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10 **A specialised pollination system using nectar-seeking thynnine wasps in
Caladenia nobilis (Orchidaceae)**

Ryan D. Phillips^{1,2,3*}, Bjorn Böhman⁴, Graham R. Brown^{5,6,7}, Sean Tomlinson^{2,8}, Rod Peakall³

15 ¹ Department of Ecology, Environment and Evolution, La Trobe University, Vic. 3086, Australia.

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

20 ³ Ecology and Evolution, Research School of Biology, The Australian National University, Canberra, ACT 2600, Australia.

⁴ School of Molecular Sciences, The University of Western Australia, Crawley, WA 6009, Australia.

⁵ Museum and Art Gallery of the Northern Territory, GPO Box 4646, Darwin, NT 0801, Australia.

25 ⁶ CSIRO Land and Water, Private Mail Bag 44, Winnellie, NT 0822, Australia.

⁷ Research Institute for Environment and Livelihoods, Charles Darwin University, Darwin, NT 0909, Australia.

⁸ School of Molecular & Life Sciences, Curtin University Bentley 6102, Western Australia.

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Running head: pollination by nectar-seeking thynnine wasps

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ABSTRACT

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- *Caladenia* is a diverse Australian genus that is exceptional among orchids in having both species pollinated by food-seeking and sexually deceived insects. Here, we investigated the pollination of *Caladenia nobilis*, a species predicted to be food-deceptive due to its large, cream-coloured, and apparently nectarless flowers.

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- Pollinator observations were made using experimental clumps of flowers. Measurements of floral colour were undertaken with a spectrometer, nectar was tested for using GC-MS, and reproductive success was quantified for two years.

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- While *C. nobilis* attracted nine species of insect, only males of the thynnine wasp *Rhagigaster discrepans* exhibited the correct size and behaviour to remove and deposit pollen. Male *R. discrepans* attempted to feed from the surface of the labellum, often crawling to multiple flowers, but showed no evidence of sexual attraction. Most flowers produced little or no nectar, although some may provide enough sucrose to act as a meagre reward to pollinators. Floral colouration was similar to a related *Caladenia* species pollinated by sexual deception, though the sexually deceptive species had a dull-red labellum. Reproductive success was generally low, and highly variable between sites and years.

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- In addition to most visitors being of inappropriate size for pollinia removal, the lack of response to the orchid by several co-occurring species of thynnine wasps suggests filtering of potential pollinators at the attraction phase. Our discovery of a pollination strategy that may be intermediate between food deception and food reward raises the question, how many putatively rewardless orchids actually produce meagre amounts of nectar?
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Key-words: orchids, specialisation, pollination, thynnine wasps, *Caladenia*, deception, nectar

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INTRODUCTION

The Orchidaceae is exceptional among plant families in that approximately 30% of all orchid species achieve pollination by deception rather than by the provision of rewards such as nectar or pollen (Dressler 1981; Ackerman 1986). Across the orchids, an immense diversity of deceptive pollination strategies have been discovered, including mimicry of brood sites (van der Niet *et al.* 2011; Martos *et al.* 2015), mimicry of potential shelter sites (Dafni *et al.* 1981), and the sexual deception of pollinators via the mimicry of a female insect (Pouyanne 1917; Coleman 1928; Schiestl *et al.* 1999; Bohman *et al.* 2016). However, the most widely used rewardless orchid pollination strategy is food-deception (Jersáková *et al.* 2006).

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In some food-deceptive species the attraction of food-seeking pollinators is achieved by mimicry of rewarding model flowers (Nilsson 1983; Johnson *et al.* 2003; Newman *et al.* 2012). Alternatively, many orchids use generalised food deception where the plant uses floral signals that pollinators associate with a reward, without precise mimicry of a specific floral model (Ackerman 1981; Steiner 1998; Johnson & Schiestl 2016). Floral mimicry is thought to be the more specialised of these two strategies, with the orchid often reliant on just a single primary pollinator species (Johnson & Schiestl 2016). However, the gullet shaped flowers of many orchid species requires the pollinator to exhibit a precise morphological fit, meaning that some orchids

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pollinated by generalised food deception can also exhibit surprisingly high levels of ecological specialisation (Li *et al.* 2008; Peter & Johnson 2013).

100 *Caladenia* is a diverse genus (350+ species) of Australian orchids (Phillips *et al.*
2009a; Backhouse 2018) that, due its exceptional range of floral forms and pollination
strategies, offers the potential to yield considerable evolutionary insights. Numerous
Caladenia species are pollinated by sexual deception (Stoutamire 1983; Phillips *et al.*
2017), while there are a few known cases of nectar reward (Faast *et al.* 2009; Reiter *et*
105 *al.* 2018, 2019) and self-pollination (Hopper & Brown 2001). Many other *Caladenia*
species with bright colouration and a prominent floral display are thought to be
pollinated by generalised food deception (Stoutamire 1983; Phillips *et al.* 2009b,
2014), but detailed studies of this group are lacking. Based on largely anecdotal
observations, putatively food-deceptive *Caladenia* are believed to be pollinated by a
110 range of wasps, bees and flies (reviewed in Phillips *et al.* 2009b). However, the
degree of specialisation of these pollination systems and the role of floral scent, floral
colours and floral architectures in pollinator attraction remains unknown.

The *Caladenia filamentosa* complex is a group of approximately 45 species with
115 diversity centred on semi-arid areas of south-western Australia (Phillips *et al.* 2009a).
The floral form of the group is characterised by long, filamentous petals and sepals
(Hopper & Brown 2001). However, the primary floral colour varies considerably
between species, including white, yellow, pink and red. Floral scents are also variable
among species, being described as ‘like burnt metal’, ‘sweet’ or ‘fetid’ (Hopper &
120 Brown 2001). Based on the combination of prominent floral display, bright
colouration, scented flowers and lack of visible nectar, Phillips *et al.* (2011) predicted
that most members of this group are likely to be pollinated by food deception. Indeed,
the few observations of floral visitors in this group suggest that this is likely to be the
case (Erickson 1965; Kuitert 2017). However, unexpectedly it was recently shown that
125 the cream-coloured *Caladenia abbreviata* is pollinated by sexual deception of
thynnine wasps (Phillips & Peakall 2018), raising the possibility that in this group
food deception may not be as widespread as previously thought.

Here we investigate the pollination biology of *Caladenia nobilis* Hopper & A.P.Br., a
130 geographically widespread member of the *C. filamentosa* complex that has large,

white flowers without a nectar reward that is visible to humans, conforming to the floral traits expected for a food-deceptive orchid (Fig. 1). We addressed the following specific questions: (i) How many pollinator species are involved? (ii) Is nectar absent or present on the labellum, and if so what are the sugars secreted? (iii) Does floral colour differ from a closely related sexually deceptive species? (iv) How do levels of reproductive success compare to related species? Following evidence that *C. nobilis* had a single pollinator species within the study region, we also built Species Distribution Models for both orchid and pollinator to test if the distribution of this specific pollinator is likely to encompass the geographic range of the orchid.

MATERIALS AND METHODS

Study species

Caladenia nobilis is widespread along the western margin of the South-west Australian Floristic Region (SWAFR; *sensu* Hopper & Gioa 2004; see Brown *et al.* 2013 for distribution). Across its geographic range, *C. nobilis* occurs in a variety of habitats including red loams in arid acacia scrub, moist depressions in semi-arid sandplain heath, and forests with well-drained calcareous soils (Hopper & Brown 2001). *Caladenia nobilis* flowers from late July-September, with northern populations the first to come into flower (Hopper & Brown 2001). Individual populations exhibit a flowering period of approximately three to four weeks. Typically, a solitary flower is produced per year per scape (Brown *et al.* 2013). Sepals can reach up to 15cm in length, making *C. nobilis* one of the largest flowered members of the *C. filamentosa* complex (Hopper & Brown 2001). While *Caladenia nobilis* requires a pollen vector to achieve pollination, like other members of the *C. filamentosa* complex, it is clonal through vegetative reproduction and can form dense clumps of up to 15 flowering plants with numerous, non-flowering individuals. However, vegetative clones are only formed over a distance of centimetres, meaning that they are reliant on sexual reproduction and seed dispersal for colonisation of new areas.

Study sites

The study was undertaken in the vicinity of Lake Pinjarrega Nature Reserve,
165 Watheroo National Park, and adjoining areas of natural bushland. This region lies in
the centre of the species' geographic range and provides study sites within intact
vegetation communities inside large areas of bushland. The study area is dominated
by semi-arid shrublands growing on well-drained sandy soils. Drainage lines often
contain stands of *Eucalyptus loxophleba* (Myrtaceae). Extensive searches revealed
170 that within this region populations of *C. nobilis* are very patchy in the landscape,
being either confined to areas of shrubland that are relatively low in the landscape, or
the margins of the *E. loxophleba* woodland. Details of study locations and voucher
specimens of *C. nobilis* are provided in Table 1. Five of these sites are in relatively
close proximity, separated by a maximum of nine kilometres, and joined by
175 continuous stretches of natural vegetation.

Pollinator observations

Pollinator observations were undertaken on the 27-28th of August 2017, the 8th and
180 15th of September 2017, and the 2nd-3rd of September 2018. Observations were
restricted to periods of sunny weather with temperatures exceeding 20°C. A modified
version of the pollinator baiting method originally developed for sexually deceptive
orchids (Stoutamire 1974; Peakall 1990) was used as the basis of pollinator
observations. In this method, typically just one or two picked flowers are moved to
185 new positions in the landscape after 2-3 mins, often leading to the rapid attraction of
sexually deceived male pollinators. However, in the present study, 8 to 10 picked
flowering scapes of *C. nobilis* that were presented together in a vial, thereby
replicating the clonal clumps often seen in this species. These artificial clumps of bait
flowers were moved through the landscape in trials of 6-minute duration, with the
190 position of each new trial separated by at least 20 metres. The bait flowers were
exposed in a total of 188 trials, focussing on sites where *C. nobilis* is known to occur
(Table 1). Two additional sites of suitable habitat for the orchid were also
investigated, giving a total of seven sites. This baiting with clumps of flowers was a
similar approach to that recently used successfully in the nectar-producing *Caladenia*
195 *colorata*, where pots of shadehouse grown plants were used to attract and study
pollinators (Reiter *et al.* 2018).

Species distribution models of orchid and pollinator

200 To investigate if the pollinator species responsible for pollination of *C. nobilis* in our
study area was likely to be involved in other parts of the geographic range of the
orchid, we generated Species Distribution Models for both orchid and pollinator.
Modelling of the present geographic range of both *C. nobilis* and the primary
pollinator species was undertaken using the maximum entropy algorithm
205 implemented in MaxEnt version 3.3.3a (Phillips *et al.* 2006). We defined the spatial
extent of our training and projection layers using the bioregional domains of the
Interim Biogeographic Regionalisation for Australia version 7 (IBRA; Australian
Government Department of the Environment and Energy 2012). The training regions
were defined by spatially intersecting the distribution records with IBRA polygons
210 that encompassed these known occurrence locations. We defined our projection
region by identifying all the IBRA regions adjacent to the IBRA polygons
contributing to the training regions. Known occurrence data for *C. nobilis* came from
herbarium records in the Western Australian Herbarium, with old or imprecise records
removed from the dataset. Pollinator records came from searches of the entomology
215 collections of Australian museums. Due to the relatively small number of location
records for both species, we did not apply any constraints on extreme observations to
more accurately represent the critical range of either species (Morueta-Holme *et al.*
2010). Duplicate species records within single grid cells were removed (Newbold
2010; Elith *et al.* 2011).

220 Bioclimatic variables were obtained for Australia at 30 arc-s resolution using
ANUCLim (Xu & Hutchinson 2013). Since *C. nobilis* is a perennial geophyte, with a
leaf above ground from late autumn to mid-spring, the period of lowest temperatures
and highest rainfall in the SWAFR, we included variables that were likely to affect
225 growth during the winter period (Supplementary Table 1). For the pollinator, we used
variables that are likely to effect the overall habitat (Supplementary Table 1), as
environmental suitability of thynnine wasps is likely to be affected by a large range of
different factors such as the availability of food in the form of nectar plants and the
sugary secretions of insects, and suitable host species for larvae (Ridsdill-Smith 1970;
230 Brown & Phillips 2014). Following an initial run, the constrained distribution model
of the orchid was based on Bio11, Bio16, Bio24 (reduced number of variables based

on those that had predictive power in the initial run), while the constrained distribution model of the orchid was based on Bio01, Bio10, Bio12, Bio28 (Supplementary Table 1). To explore the patterns of extrapolation in the resulting
235 model projection, we measured similarity based on the Mahalanobis distance (Mesgaran *et al.* 2014) using the *ecospat* package (Di Cola *et al.* 2017) in the R statistical environment (R Core Team 2016) to compare the model backgrounds with the projection to the wider project area.

240 **Co-occurring thynnine wasp community**

Following evidence that *C. nobilis* was pollinated primarily by thynnine wasps, surveys were undertaken in 2017 to determine the diversity of the community of thynnine wasps that are active during the flowering period of *C. nobilis*. This
245 approach allows comparison of the total pool of potential pollinators relative to those actually attracted to the orchid. Surveys involved 15 minutes of sweep netting and observations of the thynnines feeding on *Hakea trifurcata*, *Hakea platysperma* and *Hakea circumulata* (Proteaceae), which appeared to be the main nectar source for at least large-bodied thynnids at these study sites. Three surveys were undertaken at
250 Wilcocks 1.1, while two surveys were undertaken at the Wilcocks CG junction (see Table 1). Any species of thynnine wasp not encountered during these surveys but witnessed opportunistically was also collected. Thynnine wasps were identified with the aid of a series of unpublished keys to the Australian thynnine wasp fauna (G. Brown, unpublished data).

255 **Nectar derivatisation and GC-MS analysis**

Given that other species of *Caladenia* previously assumed to be nectarless due to a lack of visible nectar to human observers have been shown by Gas-chromatography
260 based analysis to produce meagre amounts of sucrose on the labellum (Reiter *et al.* 2018, 2019), we tested if *C. nobilis* produces nectar. An attempt to sample nectar from the labellum was made for six individuals in 2017 and four individuals in 2018, all from the Carger Rd site. As a comparison with a known nectar producing *Caladenia* (see Dixon & Tremblay 2009), samples were also collected for six individuals of
265 *Caladenia paludosa*. This species is one of the very few Western Australian

Caladenia known to produce nectar, but interestingly also attracts thynnine wasps via sexual deception (Phillips *et al.* 2017).

270 Attempts to sample nectar were undertaken in sunny conditions in the field, and the
samples transported directly back to a -80°C freezer for storage. Sampling and
analytical procedures followed that of Reiter *et al.* (2018). Briefly, a solution of
Ribitol (an internal standard to allow quantification, 0.2 mg/mL in water) was
prepared and 5µL added to the upper surface of the labellum. The aqueous extract was
275 then collected with a 5µL microcapillary tube and immediately transferred to a 2 mL
GC vial with a 50µL insert for storage. Identification and quantification of sugars
were undertaken using GC-MS, with identifications confirmed by co-injection with
synthetic standards. For specific details of sample preparation and GC methodology,
see Reiter *et al.* (2018). Quantification of the carbohydrates glucose, fructose and
sucrose was achieved by comparison of peak areas of total ion chromatograms (TIC)
280 of nectar samples with the known amount of internal standard, taking response factors
into account.

Colour measurements

285 We tested whether floral parts differ in colour between *C. nobilis* and *Caladenia*
radialis, a co-occurring sexually deceptive species pollinated by *Zeleborea* thynnine
wasps (Phillips *et al.* 2017). *Caladenia radialis* was selected for colour analysis
because it is the only species in the study region that is both sexually deceptive and
allied to the *C. filamentosa* complex to which *C. nobilis* belongs. Floral colour was
290 quantified by spectrophotometry, using an Ocean Optics (Dunedin, FL, USA) USB
4500 spectrometer and 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). For six individuals per species, two measurements were taken of the
lateral sepals, petals and labellum tip. In addition, for *C. radialis* the upper surface of
295 the base of the labellum was measured, as the colour is different to the labellum tip.
The two measurements of each floral part were averaged prior to analysis. Spectral
reflectance curves for each floral part were plotted for comparison across the
hymenopteran visual range (300 and 700 nm, Chittka & Kevan 2005). Measured
spectra were analyzed using the Chittka model to derive colour loci in the bee visual

300 space (Chittka 1992, Chittka & Kevan 2005). While there are no data on the
photoreceptor spectral sensitivity for any species of thynnine wasp, 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 likely to be a
305 reasonable approximation of the colour loci for thynnine wasps.

Pollination success

The removal and deposition of pollen was recorded for all flowering individuals of *C.*
310 *nobilis* at five sites in 2013 and 2014. The number of flowering plants in a population
in a given year ranged between 12 and 117. These data were used to calculate
pollination rate (number of flowers with pollen deposition / total number of flowers)
and pollen transfer efficiency (PTE; number of flowers with pollen deposition /
number of flowers with pollinia removed) (Scopece *et al.* 2010), and their average
315 across the five populations. To quantify the variation in pollination rate and PTE
between years, the average difference between the value in 2013 and 2014 was
calculated across populations. To test if *C. nobilis* has resource limited fruit set, in
2013 ten plants were hand-pollinated at Marchagee and checked for fruit set after the
flowering season.

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RESULTS

Pollinator observations

325 Males of the thynnine wasp *Rhagigaster discrepans* Turner (Thynnidae: Thynninae).
Rhagigaster discrepans where the only insects observed to remove and deposit pollen
of *C. nobilis* across the baiting experiments (Table 2). Furthermore, pollination by this
species was observed at three of the seven study sites (Marchagee, Wilcocks 1.1, CG
junction). Of 15 individuals responding to *C. nobilis*, 13 landed on the flower, six
330 contacted the column, four removed pollinia, and two deposited pollen on the stigma.
In all cases the pollinia were attached to the dorsal side of the thorax. In five instances
the wasp moved between flowers within the artificial clump, resulting in two transfers
of pollen. *Rhagigaster discrepans* tended to approach the flower through what

appeared to be prolonged odour-tracking behaviour. However, rather than the
335 sustained zig-zagging at the height of the flower seen in thynnines responding to
sexually deceptive orchids (Stoutamire 1983), the approach of *R. discrepans* often
involved prolonged circling and investigation of the vegetation approximately a metre
off the ground before descending to the flowers, which would likely have been
obscured from view during the approach flight. Those wasps landing on the flower
340 tended to move onto the upper surface of the labellum, before gradually moving to the
base of the column. Clear attempts to feed from the flower were observed for seven
individuals, typically from the upper surface of the labellum, but in one case from its
margin. When moving between flowers within the clump, wasps usually crawled
between flowers. Unlike thynnines visiting sexually deceptive *Caladenia*, there were
345 no attempts to copulate with the flower.

In addition to *R. discrepans*, a total of 19 visits to *C. nobilis* were recorded from other
insects. The thynnine wasps *Dimorphothynnus simillimus* (six visits) and nr *Zeleboria*
sp. (three visits) were also attracted to *C. nobilis* and attempted to feed from the
350 labellum, but were too small to remove or deposit pollinia. Two species of
unidentified bombyliid fly, a species of *Eufoneus* wasp (Gasteruptidae), a species of
Gasteruption wasp (Gasteruptidae), an unidentified species of scarab beetle, and an
unidentified species of bee were attracted to the flower. Of these, one species of
bombyliid and the *Eufoneus* sp. attempted to feed from the labellum. One individual
355 of the *Eufoneus* sp. moved to the base of the column in the position required for the
removal of pollinia, but was too small to do so (Table 3).

Species distribution models of orchid and pollinator

360 Based on herbarium records for *C. nobilis* ($N = 55$) and museum records for *R.*
discrepans ($N = 18$), the species distribution models for *C. nobilis* and *R. discrepans*
were broadly congruent, with distribution centred along the southern west coast of
Australia (Fig. 2; Supplement 1). For *C. nobilis* the variables with the greatest
contribution to the model were Bio16 – precipitation of the wettest quarter, and Bio11
365 – mean temperature of the coldest quarter. For *R. discrepans* the variables with the
greatest contribution to the model were Bio19 – precipitation of the coldest quarter,
and Bio01 – annual mean temperature.

Co-occurring thynnine wasp community

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In addition to the three species of thynnine wasp that visited flowers of *C. nobilis* (*R. discrepans*, *D. simillimus*, nr *Zeleboria* sp.), eight other species of thynnine wasp were recorded. These species were: *Catocheilus senex*, *Chilothynnus* sp., a member of the *Dimorphothynnus apicalis* complex, *Macrothynnus watherooensis*,
375 *Phymatothynnus* sp., *Pogonothynnus* sp., *Zaspilothynnus lignatus*, and *Zaspilothynnus seductor*.

Nectar derivatisation and GC-MS analysis

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In 2017, none of the six samples from *C. nobilis* contained any nectar. However, in 2018 the four individual flowers tested yielded 2.7, 5.1, 1.3 and 1.4 µg of sucrose from the labellum (mean = 2.6 ± 0.9 µg), with the first of these flowers also having 1.1 µg of fructose and 1.0 µg of glucose. The samples collected from *C. paludosa*, a known nectar producing species, contained on average 51.2 ± 16.6 (S.E.) µg of
385 sucrose, 10.7 ± 5.0 µg of glucose and 10.9 ± 5.2 µg of fructose. A comparison with previously published data on nectar producing *Caladenia* (Table 3) shows that flowers of *C. nobilis* had the least sugar of any nectar-producing *Caladenia* so far studied.

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Colour measurements

While the amount of spectral reflectance varied between floral parts, for most floral parts the reflectance curve showed a plateau from approximately 400nm upwards (Supplementary Fig. 2). The single exception to this was the labellum tip of *C. radialis*, which showed little or no reflection across most wavelengths, but a peak in
395 the red wavelengths at 700nm. Accordingly, in bee visual space all floral parts clustered near each other except for the labellum of *C. radialis*, which was close to the centre of the hexagon (Fig. 3).

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Reproductive success

For *C. nobilis* populations, the average pollination rate across years ranged between 9.4 and 24.7% (Table 4), with an average of $16.0\% \pm 2.5\%$ (S.E.). Pollination occurred at some sites where we did not observe *R. discrepans*, presumably because
405 of low pollinator abundance or response rates meant that the chances of observing pollinator visitation were low. All ten hand-pollinated plants subsequently formed fruit, confirming pollen-limited rather than resource-limited fruit set within a flowering season.

410 At any given site pollination rate was highly variable between years, with the difference for any given site between 2013 and 2014 ranging from 2.3 – 33.3%, with an average difference of $19.2\% \pm 5.3\%$. The average PTE across years for any given population ranged between 0.178 and 0.408, with an average of 0.270 ± 0.039 . At any given site PTE was also highly variable between years, with the difference between
415 2013 and 2014 ranging from 0.13 to 0.48, with an average difference of 0.268 ± 0.080 .

DISCUSSION

420 **Pollination by nectar-seeking thynnine wasps**

Observations of floral visitors to *C. nobilis* revealed that while several species of insect were attracted to the flower, only males of the thynnine wasp *R. discrepans* were of appropriate size and exhibited the necessary behaviour to achieve pollination.
425 In thynnine wasps only the males are volitant, with the winged male carrying the flightless female *in copula* and either feeding her directly or allowing her to forage on a food source (Alcock 1981). Despite the absence of any reward in some flowers of *C. nobilis*, *R. discrepans* regularly attempted to feed from the surface of the labellum. In the first year of the study the flowers of *C. nobilis* no sugars were detected in the
430 nectar analysis of six flowers, indicating a food-deceptive pollination strategy. However, in the second year meagre quantities of sugar were detected in the four flower tested, though only one flower had more than 5 μg of total sugar. While sample sizes will need to be much larger to tease apart patterns of nectar secretion in *C. nobilis*, these combined results suggest that it may largely operate as a deceptive

435 flower, though individual flowers may offer sufficient nectar to aid pollination in
some cases.

The floral form of *C. nobilis* with its long filamentous petals and sepals does not
resemble the flowers of any other genus in the south-western Australian flora,
440 suggesting that nectarless flowers of *C. nobilis* are most likely attracting pollinators
via generalised food deception rather than floral mimicry. The main food plant of the
thynnine wasps in these vegetation communities appear to be several species of
Hakea, all of which are shrubs with comparatively small, cream-coloured flowers.
Similarly, there are no known co-flowering and nectar producing *Caladenia* as
445 potential models. The combination of a lack of attempted copulation, multiple species
of thynnine wasps responding to flowers, and slow response rates to bait flowers,
suggests that sex pheromone mimicry is not being used to attract pollinators (see
Phillips *et al.* 2009b, 2017).

450 Our observations of the pollination of *C. nobilis* suggest that a similar pollination
niche of food seeking male thynnine wasps may be exploited by some species in both
Caladenia subgenus *Phlebochilus* (to which *C. nobilis* belongs) and *Caladenia*
subgenus *Calonema*. In *Caladenia colorata* (*Caladenia* subg. *Calonema*), which
typically has large, creamy flowers (though sometimes with a red tip to the labellum
455 or pink colouration), pollination is by thynnine wasps that forage on small amounts of
nectar produced on the surface of the labellum (Reiter *et al.* 2018). As such, there is
evidence that in *Caladenia* large, cream or white coloured flowers are associated with
pollination systems primarily reliant on nectar-seeking thynnine wasps. Indeed, the
large distance between the column and labellum in many large, white *Caladenia* in
460 subgenus *Calonema* (Hopper & Brown 2001) suggests that in these plants there may
be few species of nectar-seeking insects that are capable of effecting pollination other
than large-bodied Tiphiidae.

Specialisation of the pollination system

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Observations suggest that in our study region *C. nobilis* exhibits high ecological
specialisation (see Ollerton *et al.* 2007; Johnson 2010; Armbruster 2017 for
definitions of specialisation), with pollination achieved by a single species. However,

the attraction of multiple species of insect, including three species of thynnine wasp,
470 suggests that the signals used to attract pollinators in *C. nobilis* are somewhat
generalised and attractive to a range of insect species. As such, when *C. nobilis* occurs
in different habitats it is likely to have the capacity to be pollinated by different
pollinator species depending on the composition of the local thynnine wasp
community. Nonetheless, it is possible that populations of *C. nobilis* could be
475 pollinated at least in part by *R. discrepans* across much of its geographic range. A
species distribution model for *R. discrepans* revealed that its predicted geographic
range overlaps with the majority of the known geographic range of *C. nobilis* (Fig. 2).
Indeed, based on current knowledge of their distribution within the south-west
Australian floristic region, to which *C. nobilis* is restricted, the geographic ranges of
480 the two species are surprisingly well correlated. This finding is comparable to that
seen in some specialised hummingbird pollination systems, where the geographic
ranges of plants almost entirely overlap with the pollinator species, while pollinators
are often more geographically widespread (Abrahamczyk *et al.* 2017). Similarly, in
South African plants with specialised pollination systems, Duffy & Johnson (2017)
485 showed the distribution of the pollinator was an important predictor of the niche of
plants in approximately two-thirds of species.

The highly specialised pollination system seen in *C. nobilis* occurs despite a
moderately diverse community of thynnine wasps occurring at the study sites (12
490 species recorded compared with 28 species in Menz *et al.* 2015). In the case of
Dimorphothynnus sp. and nr *Zeleboria* sp., which also visited the flower, they were
too small to effect pollination – their thorax did not contact the column while they
attempted to feed from the surface of the labellum. However, other processes must
also be contributing to the specialisation at the attraction phase. In addition to the
495 three wasp species visiting *C. nobilis*, there were eight other species of thynnine
wasps in this community that were not attracted to the orchid. There are several
possible explanations that could concurrently contribute towards this result: (i) in this
habitat the large-bodied thynnines, such as members of *Zaspilothynnus*,
Pogonothynnus and *Catocheilus*, only feed higher up in the shrub layer rather than the
500 herb layer with *C. nobilis*; (ii) while comparatively small thynnines may forage on
small nectar sources, these may be ignored by large-bodied thynnines; and (iii)

different genera/species of thynnines may have different dietary preferences and respond to different floral traits when searching for food.

505 **Floral colour and shifts in pollination strategy**

Comparison of the petals and sepals of the *C. nobilis* with the sexually deceptive *C. radialis* revealed that the two species showed a strong similarity of colouration, and similar levels of reflectance. While the labellum of *C. nobilis* was similar in
510 colouration to the petals and sepals, the labellum of *C. radialis* was in the centre of the bee visual space and is likely to be perceived by hymenopteran visitors as almost colourless. These findings suggest that a shift in pollination strategy may be associated with only subtle changes in floral colouration to the primary floral display. Interestingly, the presence of both pollination by food-seeking and sexually deceived
515 thynnine wasps within the *C. filamentosa* complex and allies means that shifts in pollination strategy could arise without a shift in pollinator functional group.

Reproductive success

520 Given that all hand pollinated flowers in *C. nobilis* set seed, the mean pollination rate of *Caladenia nobilis* (16.7%) is comparable to the mean of 20.7% fruit set from averaging across 130 species of deceptive orchid (Tremblay *et al.* 2005). The pollination rate in *C. nobilis* is much lower than the value of 36% fruit set reported for five species of food-deceptive *Caladenia* (Phillips *et al.* 2009b), though comparable
525 to the value of 14% for nine species of sexually deceptive *Caladenia* (Phillips *et al.* 2009b). While the overall pollination rate of *C. nobilis* was similar between years, at any given site the pollination rate showed pronounced yearly variation. To some extent this may reflect yearly variation in pollinator availability through either variation in emergence time or abundance of male wasps. Alternatively, when both
530 orchid population sizes and visitation rates are low, chance effects could be responsible for substantial variation in reproduction between years.

Pollen transfer efficiency (PTE) in *C. nobilis* was comparable to that seen in a range of food-deceptive terrestrial orchids (Scopece *et al.* 2010). Interestingly, PTE was
535 much lower than that seen in rewarding terrestrial orchids (Scopece *et al.* 2010),

sexually deceptive terrestrial orchids (Scopece *et al.* 2010), and in a nectar producing *Caladenia* pollinated by foraging thynnine wasps (Reiter *et al.* 2018). Low PTE in food-deceptive orchids has previously been interpreted as arising through lower pollinator fidelity due to a lack of reward (Scopece *et al.* 2010). The presence of
540 pollination systems based on sexual deception, and food-seeking behaviour, both involving thynnine wasps, suggests that *Caladenia* is a useful genus for understanding the how differences in fitness could contribute to shifts in pollination strategy.

Pollinator behaviour and the potential for self-pollination

545 While large floral displays can lead to the attraction of a greater number of pollinators, a potential negative consequence of achieving this through multiple flowers is an increased frequency of geitonogamous pollination (Klinkhamer & De Jong 1993). We attempted to attract pollinators by baiting with artificial clumps of
550 flowers that replicate the natural clumping habit of many members of the *C. filamentosa* complex. Interestingly, in six of thirteen visits where *R. discrepans* alighted on the flower, the wasp moved to more than one flower in the clump, on two occasions effecting transfer of pollen. Should transfer of pollen with clonal clumps be a regular occurrence, it would suggest that members of this complex are likely to
555 experience a trade-off between the likelihood of attracting pollinators and the frequency of fertilisation of ovules by pollen from the same genet. It is possible that having flowers with no or little nectar may be a mechanism that reduces levels of pollen transfer within clumps, as seen in some experiments with multi-flowered species of terrestrial orchids (Johnson & Scheistl 2016; though see Hobbhahn *et al.*
560 2017). This hypothesis could readily be evaluated through combining nectar addition with pollen staining experiments (Peakall 1989; Johnson & Nilsson 1999).

Conclusions

565 Our observations of pollination of *C. nobilis* by a single species of thynnine wasp add support to the findings that orchids pollinated by food seeking insects can be highly specialised. While part of this specialisation arises through morphological fit, there is evidence in *Caladenia* that some species are particularly attractive to nectar-seeking thynnine wasps, raising the question of which floral signals are involved in the

570 attraction of thynnines. Interestingly, in a proportion of flowers of *C. nobilis* no sugars
were detected, while others produced minute (<5µg) quantities of sugar. From a
theoretical standpoint, there are some possible advantages to offering only meagre
amounts of nectar to pollinators. While only incurring a low cost to the plant, the
nectar may encourage pollinators to linger on the flower or continue to move down
575 the labellum searching for food (as required to achieve pollination), but without
visiting numerous flowers belonging to the same plant or clump. Given the sensitivity
of our GC-based analysis for detecting minute amounts of sugar, it is possible that
widespread application of this technique may reveal that many other species of orchid
assumed to be nectarless, may actually be producing minute amounts of sugar.

580

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825

List of figure captions

Fig. 1. Flowers of *Caladenia nobilis* (top left, bottom left) and the related sexually
deceptive species *Caladenia radialis* (top right, bottom right). In some individuals of
830 *Caladenia nobilis* meagre quantities of nectar are secreted on the upper surface of the
labellum (a modified petal).

Fig. 2. Species distribution models of a) *Caladenia nobilis*, and b) *Rhagigaster
discrepans* at 30 arc-sec resolution. Colours refer to probability of occurrence.

835

Fig. 3. Floral colour of *Caladenia nobilis* (pollinated by food-seeking thynnine wasps;
CN) and *Caladenia radialis* (pollinated by sexually deceived thynnine wasps; CR)

840 plotted in bee visual space. red square = labellum tip of CR; white square = labellum
of CN; black circle = labellum base of CR; black square = petal of CR; grey square =
petal of CN; black triangle = lateral sepal of CR; grey triangle = lateral sepal of CN.

845

Table 1. Study sites used for investigating the pollination of *Caladenia nobilis*. *N*
refers to the number of 6-minute pollinator observation trials undertaken at a given
site. Voucher specimens are lodged in the Western Australian herbarium (PERTH).

850

| Study site | Orchid present | Fitness data | Pollinator baiting (<i>N</i> trials) | Orchid voucher details | Latitude/ longitude |
|--------------|----------------|--------------|--|-----------------------------|-------------------------------------|
| Carger Rd | x | x | 14 | RDP 0268; PERTH 08645574 | 30° 5' 52.7" S 115° 49' 22.8" E |
| WCGJ | | | 28 | | 30° 02' 09.0" S 115° 44' 31.7" E |
| CG W | x | x | 7 | RDP 0270; PERTH 08645590 | 30° 02' 5.5" S 115° 45' 8.2" E |
| CG E | x | x | 14 | RDP 0310; PERTH 08739943 | 30° 02' 5.1" S 115° 45' 41.0" E |
| 7KS | x | | 21 | | 30° 06' 40.1" S 115° 44' 33.2" E |
| Wilcocks 1.1 | | | 61 | | 30° 02' 43.2" S 115° 44' 32.4" E |
| Marchagee | x | x | 43 | RDP 0266; PERTH 08645566 | 30° 06' 43.8" S 115° 53' 51.2" E |
| Boothenderra | x | x | - | RDP 0207; PERTH 08643164 | 30° 15' 21.9" S 115° 39' 41.8" E |

Table 2. A list of visitors recorded to *Caladenia nobilis*, with records of pollen

855 deposition, pollen removal and feeding behaviour. Approach = approached closely to
the flower; Land = landed on the flower; Column = contacted the column; Deposit =
deposited pollen; removal = pollen removed; feed = clear feeding behaviour was
observed.

| family | species | sites | approach | land | column | deposit | removal | feed |
|---------------|-------------------------------------|--|----------|------|--------|---------|---------|------|
| Thynnidae | <i>Rhagigaster discrepans</i> | Wilcocks 1.1; WCGJ; Marchagee | 15 | 13 | 6 | 4 | 2 | 5 |
| Thynnidae | <i>Dimorphothynnus simillis</i> | Wilcocks 1.1; WCGJ | 6 | 5 | 0 | 0 | 0 | 2 |
| Thynnidae | Nr <i>Zeleboria</i> sp. | WCGJ; CG E | 3 | 2 | 0 | 0 | 0 | 1 |
| Gasteruptidae | <i>Eufoneus</i> sp. | CG E; Wilcocks 1.1 | 2 | 2 | 0 | 0 | 0 | 1 |
| Gasteruptidae | <i>Gasteruption</i> sp. | Wilcocks 1.1 | 1 | 1 | 0 | 0 | 0 | 1 |
| Bombyliidae | Unidentified species 1 | 7KS, CGE | 4 | 3 | 0 | 0 | 0 | 3 |
| Bombyliidae | Unidentified species 2 | 7KS | 1 | 1 | 0 | 0 | 0 | 0 |
| Scarabaeidae | Unidentified | 7KS | 1 | 1 | 0 | 0 | 0 | 0 |
| unknown | Unidentified bee | Marchagee | 1 | 0 | 0 | 0 | 0 | 0 |

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865

Table 3. A summary of records of nectar production in *Caladenia* where carbohydrates have been quantified and/or identified. Numbers are mean \pm the standard error.

| Species | Strategy | Nectar quantity | Reference |
|------------------------------|--|--|-----------------------------------|
| <i>Caladenia arenaria</i> | nectar seeking thynnine wasps | 5.3 \pm 2.7 μ g of sucrose | Reiter <i>et al.</i> in press. |
| <i>Caladenia colorata</i> | nectar seeking thynnine wasps | 16.61 \pm 3.94 μ g of sucrose | Reiter <i>et al.</i> (2018) |
| <i>Caladenia concolor</i> | nectar seeking thynnine wasps | 31.5 \pm 12.1 μ g of sucrose | Reiter <i>et al.</i> in press. |
| <i>Caladenia nobilis</i> | nectar seeking thynnine wasps | 2.6 \pm 0.9 μ g of sucrose, 1 flower had 1.1 μ g of fructose and 1.0 μ g of glucose | present study |
| <i>Caladenia paludosa</i> | sexual deception of thynnine wasps | 51.2 \pm 16.6 μ g of sucrose, 10.7 \pm 5.0 μ g of glucose and 10.9 \pm 5.2 μ g of fructose | present study |
| <i>Caladenia rigida</i> | nectar seeking hymenoptera and diptera | Not quantified, primarily sucrose, some glucose, 1 flower had fructose | Faast <i>et al.</i> (2009) |
| <i>Caladenia tentaculata</i> | sexual deception of thynnine wasps | < 0.01 ng | Reiter <i>et al.</i> (2018) |
| <i>Caladenia versicolor</i> | nectar seeking colletid bee | 3.6–4.7 μ g saccharides per flower, with > 95% sucrose | Reiter <i>et al.</i> (2019) |

Table 4. Reproductive success for *Caladenia nobilis*. *N* flowers = number of flowers in the population; PTE = pollen transfer efficiency. Some study site names have been abbreviated: Booth. = Boothenderra; March. = Marchagee; Carger = Carger Rd.

880

| | | Booth. | March. | CG W | CG E | Carger | Mean |
|------|------------------|--------|--------|-------|-------|--------|-------|
| 2013 | <i>N</i> flowers | 13 | 12 | 18 | 16 | 27 | |
| | Pollination (%) | 0 | 16.7 | 11.1 | 6.3 | 14.8 | 9.8 |
| | Removal (%) | 30.8 | 41.7 | 66.7 | 85.7 | 66.7 | 58.3 |
| | PTE | 0 | 0.4 | 0.167 | 0.083 | 0.167 | 0.163 |
| 2014 | <i>N</i> flowers | 15 | 45 | 34 | 24 | 120 | |
| | Pollination (%) | 33.3 | 2.2 | 38.2 | 25.0 | 12.5 | 22.3 |
| | Removal (%) | 93.3 | 28.9 | 58.8 | 50 | 42.4 | 54.7 |
| | PTE | 0.357 | 0.077 | 0.65 | 0.5 | 0.3 | 0.377 |
| Mean | Pollination (%) | 16.7 | 9.4 | 24.7 | 15.6 | 13.7 | 16.0 |
| | Removal (%) | 62.1 | 35.3 | 62.7 | 67.9 | 54.5 | 56.5 |

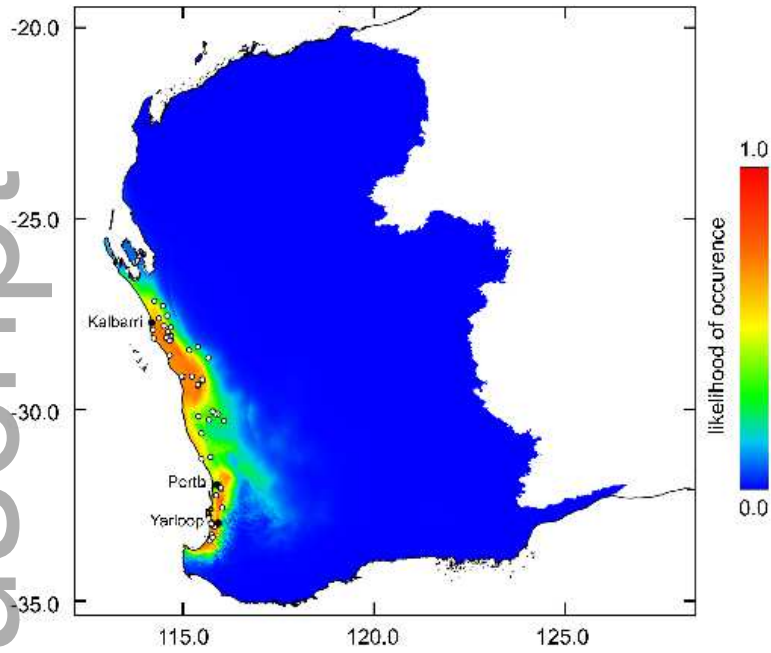
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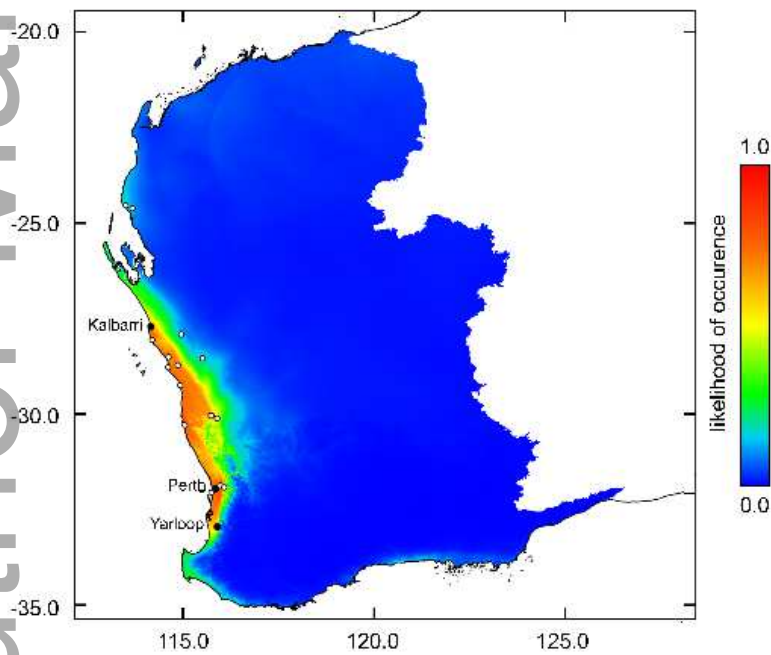
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a) *Caladenia nobilis*



b) *Rhagigaster discrepans*



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