Ecological factors driving pollination success in an orchid that mimics a range of Fabaceae

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Rewarding plants can enhance the pollination success of co-occurring plants pollinated by food mimicry. However, it is not always possible to readily discern between the effect of model and magnet species. Here, we tested for mimicry of co-occurring Fabaceae by the rewardless *Diuris magnifica* (Orchidaceae) and whether the number of flowers of Fabaceae, habitat remnant size and frequency of conspecifics, influenced the pollination success of *D. magnifica*. *Trichocolletes* bees were the primary pollinators of *D. magnifica*, on which they displayed similar behaviour as seen when feeding on Fabaceae. Quantification of spectral reflectance suggested that flowers of *Bossiaea eriocarpa*, *Daviesia divaricata* and *Jacksonia sternbergiana* may represent models for *D. magnifica*, whereas *Hardenbergia comptoniana* strongly differed in colour. Orchid pollination success was not directly affected by the number of model flowers, but the pollination rate was enhanced by increased numbers of *Hardenbergia* flowers. Pollination success of the orchid decreased with higher density of conspecifics, but did not exhibit a significant relationship with *Trichocolletes* occurrence, possibly because of the contribution of sub-optimal pollinator species. Fruit set of the orchid was greater in larger habitat remnants. Overall, pollination success of *D. magnifica* is affected by ecological factors related to the effectiveness of mimicry, numbers of co-flowering plants and anthropogenic landscape alteration.

ADDITIONAL KEYWORDS: Colletidae – *Diuris magnifica* – Faboideae – food deception – habitat fragmentation – mimicry – plant fitness – pollinator behaviour.

INTRODUCTION

Rather than providing a reward to their pollinators, it has been estimated that one-third of orchid species are pollinated via deception (van der Pijl & Dodson, 1966; Renner, 2005). Among orchids a diversity of deceptive strategies is employed including mimicry of floral rewards (Dafni, 1984), females of the pollinator species (Coleman, 1928; Schiestl *et al.*, 1999, 2003; Mant, Peakall & Schiestl, 2005), brood sites (Martos *et al.*, 2015; Suetsugu, 2018) and alarm pheromones (Brodmann *et al.*, 2009). In deceptive orchids, the most common pollination strategy is food deception, in which the rewardless orchid displays floral signals typically associated with rewarding plants (Jersáková, Johnson & Kindlmann, 2006). Pollination by food deception ranges between generalized food deception, in which

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the orchid uses floral signals that are attractive to pollinators but without closely resembling any specific model species, and floral mimicry, in which the orchid mimics one or more species of model food plants (Roy & Widmer, 1999; Johnson, 1994, 2000). Aside from similarity of floral traits to lure pollinators, in floral mimicry systems it is expected that pollinators will exhibit the same foraging behaviour on model and mimic (e.g. Scaccabarozzi *et al.*, 2018; De Jager & Anderson, 2019). Further, floral mimicry is expected to be more effective with a higher ratio of model to mimic flowers (Anderson & Johnson, 2006) through reduced opportunity for pollinator learning (Dafni & Ivri, 1981; Ruxton, Sherratt & Speed, 2004).

Although there is evidence that fitness of mimics is higher when they are scarce relative to the models (Johnson, 1994; Anderson & Johnson, 2006) or when they occur in populations at low density (Ackerman, Meléndez-Ackerman & Salguero-Faria, 1997, Ferdy *et al.*, 1999; Smithson & Gigord, 2001, Pellegrino *et al.*, 2005), other factors can influence pollination success in food deceptive systems. In particular, the presence of rewarding plant species can increase the pollination success of non-rewarding or less rewarding co-flowering plant species in the floral community through increasing the local abundance of pollinators (i.e. the magnet species effect; Thomson, 1978; Feinsinger, 1987; Laverty, 1992). For example, the pollination success of the orchid *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase was enhanced by the presence of the rewarding *Geum rivale* L. (Rosaceae) and *Allium schoenoprasum* L. (Amaryllidaceae) (Johnson *et al.*, 2003). Alternatively, other studies have found evidence for competition, where rewarding neighbouring plants draw pollinators away from plants with no or meagre reward (Free, 1968; Waser, 1983; Lammi & Kuitunen, 1995). In the deceptive *Dactylorhiza sambucina* (L.) Soó, pollination success was reduced by the presence of the rewarding *Muscari neglectum* Ten. (Asparagaceae), which displays dissimilar floral colour and shape (Internicola *et al.*, 2006). As such, deceptive orchids can be subject to both facilitation and competition from co-occurring rewarding plants, and the effect of magnet plants on their pollination success is far from generalizable (Peter & Johnson, 2008).

As one of the leading drivers of biodiversity decline (Pimm & Raven, 2000; Goddard, Andrew & Benton, 2010), habitat fragmentation can reduce abundance of pollinators in small and/or isolated habitat remnants (Cunningham, 2000; Aguilar *et al.*, 2006) and lead to low plant reproductive success via pollination limitation (Nayak & Davidar, 2010; Pauw & Bond, 2011). The impact of habitat fragmentation may often be most severe on plants with specialized pollination systems, as they are vulnerable to loss of just a single

pollinator species. For example, in a guild of orchids pollinated by oil-collecting bees, there has been a gradual extinction of species from urban remnants following decline of the pollinator species, with more clonal species showing greater persistence (Pauw & Bond, 2011; Pauw & Hawkins, 2011). Nonetheless, the ability of some species to persist in the face of pollinator decline highlights that the full effects of habitat fragmentation on many orchid populations are yet to be witnessed (Murren, 2002; Phillips *et al.*, 2015), and that more studies are needed to test the potential effect of habitat fragmentation on plant-pollination interactions, especially in the long-term (Xiao *et al.*, 2016). In particular, there has been relatively little research on the response of orchids pollinated by food mimicry to habitat fragmentation, but given that these orchids are often specialized on few pollinator species (Newman, Anderson & Johnson, 2012; Johnson & Schiestl, 2016), they are expected to be highly vulnerable.

Based on visual similarity in colour and morphology, several species in the Australian orchid genus *Diuris* Sm. are predicted to be engaged in mimicry of a guild of species of Fabaceae (Beardsell *et al.*, 1986; Dafni & Bernhardt, 1990). Although this hypothesis was supported by observations of pollinator sharing between *Diuris* and species of Fabaceae and by similar patterns of UV reflection (Beardsell *et al.*, 1986; Indsto *et al.*, 2006), food mimicry in *Diuris* has only recently been tested in detail. In *Diuris brumalis* D.L.Jones, it was shown that not only do models and mimic show overlap in floral colour and exhibit greater morphological similarity than the remainder of the plant community, but that the bee pollinator exhibits the same stereotyped foraging behaviours on both model and mimic (Scaccabarozzi *et al.*, 2018). In *D. brumalis*, fitness increased with frequency of model plants, although this is likely to be through a facilitation effect as the bee pollinator feeds almost exclusively on *Daviesia* Sm. (Fabaceae) and was scarce at sites where *Daviesia* were rare or absent (Scaccabarozzi *et al.*, 2018; 2020a).

Here we investigate the pollination of *Diuris magnifica* D.L.Jones, a species hypothesized to be a Fabaceae mimic based on similarity in flower shape and colour of co-occurring Fabaceae (Brown, 1991). Based on morphology, *D. magnifica* is believed to be closely allied to *D. brumalis* (Brown *et al.*, 2013), but it grows in areas with several co-flowering species of Fabaceae, and now mostly occurs in habitat remnants within an urban environment. First, we investigated the possibility of floral mimicry in *D. magnifica*, testing the following predictions: (1) *D. magnifica* shares a pollinator with co-occurring

Fabaceae; (2) *D. magnifica* overlaps in floral colour with the putative models; (3) the flowering phenology of the mimic overlaps with the models and (4) the pollinator exhibits with the mimic the foraging behaviour normally associated with the model. Second, we tested whether the pollination success of *D. magnifica*: (1) increases with the frequency of model species; (2) increases with the frequency of non-model food plants; (3) decreases at higher orchid density and (4) decreases in small habitat remnants.

MATERIAL AND METHODS

STUDY SPECIES

Diuris magnifica is endemic to the Swan Coastal Plain in Western Australia, with its main distribution centred on the Perth metropolitan area (Fig. 1; Brown *et al.*, 2013). Flowering occurs from late winter to early spring, with between three and nine yellow-purple flowers per inflorescence (Brown *et al.*, 2013), which are odourless to the human nose. *Diuris magnifica* is a tuberous perennial that is capable of extensive vegetative reproduction through the generation of daughter tubers (Dixon *et al.*, 1989). The primary habitat of *D. magnifica* is mixed *Banksia* L.f., *Allocasuarina* L.A.S.Johnson and *Eucalyptus* L'Her. woodland (Brown *et al.*, 2013), where it co-occurs with a range of co-flowering perennial species of Faboideae that are a conspicuous flowering component of the understory vegetation: *Daviesia divaricata* Benth., *Bossiaea eriocarpa* Benth., *Hardenbergia comptoniana* (Andrews) Benth., *Jacksonia sternbergiana* Benth., *Isotropis cuneifolia* (Sm.) Benth. ex Heynh. (Marshall, 1995) (Fig. 2). Except for *H. comptoniana*, which is violet, all the other species of Fabaceae in this study have broadly similar coloration to *D. magnifica* to the human eye (yellow-orange-purple; Fig. 2). These Fabaceae produce floral nectar (Supporting Information, Appendix S1), whereas *D. magnifica*

Figure 1. Bushland remnants where populations of *Diuris magnifica* were studied in the metropolitan area of Perth, Western Australia. The circles denoting study sites provide an estimate of the relative area of bushland remnants. Remnant sizes are reported in Supporting Information. Table S1B.

Figure 2. A, *Diuris magnifica*. Co-flowering Fabaceae: B, *Daviesia divaricata*; C, *Bossiaea eriocarpa*; D, *Isotropis cuneifolia*; E, *Jacksonia sternbergiana* and F, *Hardenbergia comptoniana*. Scale bar: 5 mm.

does not (Newman *et al.*, 2013). As for the related *D. brumalis*, a vector is required for pollination of *D. magnifica* (Scaccabarozzi *et al.*, 2018) and the flowers are self-compatible (Supporting Information, Appendix S1). Pollen deposition is the primary limitation to reproduction in *D. magnifica*, as most or all flowers on a scape produce fruit when hand pollinated (Newman *et al.*, 2013).

STUDY SITES

We surveyed populations of *D. magnifica* for pollination success during 2015 and 2017, and we carried out pollinator observations in 2015 and 2016. The orchid populations were distributed across ten bushland remnants within the metropolitan area of the city of Perth (Supporting Information, Table S1A, B). *Diuris magnifica* was the only *Diuris* sp. observed in flower at the study sites during the study period. All the study species were vouchered and identified at the Herbarium of Western Australia in Perth (Supporting Information, Table S2).

OBSERVATION OF POLLINATORS ON *DIURIS MAGNIFICA*

To identify the pollinators of *D. magnifica* and quantify their behaviour, observation periods of insects visiting flowers were performed at two sites in remnant A, Koondoola bushland (S 31°50'06.8" E 115°51'83.4") and at one site in remnant E, Kings Park (S 31°57'25.9" E 115°49'89.9") between the 26 August and 28 September 2015 and 24 August and 13 September 2016. These sites were selected as they are two of the largest woodland remnants in the study region and have relatively intact vegetation communities, and *D. magnifica* was common. We conducted 248 15-min observation periods (a total of 3720 min of observation), recording insect behaviour with an EOS M video camera (Canon, Tokyo, Japan) for subsequent examination in slow motion. Observations were conducted between 9.00 a.m. and 5.30 p.m., with air temperatures ranging between 14 °C and 30 °C, as measured with a Smartsensor AR827 set at 20 cm above the ground. Observations were made by using arrays of picked orchid flowers (two inflorescences per vial, each inflorescence with four to six flowers, three

vials per trial, 10–20 cm apart; Scaccabarozzi *et al.*, 2018; 2020b) placed 1–2 m from flowering individuals of *Daviesia divaricata*, *B. eriocarpa*, *J. sternbergiana* and *I. cuneifolia*.

For each insect visiting a flower of *Diuris* and/ or Fabaceae, the behaviour was recorded in eight categories following Scaccabarozzi *et al.*, 2018 (Supporting Information, Table S3). Due to the rapid approaches of pollinators, behaviour was only recorded for the first flower visited, as tracking accurately the subsequent visit was often impossible. Insects bearing the visible white pollinaria of *D. magnifica* (Fig. 3) were collected for identification while visiting *D. magnifica* or foraging on surrounding Fabaceae. All collected specimens were submitted to the Western Australian Museum as voucher specimens (Supporting Information, Table S4).

BEHAVIOURAL COMPARISON OF INSECTS VISITING *DIURIS MAGNIFICA* VS. FABACEAE

Observations of *Trichocolletes* visiting Fabaceae were conducted in Koondola bushland and Kings Park between 26 August and 28 September 2015 and between 24 August and 14 October 2016 from 9.00 a.m. to 5.30 p.m. We estimated the frequency of *Trichocolletes* (number of visits per trial) on the co-flowering Fabaceae to test whether *D. magnifica* share pollinators with co-occurring Fabaceae and make comparisons of the bee behaviour when visiting *D. magnifica* and Fabaceae. These observations were made during 20-min observation periods (between 16 and 19 trials per species of Fabaceae); pollinator visits were recorded with the same video camera as

described above (see Scaccabarozzi *et al.*, 2018 for details). Two of the eight behavioural categories recorded, landing and manipulation (keel-parting behaviour using the fore-middle legs, when attempting to forage for either nectar or pollen), were selected for a formal comparison of pollinator behaviour on the orchid and on Fabaceae. We used a generalized linear model assuming a Bernoulli distribution of the response variable (i.e. presence or absence of a certain behaviour for each insect observed) in R v.3.3.3. The species of Fabaceae was treated as a fixed effect. We tested the difference between *D. magnifica* and co-occurring Fabaceae (*B. eriocarpa*, *Daviesia divaricata*, *H. comptoniana* and *J. sternbergiana*) in the proportion of (1) bees landing on the flower and (2) among landing insects, bees manipulating the tri-lobed labellum (*Diuris*)/ keel (Fabaceae). The latter category included attempts to forage nectar and pollen. Because of the multiple comparisons involving *D. magnifica* and different Fabaceae (four comparisons in total), the threshold for the significance was considered to be 0.0125 through the Bonferroni correction. *Isotropis cuneifolia* (Sm.) Heynh. was not included in the analysis because it was not visited by *Trichocolletes* and *B. eriocarpa* was not included because of the low number of bee observations.

IDENTIFICATION OF POLLEN LOADS OF FLORAL VISITORS

As a complementary approach to resolving the food plants of the floral visitors, and therefore potential model plants, pollen was identified from the bodies of insects caught while visiting *D. magnifica* and
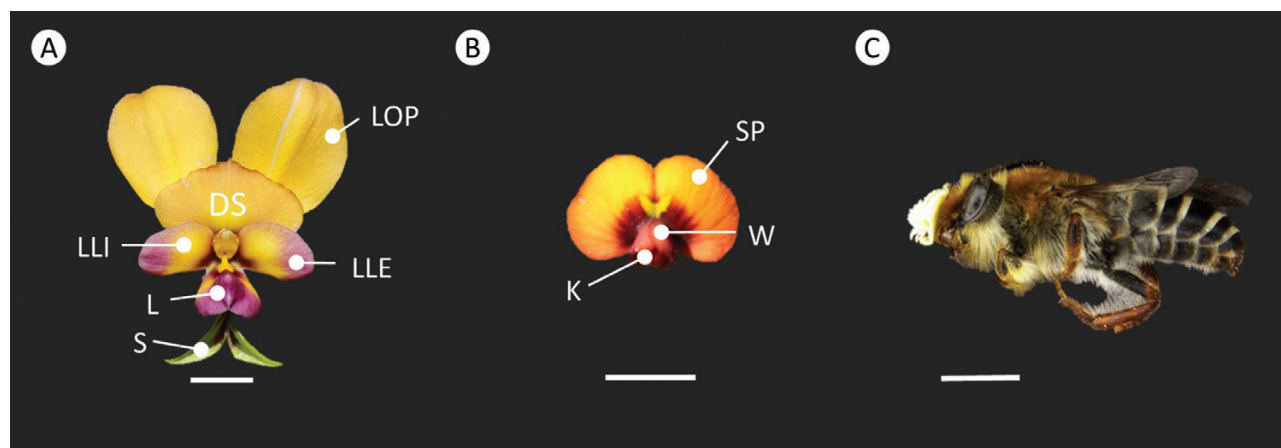
Figure 3. A, Floral morphology of *Diuris magnifica* using the terminology from Hoffman & Brown (2011): LOP (lateral outer petal), DS (dorsal sepal), LLE (external labellum lateral lobe), LLI (internal labellum lateral lobe), L (labellum), S (sepal). B, morphology of a pea flower: SP (standard petal), W (wing), K (keel). C, Male of *Trichocolletes gelasinus* with orchid pollinaria desposited on its head. Scale bar: 5 mm.

Fabaceae. Pollen observed on the tibiae or abdomen of pollinators was removed by washing the insect with distilled water, acetolysed following the methods of Erdtmann (1960) and mounted on glass microscope slides. All pollen samples were identified visually under high magnification (Olympus-BX 51 microscope with Olympus–DP71 camera, Olympus, Tokyo, Japan) by comparison with acetolysed mounted pollen samples from herbarium specimens of *B. eriocarpa*, *Daviesia divaricata*, *H. comptoniana*, *J. sternbergiana* and other commonly co-flowering plant taxa. Pollen identification was carried out for 200 pollen grains per specimen.

SPECTRAL REFLECTANCE OF MIMICS AND PUTATIVE MODELS

To test if bees were likely to be able to distinguish the colour of *D. magnifica* flowers from the co-flowering Fabaceae (*B. eriocarpa*, *Daviesia divaricata*, *H. comptoniana*, *I. cuneifolia* and *J. sternbergiana*), we measured and analysed floral spectral reflectance by using the colour hexagon model, based on the sensitivities of photoreceptors of the bee *Apis mellifera* (Chittka, 1992; Chittka & Kevan, 2005). Additionally, spectral reflectance was also measured for other common rewarding species occurring at all sites, i.e. the yellow-flowered *Hibbertia hypericoides* (DC.) Benth. (Dilleniaceae), *Acacia pulchella* R.Br. (Fabaceae), *Conostylis aculeata* R.Br. (Haemodoraceae) and the pink-flowered *Hypocalymma robustum* Schauer (Myrtaceae). Two flowers per plant from six randomly chosen individuals of each species were selected for measuring spectral reflectance using a spectrometer (Ocean Optics Jaz, DH-2000 UV-VIS-NIR Light source) with an integration time of 50 ms. In *D. magnifica*, spectral reflectance measurements were undertaken from the lateral outer petal (LOP), the centre of the dorsal sepal (DS), the labellum (L), and the internal (LLI) and external (LLE) parts of the lateral labellum lobe. For Fabaceae, the spectral reflectance measurements were taken from the standard (SP) and wing (W) petals (Fig. 3A, B). For the other co-occurring species measurements were taken from the corolla or stamens (for *A. pulchella* only). Distances between colour loci were quantified using Euclidean distance in the colour hexagon model. These values were used as the distance matrix in a PERMANOVA to test if floral parts differed in colour loci (using the vegan package in R v.3.3.3). A Bonferroni correction has been applied because of multiple comparisons and the *P* value threshold was set at 0.001.

FLOWERING PHENOLOGY OF *DIURIS MAGNIFICA* AND FABACEAE

Flowering time was quantified across the study period for *D. magnifica* and the co-occurring Fabaceae. For each species, weekly counts of open flowers were undertaken in 30 × 30 m fixed quadrats at three sites (two in Koondola and one in Kings Park) from 28 June to 18 October 2017. Due to the high number of flowers on individual Fabaceae, every week we scored the total number of flowers per quadrat as binned categories from 1 (100 flowers) to 25 (2500 flowers) by increments of 100 flowers. However, in the case of *D. magnifica*, due to the small number of flowers per inflorescence, we counted the total number of flowers on each plant.

POLLINATION SUCCESS OF *D. MAGNIFICA* IN RELATION TO THE ABUNDANCE OF FABACEAE MODELS

In 2015 and 2017, at the end of the flowering period, the proportion of *D. magnifica* flowers with pollinarium removal and the proportion of flowers forming fruits was quantified at 15 sites (populations) in a single 30 × 30 m quadrat. These 15 sites were split between five large remnants. We focused on these large remnants to minimize the effect of habitat fragmentation when attempting to understand the role of food plants on pollination success of *D. magnifica*. At the flowering peak of *D. magnifica* we recorded: (1) the estimated number of flowers for each species of Fabaceae; and (2) the number of *D. magnifica* plants and flowers. For Fabaceae, variable (1) was estimated by averaging the number of flowers per stem for ten stems and multiplying it by the number of stems (Scaccabarozzi *et al.*, 2018).

We analysed the relationship between the proportion of pollinarium removal and proportion of fruit set with the following independent variables: (1) number of flowers of putative model Fabaceae; (2) number of flowers of the non-model *H. comptoniana* and (3) number of *D. magnifica* plants per quadrat. *Isotropis cuneifolia* was excluded from the quantification of co-flowering plants because it was not visited by the primary pollinators of *D. magnifica*. Pearson correlation confirmed that these variables were not collinear and were therefore included in the same model. Data were analysed using a GLMM (Generalized Linear Mixed effect Model) in R v.3.3.3 through lme4 and nlme packages. The model was a two-way nested GLMM that included identity of the habitat remnant and site as random effects. The response variables (proportion of pollinarium removal and proportion of fruit set) were assumed to be binomially distributed.

In the case of a binomial model the average value of the response variable is equal to

$$e^{(\text{intercept}+\text{BX})}/1 + e^{(\text{intercept}+\text{BX})}.$$

Therefore, the relationship, if significant, is shaped as an exponential. Year was originally included as covariate but, due to a lack of significant effect and

the tendency to increase the AIC, it was removed from the final model. However, the repeated surveys across years (2015 and 2017) were accounted for by having site and remnant as a random effect.

Pearson type residuals were extracted from the model and were tested as a response variable in a generalized additive model to check for any non-linear patterns. When testing the effect of the number of *H. comptoniana* flowers on residuals from the GLMM, proportion fruit set 34.2% of the deviance of the residuals from the model was explained by non-linear patterns of the number of *H. comptoniana* flowers. As such, we repeated the analysis using a GAMM (General Additive Mixed effect Model) in R v.3.3.3 by using the gamm4 package. The GAMM approach provides a better estimate of the trend when the residuals from the linear model show a clear non-linear pattern (Zuur, 2012), as found here. For the GAMM analysis, we considered the same covariates (with the addition of a smooth term for the number of *H. comptoniana* flowers) and random effects. The use of the GAMM rather than GLMM lead to a decrease in the AIC value of > 11 points (from 128.8 to 117.4) suggesting a better fitting model.

POLLINATOR OCCURRENCE, HABITAT REMNANT SIZE AND ORCHID POLLINATION SUCCESS

To test whether the habitat remnant size and the presence of *Trichocolletes* affected the pollination success of *D. magnifica*, in 2017 we quantified plant-pollination success (pollinarium removal, fruit set) for an additional five sites (in addition to the 15 previously studied), located in five small habitat remnants (Supporting Information, Table S1B). We carried out two observation transects (100 m in length) for all 20 sites, from 5 to 15 September 2017, by recording the occurrence of *Trichocolletes* along transects centred on the same quadrats used to quantify pollination success of *D. magnifica*. Each transect took 40 min to complete, with an average of approximately 3 min of observations per flowering plant. Transects were repeated 1 week after the initial survey, following the same route. For the analyses, *Trichocolletes* occurrence was expressed as presence/absence, to reflect that the survey may not have provided accurate quantification of their abundance. Sizes of habitat remnants were taken from those reported in *Bush Forever* (Government of Western Australia, 2000). For both the analysis of pollinaria removal and fruit set, to avoid collinearity separate GLMMs were undertaken for the variables *Trichocolletes* occurrence and remnant size. For both analyses, bushland remnant was treated as a random effect to take into account the multiple sites within the larger remnants.

RESULTS

POLLINATORS OF *DIURIS MAGNIFICA*

In total, 248 insects were observed visiting experimental arrays of *D. magnifica*. Of the total visits, 98 were by *Trichocolletes* spp. (Colletidae, Hymenoptera), 65 by the introduced honey bee *Apis mellifera* (Apidae; Hymenoptera), 19 by *Neophyllotocus* sp. (Scarabeideae; Coleoptera), 11 by Syrphidae (Diptera), 47 by *Pollanisus* sp. (Zygaeinidae; Lepidoptera), seven by *Lassioglossum* sp. (Halictidae; Hymenoptera) and one by *Leioproctus* sp. (Colletidae; Hymenoptera). Only *Trichocolletes* spp., *Apis mellifera* and *Neophyllotocus* sp. were observed removing pollinaria of *D. magnifica*. In each case, pollinaria were attached to the frontal region of the head (Fig. 3C). Twenty-five *Trichocolletes* were observed to remove pollinaria, four were observed to arrive at the flowers already carrying pollinaria and two individuals deposited pollinia on the stigma. On three occasions, *A. mellifera* and *Neophyllotocus* sp. were observed to remove and deposit orchid pollinia on the stigma of the same flower.

During observations, ten *Trichocolletes* and two *A. mellifera* individuals were caught carrying pollinaria of *D. magnifica* while visiting the orchid (Supporting Information, Table S4), and six *Trichocolletes* were observed carrying *Diuris* pollinaria while foraging on either *Daviesia divaricata*, *J. sternbergiana* or *H. comptoniana* (Supporting Information, Video). No other insect species was observed carrying pollen of *D. magnifica* when foraging on other plant species. Fifteen individual insects (nine *Trichocolletes gelasinus*, two *A. mellifera*, two *Neophyllotocus* sp., one *Pollanisus* sp., one Syrphidae) were caught for identification on arrays of orchid flowers and 34 during observations of Fabaceae (Supporting Information, Table S4). The individuals of *Trichocolletes* spp. caught on *D. magnifica* and on Fabaceae, included females (four) and males (six). One *Trichocolletes platyprosopis* was identified in 2015 carrying *Diuris* pollinaria when foraging on *Daviesia divaricata*, whereas nine *T. gelasinus* were identified in 2016 and 2017 on arrays of orchid flowers (Supporting Information, Table S4).

DESCRIPTION OF POLLINATOR BEHAVIOUR

Male and females of *Trichocolletes* spp. visited individual flowers of *D. magnifica* for 1–2 s. Some visits included apparent patrolling behaviour by males inspecting multiple flowers without landing. Of the *Trichocolletes* visiting (*N* = 98) *D. magnifica*, 50 % landed on the flowers, in each case with the body aligned along the centre of the labellum and the head facing towards the column. Of the *Trichocolletes* that

landed (N = 49), 98% attempted to manipulate the labellum, with repeated movements of fore-middle legs, as observed on Fabaceae, facing with the head at the base of the corolla when probing for nectar (Supporting Information, Video; Fig. S1). Due to the short duration of visits, we only recorded the behaviour of *Trichocolletes* that landed for more than 1 s. Of the insects attempting to manipulate the labellum (N = 48), 52 % removed the pollinaria, 4.2% deposited the pollinia on the stigma and 20.8% visited another orchid flower.

Trichocolletes were observed visiting four species of Fabaceae: *Daviesia divaricata* (mean = 3.80 ± 1.34 SD bees per trial), *H. comptoniana* (2.5 ± 2.14 SD), *J. sternbergiana* (1.60 ± 1.54 SD) and *B. eriocarpa* (0.7 ± 0.46 SD). Alternatively, no *Trichocolletes* were observed on *I. cuneifolia* (Fig. 4). The frequency of landing was influenced by the plant species that the bee was visiting (χ^2 = 29.92, P < 0.001). Significantly more visitors landed on *Daviesia divaricata* (N = 88; β = 1.5 ± 0.34; P < 0.001), *J. sternbergiana* (N = 30; β = 1.61 ± 0.53; P = 0.002) and *H. comptoniana* (N = 62; β = 1.23 ± 0.37; P < 0.001) compared with *D. magnifica* (Table 1). The frequency of manipulation of the flower by bees also varied between plant species (χ^2 = 16.75, P = 0.002). However, while the frequency of manipulation differed between species of Fabaceae, there was no significant difference in the frequency of *Trichocolletes* attempting to manipulate the keel when foraging on *D. magnifica* or *H. comptoniana* (N = 38;
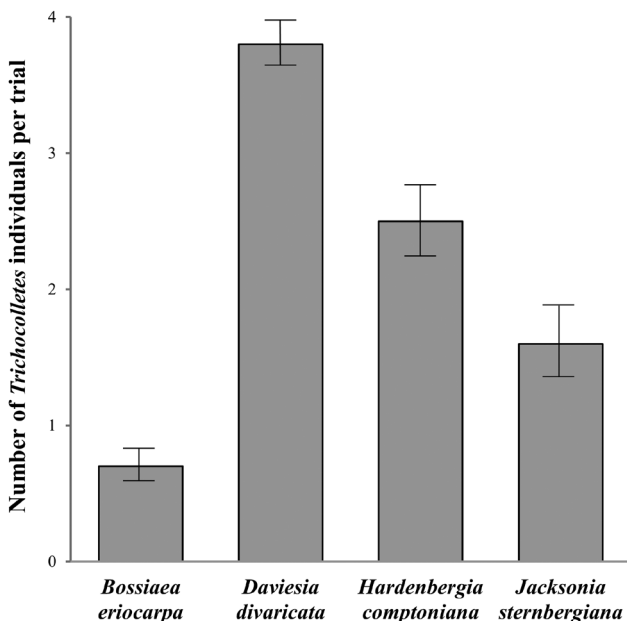
β = −2.54 ± 1.07; P = 0.018), *Daviesia divaricata* (N = 70; β = −0.32 ± 1.24; P = 0.799) and *J. sternbergiana* (N = 24; β = −0.69 ± 1.44 SE; P = 0.629; Table 1).

IDENTIFICATION OF POLLEN LOADS OF FLORAL VISITORS

Analyses of pollen from the body of eight *Trichocolletes* specimens revealed that it was mostly represented (> 80%) by pollen of the same plant species that the insect had been visiting on collection. This observation included specimens collected from *D. magnifica* carrying pollen massulae on the tibiae, *B. eriocarpa*, *Daviesia divaricata*, *H. comptoniana* and *J. sternbergiana*. In six of the specimens, additional pollen coming from co-occurring Fabaceae other than the source plant was found, in percentages varying from 1 to 18.5%. Taxa other than Fabaceae were represented in analysed pollen by percentages ranging from 0.5 to 1.5% and by traces with < 10 pollen grains per examined slide.

SPECTRAL REFLECTANCE OF MIMICS AND PUTATIVE MODELS

The average colour loci of the spectral reflectance of *D. magnifica*, *B. eriocarpa*, *Daviesia divaricata* and *I. cuneifolia* was in the UV region. The average colour loci of *H. comptoniana* corresponded to the UV-blue region (Fig. 5A). *J. sternbergiana* and *H. hypericoides* average colour loci were positioned in the UV-green region, *A. pulchella* and *C. aculeata* were situated in the green region and *H. robustum* was in the blue region. The distances in the bee vision model between mean colour loci for floral parts of *D. magnifica* and yellow-red Fabaceae were: *B. eriocarpa* = 0.07; *Daviesia divaricata* = 0.03; *I. cuneifolia* = 0.10 and *J. sternbergiana* = 0.16 (Supporting Information, Table S4). Colour loci from specific flower parts of *B. eriocarpa*, *Daviesia divaricata* and *J. sternbergiana* spatially overlapped with *D. magnifica* single colour loci (Fig. 5B). In contrast, colour loci of individuals of *I. cuneifolia* did not overlap with the colour loci of *D. magnifica* (Fig. 5B). In the PERMANOVA of the colour loci of individual floral parts, *D. magnifica* showed the most pronounced differences with *I. cuneifolia*. The most similar was *Daviesia divaricata*, followed by *J. sternbergiana* and, last, *B. eriocarpa* (Supporting Information, Table S6).

COMPARISON OF FLOWERING PHENOLOGY OF *DIURIS MAGNIFICA* AND FABACEAE

Diuris magnifica overlaps in flowering period with all the co-occurring Fabaceae (Fig. 6). Species visited by

Figure 4. The average number of *Trichocolletes* individuals per trial (mean ± SE) visiting the species of Fabaceae that co-flower with *Diuris magnifica*.

Table 1. Results of generalized linear models testing for differences in the proportion of floral visitors exhibiting behavioural categories (landing and manipulation) as per Scaccabarozzi *et al.* (2018): comparison between *Diuris magnifica* (DM) and the co-flowering Fabaceae visited by *Trichocolletes*: *Daviesia divaricata* (DV), *Jacksonia sternbergiana* (JS), *Hardenbergia comptoniana* (HC). Landing: alight on the orchid or Fabaceae flower; manipulation: attempt to manipulate the flower during the foraging behaviour for either nectar or pollen. *: indicates a significant difference between the species of Fabaceae and the orchid for a given behavioural category. **: *P* value < 0.0125 (threshold following a Bonferroni correction)

Comparison	Landing		Manipulation	
	$\chi^2 = 29.92$, $P < 0.001$		$\chi^2 = 16.75$, $P = 0.002$	
Diuris magnifica - Daviesia divaricata	DM < DD	$P < 0.001$*	DM = DD	$P = 0.799$
Diuris magnifica - Jacksonia sternbergiana	DM < JS	$P = 0.002$*	DM = JS	$P = 0.629$
Diuris magnifica - Hardenbergia comptoniana	DM < HC	$P < 0.001$*	DM = HC	$P = 0.018$

Trichocolletes spp. showed staggered flowering peaks in the following order: *H. comptoniana* (3 weeks before *D. magnifica* peak), *Daviesia divaricata* (2 weeks before *D. magnifica* peak), *B. eriocarpa* (concurrently with *D. magnifica* peak) and *J. sternbergiana* (1 week later than the *D. magnifica* peak).

ORCHID FITNESS IN RELATION TO ABUNDANCE OF MODELS AND NON-MODELS

Pollination success of *D. magnifica* was generally low across 2015 (average fruit set = 0.032 ± 0.008 SE) and 2017 (average fruit set = 0.034 ± 0.010 SE). The proportion of *D. magnifica* flowers with pollinarium removal showed no significant relationship with the number of flowers of yellow-red Fabaceae (*B. eriocarpa*, *Daviesia divaricata*, *J. sternbergiana*), which were considered as putative food models ($\chi^2 = 0.002$, $P = 0.964$). The proportion of flowers with pollinarium removal showed no significant relationship with the number of flowers of the non-model *H. comptoniana* ($\chi^2 = 0.095$, $P = 0.758$). The output from the GAMM showed a significant non-linear trend for *D. magnifica* fruit set as a function of the number of non-model *H. comptoniana* flowers (smoother term = 2.326; $P = 0.004$; Fig. 7A). The best fitting model was a non-linear curve, although two outlying points likely drove the decrease at high values of model flowers. The fruit set of *D. magnifica* increased until *c.* 700 *H. comptoniana* flowers were present, although there were large confidence intervals above 700 flowers. Finally, pollinarium removal showed no relationship with the number of orchid individuals per quadrat ($\chi^2 = 1.437$, $P = 0.231$), whereas the proportion of fruit set showed a significant negative relation with the number of orchids per quadrat ($\beta = -0.366 \pm 0.128$ SE; $P = 0.004$, Fig. 7B).

ORCHID FITNESS IN RELATION TO POLLINATOR OCCURRENCE AND PATCH SIZE

Neither pollinarium removal ($\chi^2 = 0.21$, $P = 0.647$) nor fruit set ($\chi^2 = 0.143$, $P = 0.705$) exhibited a significant relationship with the presence of *Trichocolletes*. The proportion of fruit set showed a significant positive relationship with bushland remnant size ($\chi^2 = 3.81$, $P = 0.048$; Fig. 8), but there was no significant relationship with the proportion of removed pollinaria ($\chi^2 = 2.88$, $P = 0.09$).

DISCUSSION

POLLINATION AND EVIDENCE FOR MIMICRY IN *DIURIS MAGNIFICA*

We present preliminary evidence of pollination by mimicry in *D. magnifica* by testing some of the fundamental criteria of floral mimicry such as sharing of pollinators, similar pollinator behaviour on model and mimic, overlap of flowering period and colour similarity (Roy & Widmer, 1999; Johnson & Schiestl, 2016). Based on pollinator visitation and observations of wild bees carrying orchid pollinaria, *D. magnifica* appears to be primarily pollinated by the colletid bee *Trichocolletes gelasinus*. Using direct observations and analysis of pollen loads, this bee foraged on the sympatric yellow-red Fabaceae (*B. eriocarpa*, *Daviesia divaricata*, *J. sternbergiana*) and the violet *H. comptoniana*. When foraging, individuals of *Trichocolletes* attempted to manipulate the orchid labellum using the fore and middle legs (Fig. 3C). They exhibited the same keel-parting behaviour (Supporting Information and Video) when foraging both nectar and pollen on Fabaceae (Supporting Information, Fig. S1). As previously found in *D. brumalis* (Scaccabarozzi *et al.*, 2018), this behaviour is a distinctive aspect of mimicry, in which the pollinators exhibit a behaviour with the
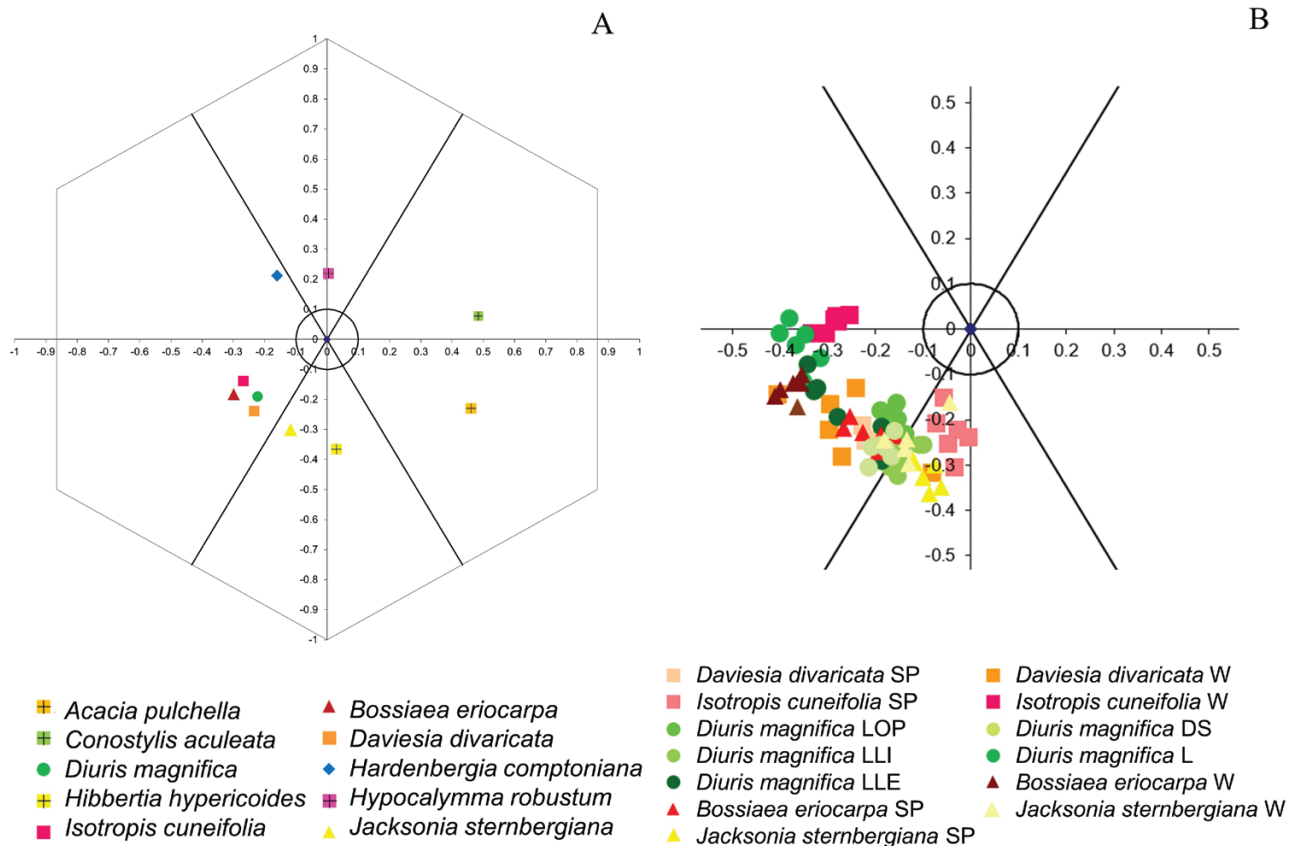
Figure 5. A, Mean colour loci in bee visual space for floral parts of the orchid *Diuris magnifica*, and the co-flowering Fabaceae *Bossiaea eriocarpa*, *Daviesia divaricata*, *Hardenbergia comptoniana*, *Isotropis cuneifolia* and *Jacksonia sternbergiana*. In addition, other yellow-flowered species present at all the sites, *Acacia pulchella* (Fabaceae), *Conostylis aculeata* (Haemodoraceae), *Hibbertia hypericoides* (Dilleniaceae) and a co-occurring pink species, *Hypocalymma robustum* (Myrtaceae) were included. B, Position of colour loci most similar to the colour of *D. magnifica*. Measurements of spectral reflectance were taken for *D. magnifica*: LOP = lateral outer petal; DS = dorsal sepal; LLE = external labellum lateral lobe; LLI = internal labellum lateral lobe; L = labellum; for species of Fabaceae: S*P* = standard petal; W = wing petal. Calculations of colour loci were made using the hexagon colour model of bee vision (Chittka, 1992).

orchid typically associated with Fabaceae. As expected in plant mimicry when pollinators learn to avoid non-rewarding flowers (Kelber, 1996; Simonds & Plowright, 2004), 'landing' was generally more frequent in the model Fabaceae than in the mimic. However, 'patrolling' behaviour of *Trichocolletes* males (see Houston, 2018) was also occasionally observed in proximity to flowers of *D. magnifica*. Given that in *Trichocolletes* this mate-searching behaviour (Barrows, 1976; Paxton, 2005) is usually observed around Fabaceae, patrolling provides further behavioural evidence of effective mimicry of these plants by *D. magnifica*.

As previously found in *Diuris brumalis* (Scaccabarozzi *et al.*, 2018), a species morphologically similar to *D. magnifica*, in the hexagon colour model developed for bee vision (Chittka, 1992; Fig. 5) the floral parts of *D. magnifica* overlapped with the colour loci of three yellow-red Fabaceae that we identified as
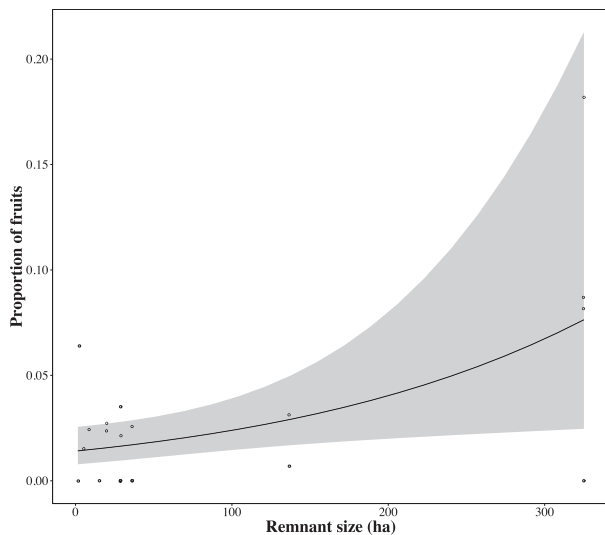
putative models (Chittka, 1992; Fig. 5A). Experiments suggest that bees that have experienced absolute conditioning are unable to distinguish colour distances < 0.06 hexagon units, but with discrimination ability improving with increased colour distances (Dyer, 2006). Whereas some individual orchids overlap with the colour of these Fabaceae, the average colour loci differences between *D. magnifica* and the rewarding *Daviesia divaricata* (0.03), *B. eriocarpa* (0.07) and *J. sternbergiana* (0.16) suggest that it depends on colour variation among individual plants as to how well the pollinators distinguish among them. Among the yellow-orange-red Fabaceae, *I. cuneifolia*, which was not visited by *Trichocolletes*, showed the most pronounced differences in dispersion of colour loci of flower parts in comparison to *D. magnifica* (Supporting Information, Table S2). Although not directly addressed here, it is likely that the plants exhibit some difference
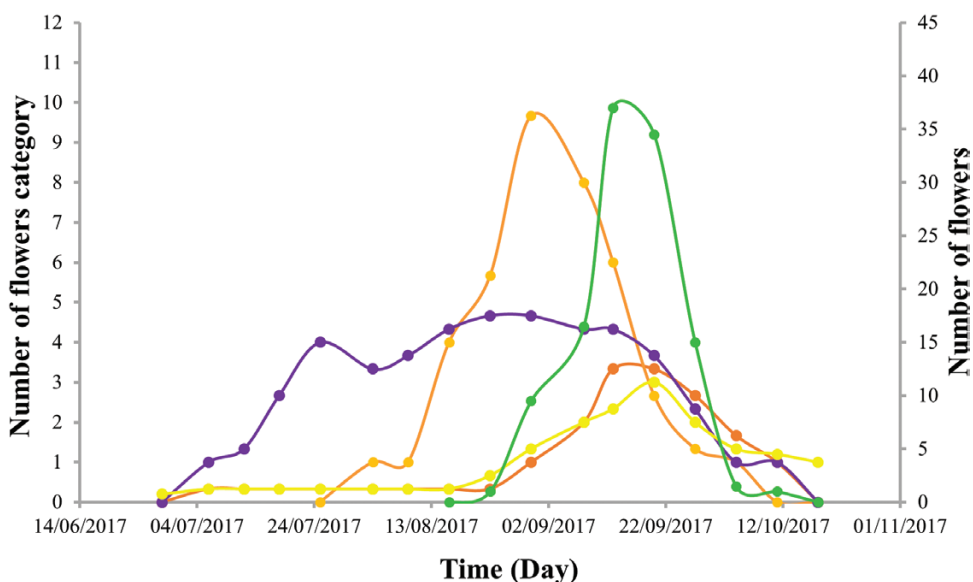
Figure 6. Flowering phenology of *Diuris magnifica* and co-occurring species of Faboideae at three sites. Phenology data were collected in a single 30 × 30 m quadrat per site. Due to the high number of flowers for *Bossiaea eriocarpa* (red line), *Daviesia divaricata* (orange line), *Hardenbergia comptoniana* (violet line) and *Jacksonia sternbergiana* (yellow line), we estimated the total number of flowers and assigned categories (primary *y* axis): (1) 1-100, (2) 101-200, (3) 301-400, (4) 401-500… up to 1100. For *D. magnifica* (green line) the number of flowers per quadrat (secondary *y* axis) was directly scored. The emergence of *Trichocolletes* approximately corresponded to the start of flowering of *H. comptoniana*.
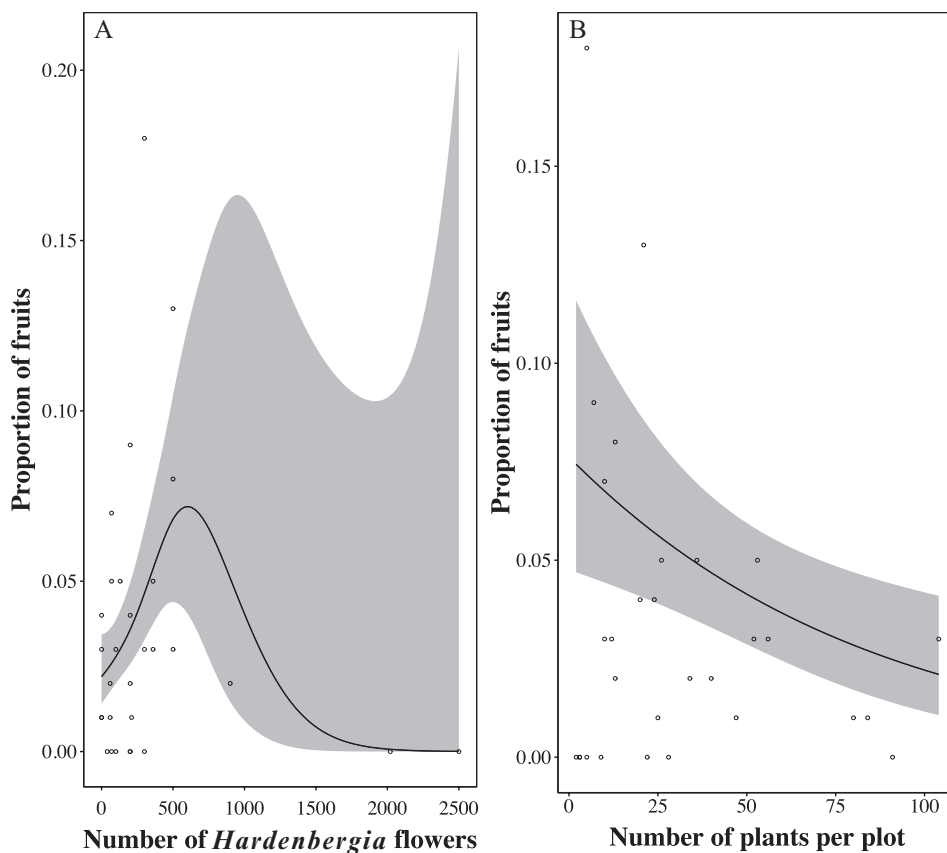
Figure 7. The proportion of flowers setting fruit in *Diuris magnifica* in quadrats in response to: A, number of flowers of *Hardenbergia comptoniana* and B, number of orchid plants per quadrat (square). Plots include data from 2015 and 2017.

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Figure 8. The proportion of flowers setting fruit in *Diuris magnifica* in quadrats in 2017 in relation to bushland remnant size.

in colour pattern, which would enhance the ability of pollinators to distinguish between models and mimics. Further, whereas parts of the flower of *D. magnifica* closely resemble the shape of a flower of Fabaceae (i.e. the labellum, dorsal sepal and lateral labellum lobes), the large LOPs (Fig. 3A) could also potentially enhance the ability for pollinators to distinguish between the orchid and similarly coloured Fabaceae. At present, the role of floral odour has not been investigated in pollinator attraction in *D. magnifica*, but unlike some food deceptive Australian orchids (including some *Diuris* spp.) they seem odourless to the human nose. It would be of interest to test if this trait has arisen as an adaptation to resemble co-occurring Fabaceae, which often have a weak scent, or a strategy to hinder pollinators learning to avoid deceptive flowers (Gumbert & Kunze, 2001).

A POTENTIAL ROLE FOR SECONDARY POLLINATORS?

Although not observed carrying pollen of *D. magnifica* while feeding on co-occurring plants, a few observations of *A. mellifera* and *Neophyllotocus* beetles removing and depositing pollinia on the same flower suggest that other visitors may occasionally contribute to the pollination of *D. magnifica*. The introduced *A. mellifera* forages on an exceptionally wide range of plant species (Paton, 1993) and frequently visited all Fabaceae in this study (Supporting Information, Table S3), so it is not surprising that it also visits *D. magnifica*. *Neophyllotocus* beetles visit several species of brightly coloured understory plants in the study area, both as a food source and as a site to congregate and mate

(Keighery, 1975; Schatral, 1996). For both *A. mellifera* and *Neophyllotocus* beetles, it remains to be confirmed if they are effective pollinators of *D. magnifica*. First, although the landing position of *Trichocolletes* was strictly aligned with the labellum as occurs when foraging on Fabaceae, these other visitors more rarely moved into the correct position for pollinarium removal and deposition. Second, they were only seen removing and depositing pollinia on the same flower meaning that they may contribute towards fruit set via self-pollination. A small number of visits were also observed by two other species of native bee (*Lasioglossum* sp., *Leioproctus* sp.), but given the rarity of these visits, it seems likely that they do not represent important pollinators of *D. magnifica*.

ORCHID POLLINATION AND CO-OCCURRING FABACEAE

In mimicry systems it is expected that the fitness of the mimic should increase relative to the local abundance of the model (Anderson & Johnson, 2006). In *D. magnifica* fruit set declined with higher density of conspecifics, suggesting either pollinator learning or the presence of a limited number of pollinators relative to the number of orchids. However, we found that in *D. magnifica* pollination success was not dependent on the total flower abundance of co-occurring yellow-red Fabaceae, the putative models for this orchid. Although this may in part be due to low pollination rate of the orchid (< 3% fruit set) that increases the chance effect in the data, there are several possible ecological explanations why this expectation was not fulfilled. First, Fabaceae may vary in their importance as model species, but the relatively modest number of study sites compared with the diversity of species of Fabaceae did not allow for testing the effect of a single species of Fabaceae on orchid fitness (as found in Juillet *et al.*, 2007; Peter & Johnson, 2008; Jersáková *et al.*, 2016). Second, multiple ecological factors could be interacting with each other (e.g. density of plants of the model species, habitat fragmentation, pollinator availability and habitat suitability, role of secondary pollinators), making it difficult to tease out trends. Third, foraging behaviour of bees towards Fabaceae and orchids may vary between sites depending on the local relative abundance of species of Fabaceae that vary in their similarity to the orchid mimic. For example, floral constancy (Waser, 1985; Chittka, Thomson & Waser, 1999), whereby pollinators may optimize their foraging activity on a single abundant species of Fabaceae at each site, could lead to changes in the effectiveness of orchid pollination system depending on which species pollinators typically forage on. To unravel the fitness dependence of *D. magnifica* on yellow-red Fabaceae, it would be of interest to investigate with experimental

arrays of orchid flowers the preference of pollinators between each of the putative models and the orchid.

Although the GLMM analysis found no significant relationships with orchid fitness, the GAMM analysis found evidence for a relationship between fruit set of *D. magnifica* and the number of flowers of the non-model *H. comptoniana*. Fruit set of *D. magnifica* initially increased with increasing number of flowers of *H. comptoniana* as expected under a magnet effect (Fig. 7A), where the local aggregation of pollinators on food plants benefit the pollination success of other nearby plants (Thomson, 1981; Laverty, 1992). At larger numbers of *H. comptoniana* flowers the relationship decreased, suggestive of a competition effect (i.e. Lammi & Kuitunen, 1995; Internicola *et al.*, 2006), whereby co-occurring rewarding species may affect negatively the pollination of the orchid. However, as a cautionary note, it must be stressed that the decrease in fitness of *D. magnifica* at higher numbers of rewarding flowers appears to be driven by two outlier sites, where there was one exceptionally large and prominently flowering individual of *H. comptoniana*. Further work is needed to test for a decreasing trend, preferably including experimental manipulation of the availability of *H. comptoniana*. Last, as expected in deceptive systems (Smithson & Gigord, 2001), we found that *D. magnifica* fruit set declined at higher density of conspecific plants (Fig. 7B).

ORCHID POLLINATION AND HABITAT FRAGMENTATION

Given that *Trichocolletes* appears to be the primary pollinator of *D. magnifica*, it was expected that pollination success of *D. magnifica* would be greater in terms of pollinaria removal or fruit set when *Trichocolletes* were present, but we detected no significant difference in pollinarium removal or fruit set for the orchid between sites where *Trichocolletes* were present/absent. However, it should be noted that fruit set was low across all orchid populations (< 3% in any given year). Although *Trichocolletes* may have remained undetected at some sites in our survey, it is possible that sub-optimal pollinators such as *A. mellifera* and *Neophyllotocus* beetles may be contributing to the pollination success of the orchid sufficiently to obscure any difference in pollination success between sites with and without *Trichocolletes*. However, due to the self-pollination observed when *A. mellifera* and *Neophyllotocus* beetles acted as pollinators, the fitness of seeds originating from pollination events by *Trichocolletes* versus those from alternative pollinators should be investigated.

As predicted, the size of bushland remnants was positively related to fruit set of *D. magnifica* (Fig. 8), in accordance with previous research in which habitat fragmentation causes lower fruit set through pollen limitation (Cunningham, 2000; Aguilar *et al.*, 2006). However, it should be noted that the sites with high fruit set were mostly in Kings Park, the largest of the remnants. Lower fruit set in small remnants could be because habitat is less suitable for pollinators, or the remnants are too small to support viable populations of *Trichocolletes*, a remnant dependent genus of bee. The proportion of flowers with pollinaria removal did not exhibit a significant relationship with any of the tested variables, although it is expected that pollinarium removal will be more affected by sub-optimal pollinators removing but not transferring pollinaria. From a conservation perspective, understanding whether secondary pollinators such as *A. mellifera* and *Neophyllotocus* are capable of maintaining populations of the orchid, or whether their visits mostly lead to self-pollination, could be fundamental for predicting the fate of *D. magnifica* in small habitat remnants.

IMPERFECT MIMICRY BY ADVERTISING A 'GENERAL PEA FLOWER IMAGE'?

There are multiple ecological interactions that potentially drive the effectiveness of mimicry and levels of pollination success in *D. magnifica*. However, based on shared pollinator behaviour between orchid and Fabaceae, and similarity of some key floral traits, this pollination system appears to involve the imperfect mimicry of several species of yellow-red Fabaceae (*B. eriocarpa*, *Daviesia divaricata*, *J. sternbergiana*; e.g. Sherratt, 2002; Gilbert, 2005). This appears to be a similar pollination system to *D. brumalis*, in which Scaccarbarozzi *et al.* (2018) showed that different *Daviesia* spp. acted as models depending on habitat, and the orchid co-flowers with other genera of yellow-red Fabaceae elsewhere in its geographical range. While in *D. brumalis* the models were *Daviesia* spp. that were similar in morphological traits and spectra reflectance (Scaccabarozzi *et al.*, 2018), in *D. magnifica* the mimicry encompasses a range of genera of Fabaceae that show variation in colour reflectance (Supporting Information, Table S6). However, *D. magnifica*, which flowers at the peak of spring rather than in late winter as *D. brumalis*, is likely to use a large range of model species at any given site. In *D. magnifica* there is also evidence for facilitation by *H. comptoniana*, a potential non-model Fabaceae that is also foraged by the primary pollinator species.

Should *D. magnifica* be using a pollination system based on multiple models, the orchid may benefit from pollinators having a 'general search image' (Goulson, 2000; Johnson & Schiestl, 2016) that encompasses all of the co-occurring Fabaceae of similar coloration. *Diuris magnifica* starts flowering later in the season than any of the putative model species and after the emergence

of the primary pollinator species. The latter were already observed at the study sites in the first week of July, 4 weeks prior to the start of flowering in *D. magnifica*, suggesting that the pollinators could be already familiar with a 'pea flower image' when *D. magnifica* begins flowering. This scenario differs from what is expected in generalized food deception systems, where the orchid is partly reliant on the exploitation of perception biases of naïve, recently emerged pollinators (Schaefer & Ruxton, 2009). However, it remains to be tested whether there is a specific model Fabaceae that is more important for supporting populations of the pollinator or for increasing the pollination success of *D. magnifica*.

The use of imperfect mimicry of multiple models makes *Diuris* a potentially powerful study system from both theoretical and conservation perspectives. Given the diversity of floral forms in the genus, *Diuris* may offer novel insights on the evolutionary transitions in pollination strategies, allowing tests of whether food mimicry has evolved from generalized food deception, as suggested for other orchid lineages (Cozzolino *et al.*, 2005). Further, the apparent variation between *Diuris* spp. and populations in the number of model species suggests that *Diuris* may be useful for the identification of those selective pressures that drive the evolution of more specialized forms of floral mimicry. From a conservation standpoint, it would be of interest to test if these species are resilient through the exploitation of multiple models or vulnerable due to their reliance on a small number of pollinator species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Floral biology of *Diuris magnifica* and co-occurring Faboideae.
Table S1. Sites (A) and pollination data for *D. magnifica* (B).
Table S2. Plant species vouchered at the Western Australian Herbarium.
Table S3. Observations of floral visitors to *D. magnifica* and co-occurring Faboideae.
Table S4. Insects caught visiting the flowers of *D. magnifica*.
Table S5. Means and standard deviation of colour loci of *D. magnifica* and co-occurring plants.

Table S6. PERMANOVA analysis of distance between colour loci of *D. magnifica* and co-occurring plants.

Figure S1. Foraging activity of *Trichocolletes gelasinus* on different species of Fabaceae.

Video: *Trichocolletes* behaviour on *Diuris magnifica* and *Daviesia divaricata.* Key behaviours illustrated: keel (model) or labellum (mimic) 'manipulation' using foreleg and middle leg.