Pollination by nectar-foraging pompilid wasps: a new specialised pollination strategy for the Australian flora

Ryan D. Phillips[1,2,3], Björn Bohman[4,5] & Rod Peakall[3]

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[1]Department of Ecology, Environment & Evolution, La Trobe University, Bundoora, VIC 3086, Australia

[2]Kings Park Science, Department of Biodiversity Conservation and Attractions, Kings Park, WA 6005, Australia

10   [3]Ecology and Evolution, Research School of Biology, The Australian National University, Canberra, ACT, 2600 Australia

[4]Department of Plant Protection Biology, Swedish University of Agricultural Science, Alnarp 23053, Sweden

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

15   6009 Australia

Correspondence: Ryan Phillips, Department of Ecology, Environment & Evolution, La Trobe University, Bundoora, VIC 3086, Australia

E-mail: R.Phillips@latrobe.edu.au

20   Phone: +61 03 94796674

Fax: +61 03 9479 1188

25   **Summary:** *Caladenia drummondii* is pollinated by males of a single species of pompilid wasp, making this the first well-documented case of a pollination system specialised on pompilids outside of Africa.

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35 **ABSTRACT**

- The Pompilidae is a cosmopolitan and diverse group of wasps, which commonly feed on nectar. However, pollination systems specialised on pompilids have not been documented in detail outside of southern Africa.

40 Here, we studied *Caladenia drummondii* (Orchidaceae), where based on floral traits and preliminary field observations we predicted pollination by sexual deception of male pompilid wasps.

- Detailed pollinator observations were undertaken using floral baiting experiments at sites spanning 375 km. Following evidence for nectar on some

45 flowers of *C. drummondii*, the sugar content on the labellum was analysed by GC-MS. Floral spectral reflectance was measured and compared with *Caladenia* using other pollination strategies.

- Males of a single species of pompilid wasp (*Calopompilus* sp.) were the only visitors capable of pollinating *C. drummondii*. Attempts to feed from the

50 surface of the labellum were frequent, and were associated with removal and deposition of pollinia. GC-MS analysis revealed larger quantities of sugar on the labellum than reported in other *Caladenia* species. While no sexual or courtship behaviour was observed, the zig-zag and circling flight on approach to the flower is suggestive of odour-based attraction. Floral spectral

55 reflectance was similar to sexually deceptive *Caladenia*.

- This study represents the first confirmation of a specialised pompilid pollination system outside of Africa. Although pollination occurs during nectar-foraging, long-distance sexual attraction cannot be ruled out as an explanation for the exclusive male visitation. The similarity in floral spectral

60 reflectance to other *Caladenia* indicates colour may not impose a constraint on the evolution of pollination by pompilids.

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INTRODUCTION

70   Floral visitation by wasps is common in relatively generalist pollination systems involving nectar rewards, where the wasps form a subset of a more diverse range of floral visitors (e.g. Nilsson 1981; Vieira & Shephard 1999; Cerana 2004; Gess & Gess 2004; Yates *et al*. 2005). In these plants, any given wasp species probably plays only a minor role in pollination. However, pollination systems involving pollination by one

75   or a few wasp species have evolved in a range of different plant families (Rosas-Guerrero *et al*. 2014). These plant species employ a diversity of pollination strategies, many of which involve chemical cues, to attract their wasp pollinators. Specialised wasp pollination strategies include the attraction of pollinators to nectar-producing flowers by a specific floral odour (Shuttleworth & Johnson 2009b), the fig-wasp

80   nursery pollination mutualism where chemical cues are crucial (Janzen 1979; Chen *et al*. 2009; Cornille *et al*. 2012), the mimicry of alarm pheromones of prey (Brodmann *et al*. 2009), and the chemical and morphological mimicry of female insects (Ayasse *et al.* 2003; Schiestl *et al*., 2003; Bohman *et al*. 2014, 2017).

85   The Pompilidae (Hymenoptera) is a cosmopolitan family of wasps with over 5,000 species and 230 recognised genera (Pitts *et al*. 2006; Waichert *et al*. 2015). Females provision each offspring with a single paralysed spider, which is consumed by the larva (Endo & Endo 1994; O'Niel 2001). There have been few systematic studies of the diet of adult pompilids (though see Punzo 2006), but in multiple geographic

90   regions they are frequently observed to feed on nectar alongside other hymenopteran species (Shimizu 1992; Evans 1997; Vieira & Shephard 1999; Cerana 2004; Yates *et al*. 2005; Punzo 2006; Waichert *et al*. 2012; Wiemer *et al*. 2012). At present, the only fully documented pollination systems specialised on members of the Pompilidae come from southern Africa (Steiner *et al*. 1994; Shuttleworth & Johnson 2012). However,

95   in two species of *Morrenia* asclepiads from South America pompilids and other solitary wasps are important pollinators (Wiemer *et al*. 2012). Pompilids are also reported to be pollinators of some *Brassia* orchids in the Neotropics (van der Pijil & Dodson 1966; Pupulin & Bogarin 2005), although detailed studies appear to be lacking. Thus, examples of known pompilid pollination seem to be surprisingly rare

100   given the taxonomic diversity and broad geographic range of the family.

The specialised pompilid pollination systems from southern Africa are almost entirely based on four members of the wasp genus *Hemipepsis* (Shuttleworth & Johnson 2012). The guild of plants specialised on these wasps includes members of the Orchidaceae (Johnson 2005), Asparagaceae subfamily Scilloideae (Shuttleworth & Johnson 2010), and several members of the Apocynaceae (Shuttleworth & Johnson 2006, 2009b, 2012). Members of this guild tend to be characterized by dull greenish- or brownish-white flowers, often with purple blotches, and a sweet spicy scent (Shuttleworth & Johnson 2012). Interestingly, specialisation in these plants arises despite having open-faced flowers where the nectar is exposed allowing easy access for nectar feeders. It has been experimentally demonstrated that, at least in some milkweeds, long-distance pollinator attraction is by chemical cues (Shuttleworth & Johnson 2009a,b), with specialization on *Hemipepsis* achieved through floral odour and distasteful nectar that deters non-pollinating visitors (Shuttleworth & Johnson 2009b, 2010). The other documented specialized pompilid pollination system in southern Africa is in an orchid species in the genus *Disa*, where at least long-distance attraction of pollinators appears to be via mimicry of the sex pheromone of the female wasp (Steiner *et al*. 1994).

In the Australian flora, the only documented cases of plants with pollination systems specialised on wasps outside of the fig-wasp mutualism are in the Orchidaceae (Armstrong 1979; Phillips *et al*. 2010). For example, several hundred species of Australian terrestrial orchids achieve pollination by the sexual deception of male thynnine wasps (Thynnidae) (Bohman *et al*. 2016; Peakall *et al*. 2020). Typically, the flowers of these species are dull-coloured, and/or diminutive with aggregations of maroon coloured labellum calli (Stoutamire 1983; Peakall 1990, Phillips *et al*. 2009, 2017). However, specialised pollination by nectar-foraging male thynnine wasps has also been demonstrated in Australian orchids (Bates 1984; Reiter *et al*. 2018, 2019b; Phillips *et al*. 2020). In these cases, the flowers tend to be more brightly coloured and have larger floral displays than their sexually deceptive counterparts, though there are exceptions to these trends (Phillips *et al*. 2017; Phillips & Peakall 2018a). Interestingly, pollination systems based on sexual deception and food-foraging behaviour are both represented in the diverse orchid genus *Caladenia* (Stoutamire 1983; Phillips *et al*. 2017, 2020; Reiter *et al*. 2018, 2019b), suggesting that it may

135   provide a powerful study system for understanding the mechanisms underpinning

shifts in pollination strategy.

During studies to resolve the pollination strategies of phylogenetically distinct

Caladenia (see Clements *et al*. 2015 for phylogeny), we found evidence for visitation

140   of pompilid wasps to *Caladenia drummondii* Benth (see the preliminary observation

in the methods). This orchid species is exceptional in that it is the only autumn

flowering member of the genus outside of some members of the distantly related

Caladenia subgenus *Caladenia* (Backhouse 2018). The dark labellum, relatively short

tepals (Figure 1; 2-4 cm total flower size) and an absence of visible nectar gives *C.*

145   *drummondii* a superficial similarity to some spring-flowering *Caladenia* pollinated by

sexual deception of thynnine wasps. As such, it has been predicted that *C.*

drummondii is pollinated by sexual deception (Phillips *et al.* 2011) but at present there

is no published data on the pollinators and their behaviour. Here, we address four

questions concerning *C. drummondii*: (i) Are pompilids the primary pollinator? (ii)

150   What is the pollination strategy? (iii) Is the species truly nectarless? (iv) Does floral

colour conform to that of *Caladenia* pollinated by food-foraging or sexually deceived

wasp pollinators?

MATERIALS AND METHODS

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Study species

Caladenia drummondii is widespread through the central and northern parts of the

semi-arid zone in the South-west Australian Floristic Region (Hoffman & Brown

160   2011). It frequently occurs in *Eucalyptus* woodland and mallee communities but can

be found in a range of vegetation associations and soil types (Hoffman & Brown

2011). Flowering occurs primarily during May and early June, approximately

coinciding with the first substantial winter rains. With the exception of the occasional

Eucalyptus (Myrtaceae), and a few species of orchid that are believed to use deceptive

165   pollination strategies (members of the genera *Eriochilus*, *Pterostylis* and

Rhizanthella), there are few or no other plants flowering at sites where *C. drummondii*

occurs during its late autumn flowering period.

Pollinator observations at experimental bait flowers

In May 2009, one hour of preliminary pollinator observations were made for *C. drummondii* at Wongamine Nature Reserve (31.4846˚ S; 116.5946˚ E) using flowers picked from a small woodland remnant south of Bejoording (representative voucher G. Brockman 1143; PERTH 06964044, Western Australian Herbarium; 31.3998˚ S; 116.5523˚ E). The pollinator baiting method was used to observe pollinators, based on the assumption that this species was likely to be pollinated by sexual deception of a species of Hymenoptera. In sexually deceptive systems, pollinator baiting involves picking a small number of flowers (usually 1 or 2) and moving them to a new position in the landscape, often leading to a rapid response of male pollinators (Stoutamire 1974; Peakall 1990). This initial trial, where flowers were repositioned every two minutes, led to the unexpected observation of a visit by a single pompilid wasp. From the 24th-25th of May 2010, a more detailed survey was undertaken to test if this initial observation could be repeated and to find sites where pollinators were sufficiently common to permit more detailed observations (Supplement Table 1). Here, six picked flowers were used to attract pollinators. At each of ten sites, six baiting periods of two minutes were conducted. The sites selected were in an arc running north from Wongamine Nature Reserve to the east of Morowa, a distance of approximately 250 km. A total of 37 visits to flowers by the same species of pompilid wasp were observed during this survey.

In May 2017, an attempt was made to make detailed observations of the behaviour of pollinators of *C. drummondii*. However, returning to sites in the north of the range of *C. drummondii* that had previously yielded frequent floral visitors (see Supplement Table 1) proved unsuccessful, possibly because of an autumn with very little rainfall. Therefore, attention shifted south to the Corrigin area, where there are several known populations of *C. drummondii*. In total, pollinator observations were attempted at ten sites in 2017 (Supplement Table 2). Two-minute baiting trials were used to attract pollinators, but up to 15 trials were used per site as pollinator abundance appeared to also be low in this region. In total, only eight floral visitors were recorded in 2017.

From the 15th to the 22nd of May 2018, detailed observations of the pollinators of *C. drummondii* were made in the north and south of its geographic range. Initial observations of pompilid wasps revealed that they appeared to engage in nectar-foraging behaviour on the flower, which had not been apparent in 2010, when the focus was on surveying for suitable experimental sites, or in 2017, when very few pollinator individuals responded. Based on the observation of Reiter *et al*. (2018, 2019a) and Phillips *et al*. (2020) that baiting with multiple flowers can attract food-foraging pollinators, a group of 10-12 picked flowers was used to bait for pollinators (flowers sourced from the S Bejoording and SE Katanning sites; 33.5620˚ S; 117.7036˚ E). We also extended the baiting period from 2 to 10 minutes to allow for the possibility that responses to the flower are slower because long-distance attraction is not based on sex pheromones. No observations were undertaken for plants *in situ* (i.e. in their natural state, rather than via baiting). For all floral visitors it was recorded if they landed on the flower, if they fed from the flower, if they contacted the column, if they removed pollinia, if they deposited pollinia, if they carried pollinia when arriving at the flower, and the time elapsed since the beginning of the trial. Because of the initial prediction that *C. drummondii* was sexually deceptive, careful attention was paid to see if pollinators attempted copulation with the flower. Recordings by a human observer were supplemented with video using a Panasonic HC-V750M camcorder. Attempts to observe pollinators were made at 11 sites (122 10-minute trials in total) but were focussed on Babakin town reserve (32.1239˚ S; 118.0190˚ E; 40 10-minute trials), where pollinators were most reliably recorded. While *C. drummondii* occurs at this site, there were few flowering plants observed during the study period. A total of 55 visits by pompilid wasps to *C. drummondii* were observed during 2018. The Spearman's rank order correlation between time since the beginning of the trial (in one-minute intervals) and the number of wasps responding was calculated in SPSS v25.0 (IBM Corp. 2017).

Observations of nectar and GC-MS analysis

Though visible nectar was not apparent on any of the flowers used in our earlier baiting studies, while collecting flowers for pollinator experiments in 2018 it became clear that at least some individuals of *C. drummondii* had droplets of nectar on the labellum calli. Therefore, at both the Katanning and Bejoording populations, 30 plants

were scored for the presence of visible nectar droplets on the labellum calli at approximately 10am (15th May 2018 at Katanning, 19th of May 2018 at Bejoording).

For ten randomly picked flowers, all without visible nectar, quantification of sugars present on the labellum was made based on the methods outlined in Reiter *et al*. (2018). In summary, a solution of ribitol (an internal standard to allow quantification) was prepared and 5 µL added to the upper surface of the labellum. The resulting extract was then collected with a 5 µL microcapillary tube and immediately transferred to a 2 mL GC-vial with a 50 µL insert for storage. Identification and quantification of trimethylsilylated sugars was undertaken using GC-MS, with identifications confirmed by co-injection with synthetic standards. For specific details of GC methodology, see Reiter *et al*. (2018). 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 trimethylsilylated ribitol used as internal standard, taking into account the differences in response factors (Reiter *et al.* 2018).

Measurement of spectral reflectance

To test if the floral spectral reflectance of *C. drummondii* more closely conforms to that of species pollinated by sexual deception of thynnine wasps, or those pollinated by nectar-seeking insects, we quantified floral spectral reflectance of *C. drummondii* and other species of *Caladenia*. In addition to the measurements in Phillips *et al*. (2020) for *Caladenia nobilis* (nectar-seeking pollinators; RDP 0268; PERTH 08645574) and *Caladenia radialis* (sexual deception; RDP 0430 PERTH 08978204), we measured spectral reflectance for four additional species where the pollination strategy has been experimentally confirmed (Stoutamire 1983, Phillips *et al*. 2009, Phillips *et al*. 2013; Swarts *et al*. 2014; Bohman *et al*. 2018); *Caladenia attingens* subsp. *attingens* (sexual deception; RDP 0343 PERTH 08739587), *Caladenia pectinata* (sexual deception; RDP 0247 PERTH 08643261), *Caladenia reticulata* (sexual deception; NHR 148 MEL 2423864A), and *Caladenia longicauda* subsp. *eminens* (nectar-seeking pollinators; RDP 0390 PERTH 08876363).

Floral spectral reflectance was quantified by spectrophotometry, using an Ocean Optics (Dunedin, FL, USA) USB 4500 spectrometer. With the aid of an internal

270   deuterium tungsten halogen light source (200–850 nm), measurements were
       undertaken using a UV–Vis 400 fiber optic reflection probe, held 5 mm from the
       center of the surface of the floral tissue at 45° by a RPH Reflection Probe Holder
       (Ocean Optics). Reference spectra were taken using a WS-1 Diffuse Reflectance
       Standard. For six individuals per species, two measurements were taken of the lateral
275   sepals, petals and labellum tip. The two measurements of each floral part were
       averaged prior to analysis. Measured spectra, averaged across the six individuals,
       were analyzed using the Chittka model to derive colour loci in the bee visual space
       (Chittka 1992, Chittka & Kevan 2005) assuming an average green leaf as adaptation
       background (the spectrum published by Gumbert *et al*. 1999) and the standard D65
280   illumination function (Gumbert *et al*. 1999). While there is no data on the
       photoreceptor spectral sensitivity for any member of the Pompilidae, evidence
       suggests that the spectral sensitivities of Hymenoptera are phylogenetically
       conservative (Briscoe & Chittka 2001). As such, the model of Chittka (1992), which
       uses the spectral sensitivity of the receptors of *Apis mellifera* (Apidae), is assumed to
285   be a reasonable approximation of the colour loci for pompilid wasps.

       Results

       Pollinator observations
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       An undescribed species of pompilid wasp *Calopompilus* sp. (between 5 and 7 mm in
       length) was the only floral visitor observed to carry, deposit or remove pollinia
       (Figure 2). Across the three years in which observations were undertaken, 100
       individuals responded to *C. drummondii* at a total of 14 sites. Wasps could only be
295   sexed with the aid of a microscope, but all 24 specimens collected for identification
       were male (Supplement Table 3). At three sites they were observed carrying pollinia
       of *Caladenia* (six individual wasps), which can be separated by eye from the pollinia
       of co-flowering orchids in the genera *Eriochilus*, *Pterostylis* and *Rhizanthella*. As no
       other *Caladenia* species with similar pollinia flowers at this time of year, this pollen
300   can be confidently attributed to *C. drummondii*. Visits to bait flowers were also
       observed by one individual of an unidentified species of syrphid fly, and the formicid
       ants *Iridomyrmex greensladii* (two sites) and *Iridomyrmex agilis* (one site). However,

none of these other visitors were large enough to contact the reproductive structures of the plant, which is required for the removal or deposition of pollinia.

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Detailed observations of the pollinator behaviour were obtained for 63 *Calopompilus* sp. wasp visitors to artificially presented bait flowers in 2017 and 2018. Of the 63 individuals responding, 51 landed on the flower, and six contacted the column, which is the behaviour needed for pollination. Of the individuals contacting the column, five

310 of them removed pollinia, which were deposited on the dorsal side of the thorax. On six additional occasions wasps moved to the position where pollination could occur, but the individual wasps were too small to contact the column. One of the two wasps arriving at a bait flower with pollinia from wild orchids successfully transferred pollen to the bait flower. While responses of the wasps continued throughout the ten-

315 minute baiting trails, the greatest number of responses was in the first two minutes (Figure 3). However, overall, there was no significant correlation between number of wasps responding and time since the trial commenced ($r = -0.345$; $P = 0.329$). As they approached the flower in flight, the wasps exhibited zig-zagging and/or circling behaviour. Some individuals perched on leaf litter as they neared the bait flower (held

320 10 cm above the ground), while others landed on the vial supporting the bait flowers before crawling onto the flowers themselves.

Unlike the wasp pollinators of sexually deceptive *Caladenia* orchids, of the 51 landing individuals, 24 attempted to feed from the surface of the labellum. Typically,

325 they began feeding among the anterior calli before gradually moving down to the base of the labellum while continuing to feed. Feeding sometimes lasted for periods of in excess of 10 seconds and occurred in flowers with no visible nectar droplets. No copulation attempts were observed. While the wasps did exhibit some wing flicking as they moved over the surface of the flower or climbed up the vial, they did not show

330 any behaviour that was suggestive of courtship. The fly and ants that visited the flowers also showed nectar-foraging behaviour.

Observations of nectar and GC-MS analysis

335 Despite no prior reports in the literature of nectar production in *C. drummondii*, at the Katanning site 14 out of 30 plants (46.7%) had visible nectar, while at Bejoording

only 2 out of 30 plants (6.7%) had visible nectar. On all occasions where nectar was visible, nectar droplets were observed on the upper surface of the calli on the labellum lamina (Figure 1). Using GC-MS for ten flowers with no visible nectar, on average

340   142.5 ± 27.0 µg (SE) of sucrose, 1.6 ± 0.5 µg of fructose, and 1.5 ± 0.4 µg of glucose was detected on the labellum.

Spectral reflectance measurements

345   The *Caladenia* species measured showed considerable variation in spectral reflectance of the lateral sepals and petals, though most species were within the BL-GR segment of the colour hexagon bee vision model (Figure 4). However, for both sexually deceptive species and *C. drummondii*, the labellum only exhibited spectral reflectance at high wavelengths (>600nm), where the photoreceptors of bees show

350   low sensitivity to light (Peitsch *et al*. 1992). This spectral reflectance profile is almost at the centre of the colour hexagon, in a region where it is likely to be perceived as achromatic to hymenopterans (Lunau *et al*. 2011). By comparison, in *C. nobilis*, which is pollinated by nectar-foraging thynnine wasps (Phillips *et al*. 2020), and *C. longicauda* subsp. *eminens*, which is thought to be pollinated by a range of nectar-

355   foraging insects (Stoutamire 1983), the labellum colour was in the BL-GR segment.

DISCUSSION

Pollination strategy in *Caladenia drummondii*

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Observations spanning multiple years and an approximately 375 km geographic range showed that *C. drummondii* is likely to be pollinated by males of a single undescribed species of *Calopompilus* pompilid wasp. Interestingly, this genus of wasp is in subfamily Pepsinae (Waichert *et al*. 2015), as are the pompilid genera involved in the

365   specialised pollination systems in South Africa and the Americas (van der Pijil & Dodson 1966; Shuttleworth & Johnson 2012; Wiemer *et al*. 2012). Contact with the reproductive structures of the orchid occurred while the wasps were foraging for nectar on the upper surface of the labellum, demonstrating that unlike most *Caladenia*, *C. drummondii* uses a rewarding pollination system. Foraging behaviour

370   was evident even when there were no visible nectar droplets on the surface of the

labellum. Despite their diminutive size, and the frequent lack of visible nectar, flowers of *C. drummondii* had substantially more sugar on the labellum than other *Caladenia* species where sugars have been quantified with the same method (Table 1; Reiter *et al.* 2018, 2019a,b; Phillips *et al.* 2020). It is possible that in *C. drummondii*

375   the nectar is very concentrated and forms a film on the surface of the labellum. However, the irregularity of which nectar droplets are visible to human observers suggests that in *C. drummondii* nectar production may only occur under certain environmental conditions, or perhaps only early in the flowering period. At present, no research has been undertaken on the environmental conditions or flower stage(s) in

380   which *Caladenia* or other related Australian orchid genera secrete nectar. Nonetheless, our observations of variation in visible nectar between days and between individuals suggest that *C. drummondii* may represent a useful system for studying the ecology of nectar production.

385   **Why is the pollination system so specialised?**

The high level of pollinator specialisation observed raises the question of why *C. drummondii* only appears to attract the one wasp species? The provision of a common floral reward in the form of sucrose suggests that the nectar of *C. drummondii* should

390   be palatable to a diversity of insect species (Percival 1961). However, in the southern African pollination guild using *Hemipepsis* pompilid wasps, there is evidence that long-distance attraction is by specific chemical cues (Shuttleworth & Johnson 2009, 2010). In *Caladenia*, sexually deceptive species have been experimentally demonstrated to attract pollinators to the flower primarily with chemical cues

395   (Stoutamire 1983; Bohman *et al.* 2017, 2018; Xu *et al.* 2017), while food-foraging thynnines show evidence of odour-tracking behaviour as they approach the flower (Reiter *et al.* 2018). The behaviour of pollinators approaching the diminutive flowers of *C. drummondii* was suggestive of an odour-based attraction, and responses occurred shortly after the commencement of baiting trials (most often within two

400   minutes), which can also be indicative of odour-based attraction (e.g. Peakall 1990; Phillips *et al.* 2014). As such, it seems likely that specific chemical cues may be responsible for attraction of this species of pompilid wasp. Unfortunately, with the exception of the Jibberding Rocks site in the 2010 pilot study, during the course of this research the wasps never responded to *C. drummondii* in sufficient numbers to

405   allow experiments testing for odour-based attraction. Ideally, these experiments would involve presenting flowers obscured from view of pollinators to test for primarily odour-based attraction (e.g. Shuttleworth & Johnson 2009b; Phillips *et al*. 2014), experimental dissections to determine the source of chemical attractant (e.g. Phillips *et al*. 2013, 2014), and subsequent chemical analysis.

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Unlike the guild of plants specialised on *Hemipepsis* wasps for pollination (Shuttleworth & Johnson 2006, 2009b), and plants visited incidentally by pompilids, all of the pompilid wasps caught visiting *C. drummondii* were males. Male-biased floral visitation could arise through males emerging prior to females, though that

415   seems unlikely in this case where observations were undertaken across multiple years, sites and over multiple weeks within the flowering period. While there was no evidence of attempted copulation on flowers of *C. drummondii*, it remains possible that the inclusion of a component of the sex pheromone in the floral odour could explain long-distance attraction to the flower (e.g. Bino *et al*. 1982), even though the

420   wasps engage in nectar-foraging. This possibility was demonstrated experimentally by Kullenberg (1961), who showed that bees attracted to the flowers of *Ophrys* via sex pheromones would switch to nectar-foraging if a reward was artificially added to the flower. Outside of the orchids, male pollinators of the daisy *Gorteria diffusa* will exhibit either feeding or attempted sexual behaviour with the flower (Ellis & Johnson

425   2010), though in this case sexual cues are visual and tactile (De Jager & Ellis 2012).

In some sexually deceptive plants, pollination may be achieved during mate searching (Phillips & Peakall 2018b) or attempted courtship with the flower (Peakall 1990) rather than necessarily requiring attempted copulation. Courtship in pompilids has

430   received very little attention in the literature, although it is evident that interspecific variation exists. For example, in some species males pursue and capture females (Alcock 1981; Shimizu 1992), while wing-scissoring and antennal vibrations during courtship have also been observed (Kurczewski 1989). During the visit to *C. drummondii* male *Calopompilus* sp. did exhibit some of the brief wing-flicking typical

435   of both male and female pompilids, but not the courtship or mating behaviour previously recorded for other species of pompilid (Alcock 1981; Kurczewski 1989; Shimizu 1992). However, the approach to the flower involved zig-zagging flight, and sometimes perching on leaf litter and crawling up the stem on final approach. This

behaviour is similar to that seen in some species of *Neozeleboria* thynnine wasps

440 when approaching sexually deceptive *Chiloglottis* orchids (e.g. De Jager and Peakall 2016) and beads spiked with sex pheromones (R. Peakall pers. obs). Unfortunately, no females of the pollinator species were observed during this study, meaning that we were unable to witness the courtship or copulatory behaviour of *Calopompilus* sp.. We therefore lack a clear expectation of what attempted sexual behaviour with a

445 flower of *C. drummondii* would look like.

If females of the pollinator species can be obtained in the future, a high research priority for testing if pollinators are sexually attracted to *C. drummondii* would be to compare the field response of the wasps to chemical extracts of the female(s) versus

450 orchid flower extracts. In the absence of females, future experiments to investigate potential courtship behaviour could use dead males of *Calopompilus* sp. (which in pompilids are similar in size to females), rendered odourless by drying or via Soxhlet-extraction (Bohman *et al*. 2020). These dummies can then be spiked with floral extracts of *C. drummondii*, in a similar manner as previously been proven effective in

455 attracting *Argogorytes* spp. pollinators to *Ophrys insectifera* extracts (Kullenberg 1961), to test if males will attempt copulation with an appropriate morphological stimulus. If the above steps provide evidence for sexual attraction, then further steps towards identifying the compounds involved in pollinator attraction could follow (see Bohman *et al*. 2020 for a review on bioassay guided semiochemical discovery, which

460 typically includes gas-chromatography with electoantennography (GC-EAD) and/or fractionation by preparative chromatography).

Shifts in floral spectral reflectance between pollination strategies

465 The spectral reflectance of the labellum of *C. drummondii* conformed to that seen in several species of sexually deceptive *Caladenia*, where the dull red colour of the labellum is likely to be perceived as essentially colourless by Hymenoptera (Lunau *et al*. 2011). Alternatively, the other tepals were highly reflective and located in the BL-GR segment of the colour hexagon. To an approaching pollinator the labellum of *C.*

470 *drummondii* would exhibit strong achromatic contrast with the other tepals, suggesting that the dark labellum could function as a target to increase the likelihood

of the pollinator coming into close proximity to the reproductive structures (e.g. Streinzer *et al*. 2009).

475   In our analysis, there was no clear differentiation in the colour of sepals and petals between species pollinated by sexual deception of thynnine wasps, those pollinated by nectar-seeking insects, and *C. drummondii*. The similarity in colouration with taxa employing other strategies, in particular the dark colour of the labellum of *Caladenia* pollinated by sexual deception of thynnine wasps, suggests that the evolution of

480   pollination by pompilids in *Caladenia* could have arisen without a substantial shift in floral colour. Given that the unusual morphological traits of *C. drummondii* suggest that it has no close extant relatives, the phylogenetic placement of this species is likely to prove critical for understanding the evolutionary history of pollination shifts in *Caladenia*.

485

Specialised pollination systems involving nectar-foraging insects

        The apparent reliance on a single species of pollinator in *C. drummondii* supports the

490   emerging evidence from other Australian orchid species for a surprisingly high degree of specialisation even when the pollination is by nectar-seeking insects (Indsto *et al*. 2006; Reiter *et al*. 2018, 2019a,b; Scaccabarozzi *et al*. 2018; Phillips & Batley 2020; Phillips *et al*. 2020). Some of these species exhibit morphological filtering on pollinator size where not all insect species visiting the flower are of the appropriate

495   size for the removal or deposition of pollinia (Reiter *et al*. 2018; Phillips *et al*. 2020). However, like *C. drummondii*, they all appear to also show a high level of specialisation at the attraction phase, where only one or few pollinator species are attracted, representing just a small fraction of the pool of potential pollinators (e.g. Reiter *et al*. 2019a, Phillips *et al*. 2020; Phillips & Batley 2020). Given the growing

500   evidence for ecological specialisation in *Caladenia* species pollinated by nectar-foraging insects (Reiter *et al*. 2018, 2019a,b; Phillips *et al*. 2020; Phillips & Batley 2020), the genus as a whole may yet prove to be characterised by high to extreme pollinator specificity, at least at the population level, irrespective of whether the species are sexually deceptive or not (Phillips *et al*. 2017).

505

Conclusions

Specialised pollination systems using pompilid wasps appear to be very rare in nature, with this paper representing the first record of such a system in the Australian flora. The species of *Caladenia* with the most similar floral traits to *C. drummmondii* are known to be pollinated by the sexual deception of thynnine wasps (e.g. Stoutamire 1983; Phillips *et al.* 2009, 2017; Swarts *et al.* 2014), suggesting that *Caladenia* is unlikely to be harbouring other pompilid-based systems. Further, there are generally few species of plants flowering during autumn in many habitats in south-western Australia, outside of some generalist Myrtaceae and some bird-pollinated species (Brown *et al.* 1997), suggesting that there is unlikely to be other co-flowering pompilid-pollinated species. As such, pollination strategies based on pompilids are likely to prove genuinely rare in the Australian flora. Sites with more abundant pollinators will need to be located to permit experimental tests to understand the mechanisms underpinning pollinator attraction in this highly specialised system, including the possibility that sex pheromone mimicry may explain the pronounced male bias in pollinator visitation.

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840 **Table 1**: A summary of species where labellum nectar sugars have been quantified and/or identified in *Caladenia*. Numbers are mean ± SE per flower.

Species	Strategy	Nectar quantity	Reference
Caladenia arenaria	nectar-seeking thynnine wasps	5.3 ± 2.7 µg sucrose	Reiter *et al.* (2019b)
Caladenia colorata	nectar-seeking thynnine wasps	16.61 ± 3.94 µg sucrose	Reiter *et al.* (2018)
Caladenia concolor	nectar-seeking thynnine wasps	31.5 ± 12.1 µg sucrose	Reiter *et al.* (2019b)
Caladenia drummondii	nectar-seeking pompilid wasps	142.5 ± 27.0 µg sucrose, 1.6 ± 0.5 µg fructose, and 1.5 ± 0.4 µg glucose	Present study
Caladenia nobilis	nectar-seeking thynnine wasps	2.6 ± 0.9 µg sucrose, 1 flower had 1.1 µg fructose and 1.0 µg glucose	Phillips *et al.* (2020)
Caladenia paludosa	sexual deception of thynnine wasps	51.2 ± 16.6 µg sucrose, 10.7 ± 5.0 µg glucose and 10.9 ± 5.2 µg fructose	Phillips *et al.* (2020)
Caladenia rigida	nectar-seeking Hymenoptera and Diptera	Not quantified, primarily sucrose, some glucose, 1 flower had fructose	Faast *et al.* (2009)
Caladenia tentaculata	sexual deception of thynnine wasps	< 0.01 ng	Reiter *et al.* (2018)
Caladenia versicolor	nectar-seeking colletid bee	3.6–4.7 µg saccharides per flower, with > 95% sucrose	Reiter *et al.* (2019a)

FIGURES

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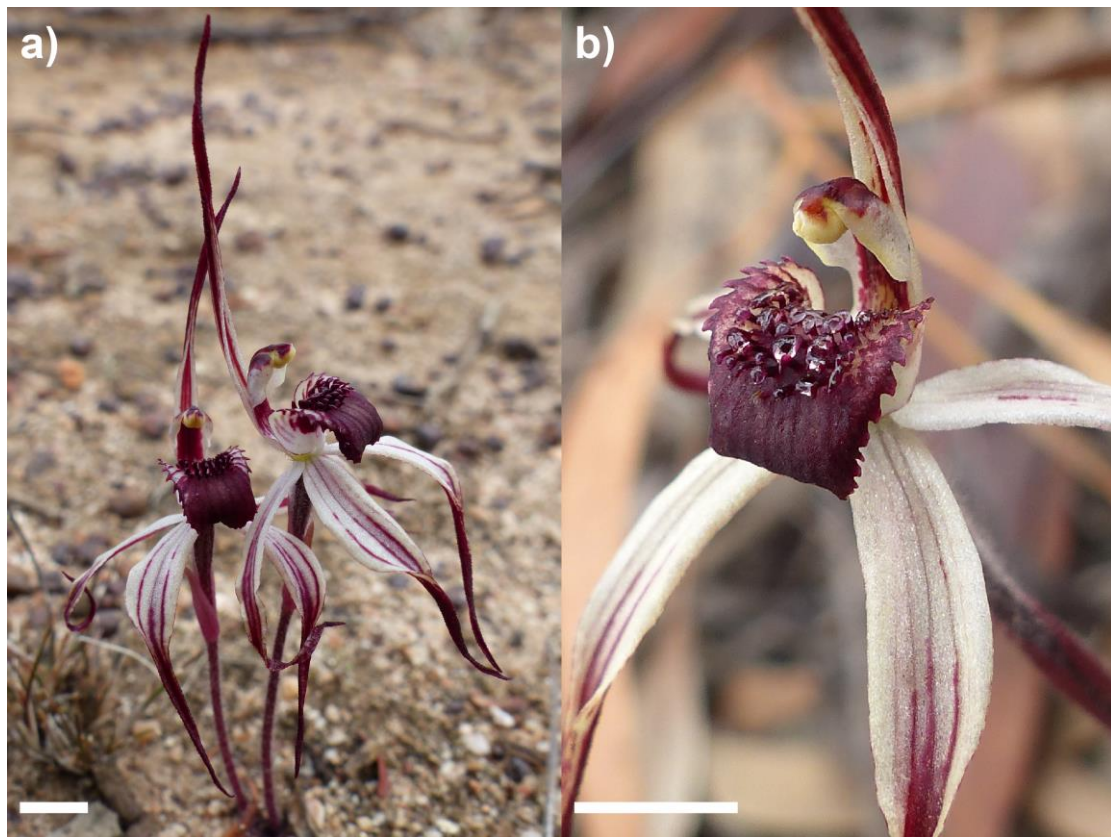
Figure 1: a) Flowering individuals of *Caladenia drummondii*. Flowers typically range in size from 2-4 cm, scape height typically ranges from 6-10 cm (Hoffman & Brown

850   2011) b) Nectar droplets on the calli of the labellum lamina of *Caladenia drummondii*. The scale bar is 4 mm.
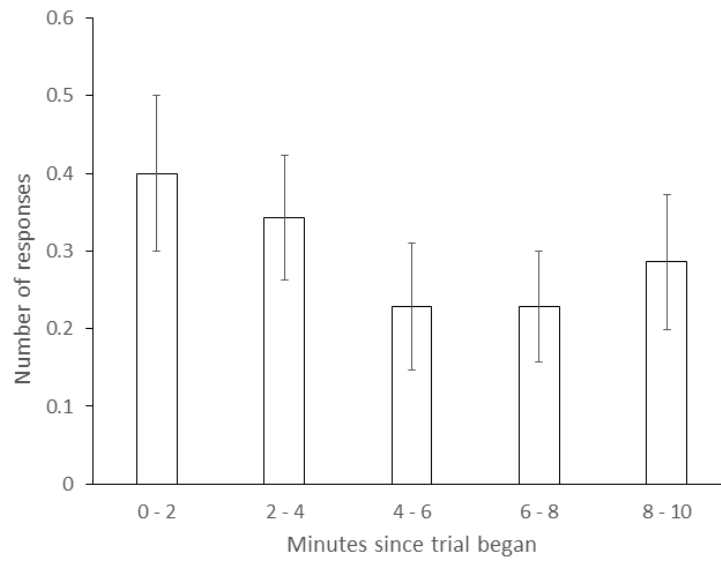
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**Figure 2:** A male of *Calopompilus* sp., the pollinator of *Caladenia drummondii*, here shown carrying pollinia of the orchid. Total body length varies between 5 and 7 mm. The scale bar is 5 mm.

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Figure 3: The number of *Calopompilus* sp. responding to flowers of *Caladenia drummondii* during two-minute intervals of ten-minute trials. Bars represented the mean number of responses across trials (± SE).
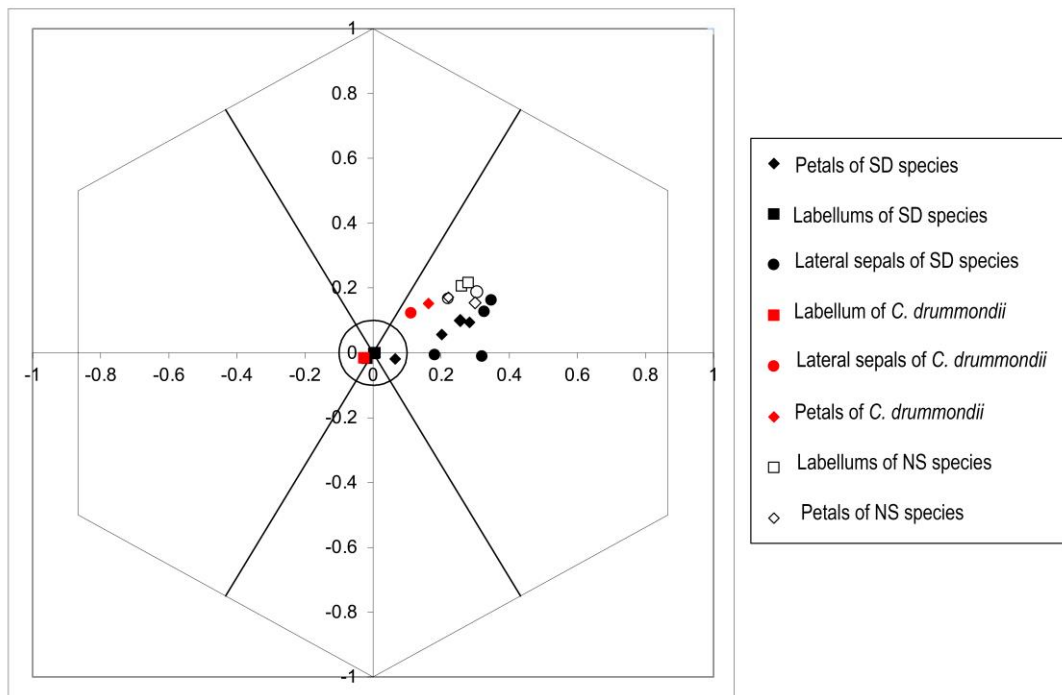
885 **Figure 4:** Floral colour of *Caladenia drummondii* plotted in bee visual space in comparison with species pollinated by sexual deception of thynnine wasps (SD; *Caladenia attingens* subsp. *attingens*, *Caladenia pectinata*, *Caladenia radialis*, *Caladenia reticulata*) and nectar-seeking insects (NS; *Caladenia longicauda* subsp. *eminens, Caladenia nobilis*).