and deposited pollen ($N=2$ and $N=1$ respectively)). *Chilothygnus* sp. and *Phymatothygnus* sp. were observed on the flower but were not seen removing or depositing pollen. The individual of *Phymatothygnus* sp. was a similar size to the *Tachynomyia* sp. nr *volatilis* and thus could not be ruled out as a potential pollinator. In the case of *Chilothygnus* sp., the wasp was too small compared to the labellum-column distance to remove pollen. Five individual male thynnine wasps (including both *A. westwoodii* and *T. sp. nr volatilis*) were observed to exhibit prolonged feeding behaviour with their mouth-parts in contact with the upper surface of the labellum (Figure 4 and 5). In addition, a male of both *T. sp. nr volatilis* and *A. westwoodii* exhibited prolonged feeding

while a female was attached *in copula* (Figure 4). Attempted copulatory behaviour with the flower was not observed by any of the individuals visiting the flowers. The thynnine visitors typically landed on the sepals (40 % of responding individuals) and crawled over the sepals and petals before flying away; or landed on the labellum (33 %), then gradually moved forwards to the base of the column attempting to feed from any part of the surface of the labellum (Figure 3). In addition to the four species of thynnines, there were visits by *Leioproctus maculatus* (Colletidae) ♀ ($N=2$), where one individual removed and deposited pollen, and the introduced honey bee *Apis mellifera* (Apidae) ($N=2$). Across all pollen vectors, in each case where pollinia were removed it was attached to the dorsal side of the thorax as the insect attempted to feed from the upper surface of the labellum. Representative specimens of the pollinator species from C, Y, M and Me were lodged at the Museum and Art Gallery of the Northern Territory NTM1008687-NTM100693.

Pollinator presence at extant populations and potential conservation translocation sites

- *Caladenia arenaria*

Both pollinator species *Aeolothynnus westwoodi* and *Tachynomia* sp.nr *volatalis* were confirmed as present at the wild sites Y and C and at two of the potential conservation translocation sites Me and M. While thynnine wasps were also observed visiting *C. arenaria* bait plants at the wild sites B, L and conservation translocation sites G and L, specimens were not caught for identification. There was no pollinator activity observed when baiting at potential conservation translocation site A. Pollinator baiting in 2017 was during a drought year, which may have affected the availability of pollinators and thus detection at the potential conservation translocation sites. At the wild sites, wasps were detected in 42 % of the baiting trials (total trials $N=64$), while at the potential translocation sites wasps were detected in 35 % of the trials (total trials $N=41$).

Nectar measurements

For *C. arenaria*, the 10 samples on average contained $5.3 \mu\text{g} \pm 2.7 \text{ SE}$ of saccharides per flower with $>95 \%$ sucrose and $< 5 \%$ monosaccharides. In one sample, no saccharides could be detected. For the 10 samples of *C. concolor*, $31.5 \mu\text{g} \pm \text{SE } 12.1$ of saccharides were detected per flower with $>95 \%$ sucrose and $< 5 \%$ monosaccharides.

Reproductive success

Across two natural populations of *C. concolor* at site B in 2017 (Number of flowers = 200), pollination rate averaged $30.5 \% \pm 7.5 \text{ SE}$, the proportion of flowers with pollen removal averaged $36.8\% \pm 0.3 \text{ SE}$, and the pollen transfer efficiency averaged $0.8 \pm 0.2 \text{ SE}$.

Discussion

Pollination by nectar-foraging thynnine wasps

While the majority of *Caladenia* pollinated by thynnine wasps are sexually deceptive (Stoutamire 1983; Phillips *et al.* 2009; Swarts *et al.* 2014; Phillips *et al.* 2017), there is only one previous account of food-foraging thynnines pollinating *Caladenia* (Reiter *et al.* 2018a). Here we document that the endangered species *C. concolor* and *C. arenaria* are also pollinated by nectar-foraging thynnine wasps, suggesting that these insects are also important pollinators of large-flowered *Caladenia* outside of sexually deceptive species. Our observation of food-foraging pollinator behaviour is supported by the detection of sucrose on the upper surface of the labellum of both species. These observations are consistent with many genera of thynnine wasps being nectar feeders (Alcock 1981; Bates 1984; Brown and Phillips 2014; Menz *et al.* 2015).

Pollination of Caladenia concolor

Caladenia concolor appears to have a highly specialised pollination system with only *C. trochanterinus* observed removing and depositing pollinia. Furthermore, there may be strong filtering at the attraction phase amongst the pool of potential pollinators, with only *C. trochanterinus* being regularly attracted to the flowers. *Caladenia concolor* is strongly scented, which may be important for long distance attraction of pollinators. Here we demonstrated that *C. concolor* secretes nectar on the surface of the labellum on which some of the wasps appeared to feed. This finding contributes to a growing body of observations of nectar secretion in members of *Caladenia* subgenus *Calonema* (Faast *et al.* 2009; Dixon & Tremblay 2009; Reiter *et al.* 2018; Reiter *et al.* 2019). Our discovery of nectar secretion in *Caladenia* that were previously assumed to be either food deceptive or sexually deceptive, suggests that nectar secretion across *Caladenia* should be evaluated more generally, including species without signs of visible nectar secretion.

In orchids, species that provide a reward to pollinators on average have a higher rate of pollination and fruit set than deceptive species (Tremblay *et al.* 2005; Scopece *et al.* 2010). Hand pollination of *C. concolor* in nursery conditions lead to 100 % capsule formation (N. Reiter pers obs), and in general, outside of drought conditions *Caladenia* show no resource limitation to fruit set within a flowering season. As such, pollination rate and fruit set data are broadly comparable for this genus. *Caladenia concolor* has a similar pollination rate of 30.5 % at natural sites compared to the mean of 37.1 % fruit set averaged across 84 species of rewarding orchids (Tremblay *et al.* 2005). The pollination rate of *Caladenia concolor* is similar to the pollination rates observed in the nectar-producing *C. colorata* (Reiter *et al.* 2018a) and *C. versicolor* (Reiter *et al.* 2019) and much higher than those seen across a range of sexually deceptive *Caladenia* (mean fruit set = 14 %, $N = 10$; Phillips *et al.* 2009). Thus,

we hypothesise that lack of pollinators and an ineffective pollination strategy is not what is driving the decline of *C. concolor* at some sites.

Interestingly, some individuals of *C. trochanterinus*, the pollinator of *C. concolor*, perched on orchid flowers overnight. Although individuals perched on the flowers in the morning were not observed to pollinate the flowers when they roused, it is possible that these individuals may on occasion contribute to the pollination of *C. concolor*. In a different group of orchids, bees have been recorded pollinating *Serapias vomeracea*, where the flowers provide a dark hole that bees sleep the night in before warming them up more quickly than ambient conditions in the morning (Dafni *et al.* 1981). Similarly, male solitary bees are known to take advantage of the warming properties of some *Iris* flowers by using them as overnight shelters and pollinating them in the process (Sapir *et al.* 2006). However, we believe this explanation is unlikely to apply in *C. concolor* as the flower does not form an enclosed space, and the wasps are often positioned where they are not exposed to direct morning light. Little is known about the roosting habits of male thynnine wasps, though various species have been recorded roosting in groups on sticks or blades of grass, under leaves or roosting individually under bark, and some species in captivity will burrow in sand at night (R. Phillips, unpublished observations). Given that regular perching on flowers overnight has not been documented in other members of *Caladenia* subgenus *Calonema*, it would be of interest to evaluate if this behaviour is related to the signals used to attract pollinators, and what role roosting individuals play in the pollination of *C. concolor*.

Pollination of Caladenia arenaria

The vast majority of visitors to *Caladenia arenaria* were thynnine wasps and of these the only species to remove or deposit pollinia were *Aeolothynnus westwoodii* and *Tachynomia*

sp.nr *volatalis*, suggesting that the pollination strategy of *C. arenaria* is largely specialised on thynnine wasps. There was one species of native bee that also pollinated *C. arenaria* at site Y, however, due to the low number of responses, bees are likely to play only a minor role as a pollinator. Nevertheless, it would be of interest to undertake pollinator observations in a season with more typical levels of rainfall, to test if additional insect species are involved in pollination, including thynnines that have been attracted to *C. arenaria* without effecting pollination. Interestingly, the detected amounts of sucrose were small in *C. arenaria* ($5.3 \mu\text{g} \pm \text{SE } 2.7$) compared to *C. concolor* ($31.5 \mu\text{g} \pm \text{SE } 12.1$), *C. colorata* ($16.6 \mu\text{g} \pm \text{SE } 3.9$) (Reiter *et al.* 2018), and *C. versicolor* ($39.5 \mu\text{g} \pm \text{SE } 37.4$) μg (Reiter *et al.* 2019). Further, nectar secretion in *C. arenaria* is also low compared with nectar-rewarding orchids outside of *Caladenia* (see references in Reiter *et al.* 2018a). This raises the question of how much of a reward thynnine wasps receive from *C. arenaria* and, while pollination occurs via food foraging behaviour, whether this is truly a rewarding system? For example, it is possible that the production of a meagre amount of sugar may be enough to stimulate a feeding response by the pollinator (Hammer 1993), but without providing sufficient reward to encourage visitation to conspecific orchids. In such a scenario, the orchid could potentially be considered deceptive, while still using sugar to more effectively position the animal for pollination.

Chilothynnus, Tachynomia and Aeolothynnus as pollinators

Pollination systems involving species of the thynnine wasp genera *Aeolothynnus*, *Chilothynnus* and *Tachynomia* are not commonly recorded within the orchid literature. *Aeolothynnus* are known to be sexually deceived pollinators of *Caldenia toxochila*, *Caladenia concinna* (Bower 2001; Hayashi 2016) and *Caladenia williamsiae* (Phillips 2017). *Chilothynnus* have been recorded as sexually deceived pollinators in two species of

Chiloglottis (Mant *et al.* 2005) and *Caladenia bryceana* (Brown *et al.* 2013). Interestingly, *C. trochanterinus*, the pollinator of *C. concolor*, is also a pollinator of *Caladenia filamentosa* (C. Bower unpublished data, referred to in Phillips *et al.* 2009), a species which shares the unusual uniform dull red colouration seen in *C. concolor*. *Tachynomia* have been recorded as pollinators of the sexually deceptive *C. macrostylis* and *C. multiclavia* (Phillips *et al.* 2017; Stoutamire 1983) in Western Australia. *Tachynomia* have also been implicated in the pollination of *C. behri* from South Australia, however, the pollinator and pollination strategy of this species remains unknown (Petit *et al.* 2005). While these genera have been recorded as being involved in pollination by sexual deception of a small number of species, our records are the first of them as food foraging pollinators.

Little is known about the food sources utilised by *Chilothygnus*, *Aeolothygnus* or *Tachynomia*. All three of these genera are rarely seen foraging on nectar compared with many other genera of thynnine wasp (Brown & Phillips 2014, Menz *et al.* 2015; R. Phillips unpublished observation). In a review of collections of flower wasps from Australian museums, *Tachynomia* had only been recorded on *Callistemon* (Brown and Phillips 2014). Earlier, in a study by Bond and Brown (1979), *Tachynomia* were found foraging on *Eucalyptus incrassata*, while Bernhardt (1987) found *Tachynomia* foraging on extra floral nectaries of *Acacia*. A study in Western Australia using systematic swabs of pollen loads on thynnine wasps found that *Chilothygnus* carried the pollen of *Agonis flexuosa* (Menz *et al.* 2015). Given that these thynnines are infrequently collected from nectar plants, it would be of interest to test if there are any floral traits exhibited by the orchids that make them particularly favourable to nectar-foraging thynnine wasps. In terms of managing pollinator populations, a dietary study of the pollinators would shed light on other plants species

required to sustain the pollinators, or if they utilise alternative sugar sources such as lerps (Brown & Phillips 2014).

Implications for conservation

Sites for conservation translocations should be selected on the basis that the pollinator species is known to be present (see Reiter *et al.* 2017). As such, the specialisation evident in the pollination system of *C. concolor*, where the populations surveyed are likely to be entirely reliant on *C. trochanterinus* for pollination, potentially has important implications for conservation. However, all wild and candidate translocation sites for *Caladenia concolor* contained the pollinator *C. trochanterinus*, suggesting that within the current range of vegetation types in which *C. concolor* is found, the pollinator is reasonably common and does not limit on the suitability of translocation sites. While *C. arenaria* was found to have multiple potential pollinators, these were not detected in one of the smaller habitat remnants (site A), highlighting that translocation failure could potentially occur through low pollinator availability in this more generalised species if inappropriate sites are selected.

In *C. concolor*, the discovery that baiting with large numbers of potted plants between 3 pm and dusk on days which reached 18 °C is an effective technique for attracting pollinators, suggests that pollinators of this species can be reliably surveyed for. Additionally, surveying wild populations on a cold morning after a day of 18° C or above, reliably found pollinators motionless on plants with a proportion of any population with a wasp on a flower. Therefore, given the large number of plants that can be cultivated with current propagation techniques (Reiter *et al.* 2018 b), our baiting technique could be modified to leave trays of *C. concolor* overnight at potential translocation sites on a warm day and return the following morning while the weather is still cold. Most likely, this technique can be applied to other *Caladenia*

closely allied to *C. concolor*, and therefore it may make a broader contribution to the conservation of a little studied group.

Due to the minimal post-zygotic barriers to reproduction within all subgenera of *Caladenia*, hybridisation is potentially a challenge in identifying suitable translocation sites. Plants that are the product of hybridisation between sexually deceptive *Caladenia* and those that are putatively pollinated by food-foraging insects, are frequently witnessed in the field (Backhouse 2011; Brown *et al.* 2013;). In the case of *C. concolor*, we now know this species shares a pollinator with *C. filamentosa*. Assuming that the pollen is placed on the thorax of the thynnine as in other *Caladenia* where pollination has been observed, pollen transfer between species would be likely, meaning that translocations of *C. concolor* into areas already containing *C. filamentosa* should be avoided. Hybridisation is of particular concern with *C. arenaria*, where hybridisation between *C. arenaria* and the sexually deceptive *Caladenia concinna* and *C. tentaculata* is so extensive in two populations (TSU 2004) that, based on floral traits, the species appear to completely intergrade with each other. While we did not observe the known pollinators of *C. concinna* and *C. tentaculata* visiting *C. arenaria* (see Bower 2001, 2009; Hayashi 2016), our finding that *C. arenaria* attracts a range of species of nectar-foraging thynnine wasps provides a mechanism by which frequent hybridisation could occur – that the same wasp species contributes to pollination in both *C. arenaria* and co-occurring sexually deceptive species. Our work identified that minimising hybridisation risk should be a criterion for conservation translocation sites in *C. arenaria*, and more broadly highlights the importance of understanding pollinator sharing when selecting sites for conservation translocations.

Conflict of Interest

The authors declare no conflicts of interest.

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Author contributions

NHR propagated the plants for baiting, designed the study, undertook pollinator baiting, and collected the nectar samples and reproductive data, MF undertook pollinator baiting, BB undertook nectar analysis, GB identified all thynnids; NHR and RDP wrote the manuscript; all authors contributed to the editing of the manuscript.

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Figures

Figure 1: Baiting trial locations for *Caladenia concolor* pollinators at three wild sites (C, NCH and B, indicated by white circles) and five potential translocation sites (three within B and two within W, indicated by white triangles). Locations where the pollinator was detected are indicated by a black circle; locations where the pollinator was not detected are indicated by a black cross.

Figure 2: Baiting trial locations for *C. arenaria* pollinators at five wild sites (LP, C, Y, B, and N indicated by white circles) and five potential translocation sites (M, G, L, Me and A) (indicated by white triangles). Locations where the pollinator was detected are indicated by a black circle; locations where the pollinator was not detected are indicated by a black cross.

Figure 3: A-C) *Chilothylnus trochanterinus*, confirmed pollinator of *Caladenia concolor*, perched on flowers of *C. concolor* at 6.30 am.

Figure 4: A) *Tachynomia* sp.nr *volatalis* foraging at base of labellum of *Caladenia arenaria*, B) pollinia removal by *Tachynomia* sp.nr *volatalis* C) *T. sp.nr volatalis* leaving following deposition of pollinia.

Figure 5: A-B) *Tachynomia* sp.nr *volatalis* feeding off the labellum of *Caladenia arenaria*,
C) A pair of *T. sp.nr volatalis* investigating *C. arenaria* while *in copula*.