

## Masquerading as pea plants: behavioural and morphological evidence for mimicry of multiple models in an Australian orchid

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- **Background and Aims** While there is increasing recognition of Batesian floral mimicry in plants, there are few confirmed cases where mimicry involves more than one model species. Here, we test for pollination by mimicry in *Diuris* (Orchidaceae), a genus hypothesized to attract pollinators via mimicry of a range of co-occurring pea plants (Faboideae).
- **Methods** Observations of pollinator behaviour were made for *Diuris brumalis* using arrays of orchid flowers. An analysis of floral traits in the co-flowering community and spectral reflectance measurements were undertaken to test if *Di. brumalis* and the pea plants showed strong similarity and were likely to be perceived as the same by bees. Pollen removal and fruit-set were recorded at 18 sites over two years to test if fitness of *Di. brumalis* increased with the abundance of the model species.
- **Key Results** *Diuris brumalis* shares the pollinator species *Trichocolletes capillosus* and *T. leucogenys* (Hymenoptera: Colletidae) with co-flowering Faboideae from the genus *Daviesia*. On *Di. brumalis*, *Trichocolletes* exhibited the same stereotyped food-foraging and mate-patrolling behaviour that they exhibit on *Daviesia*. *Diuris* and pea plants showed strong morphological similarity compared to the co-flowering plant community, while the spectral reflectance of *Diuris* was similar to that of *Daviesia* spp. Fruit-set and pollen removal of *Di. brumalis* was highest at sites with a greater number of *Daviesia* flowers.
- **Conclusions** *Diuris brumalis* is pollinated by mimicry of co-occurring congeneric Faboideae species. Evidence for mimicry of multiple models, all of which share pollinator species, suggests that this may represent a guild mimicry system. Interestingly, *Di. brumalis* belongs to a complex of species with similar floral traits, suggesting that this represents a useful system for investigating speciation in lineages that employ mimicry of food plants.

**Keywords:** *Diuris brumalis*, *Daviesia*, Faboideae, Colletidae, mimicry, food deception specialization, pollination, pollinator behaviour, plant fitness.

### INTRODUCTION

Batesian mimicry represents an interaction between model, mimic and operator (the signal receiver), in which the operator mistakes the mimic for the model leading to a fitness benefit for the mimic (Vane-Wright, 1980). Mimicry can be achieved through a diversity of cues, including visual, acoustic, chemical, tactile and possibly electrical (Stoddard, 1999; Norman *et al.*, 2001; Barbero *et al.*, 2009; Schiestl and Johnson, 2013; Bohman *et al.*, 2018). Despite mimicry in animals being well supported in multiple systems, the phenomenon remained rarely confirmed and largely controversial in plants (Ruxton *et al.*, 2004). Only in the last three decades has evidence been presented suggesting that floral mimicry may be widespread in some plant families (Johnson and Schiestl, 2016).

Orchids (Orchidaceae) are an unusual group among flowering plants in that approximately one-third of known species (6500–9000 species) are believed to attract their pollinators via deception (Dafni, 1984; Ackerman, 1986). Non-rewarding orchids exhibit a range of mechanisms to attract pollinators, including sexual deception (Coleman, 1928; Schiestl *et al.*, 1999, 2003), brood site mimicry (Van der Niet *et al.*, 2011; Martos *et al.*, 2015) and alarm pheromone imitation (Brodmann *et al.*, 2009). However, the majority of deceptive orchids attract pollinators by falsely advertising floral rewards to pollinators (Ackerman, 1986), using traits such as inflorescence shape and architecture, flower colour and brightness, scent, nectar guides and pollen marks (Kunze and Gumbert, 2001; Galizia *et al.*, 2005; Jersáková *et al.*, 2012). The most common form of food deception is generalized food deception, where food-seeking

animals are attracted by general floral signals rather than the traits of any particular rewarding species (Van der Cingel, 1995; Jersáková *et al.*, 2006). Alternatively, deceptive orchids that exhibit similar floral traits to those of a particular rewarding flower are predicted to be using Batesian mimicry to attract pollinators (Jersáková *et al.*, 2006), where the mimic receives a benefit from co-flowering plant species through increased reproductive success (Jersáková *et al.*, 2006).

The most comprehensive evidence to date for Batesian mimicry in orchids comes from research undertaken on the South African flora. For example, Peter and Johnson (2008) employed UV-manipulation experiments to show that *Eulophia zeyheriana* mimics the floral colour of nectar-rewarding *Wahlenbergia cuspidata* (Campanulaceae) to attract *Lipotriches* (Halictidae) bees. Similarly, Jersáková *et al.* (2012) demonstrated that *Disa pulchra* attracts long-proboscid tabanid flies by mimicking the rewarding iris *Watsonia lepida* through closely matching the floral reflectance spectra of the species. In these cases, and most others where floral Batesian mimicry has been hypothesized, in any given population there is evidence for mimicry of a single model species (Dafni *et al.*, 1981; Nilsson, 1983; Johnson, 2000; Benitez-Vieyra *et al.*, 2007). However, in orchids there is some evidence for guild mimicry, where a rewardless species mimics a range of model species that have similar floral traits and share the same pollinator species (Brown and Brown, 1979; Dafni and Bernhardt, 1990; Johnson and Schiestl, 2016). For example, the European orchid *Traunsteinera globosa* attracts pollinators by mimicking the colour and inflorescence shape of representatives of three morphologically similar co-occurring genera in the Dipsacaceae and Caprifoliaceae (Juillet *et al.*, 2007; Jersáková *et al.*, 2016). This strategy may be advantageous over other more specialized forms of Batesian mimicry as the mimic may receive a fitness benefit from co-flowering with a wider range of model plants.

The Australian orchid genus *Diuris* has been long hypothesized to engage in guild mimicry (Dafni and Bernhardt, 1990). Some clades of *Diuris* are superficially similar in both colour and shape to those of a guild of yellow and brown pea plants (Faboideae). Floral mimicry of Faboideae was first tested in the eastern Australian species *Diuris maculata* (Beardsell *et al.*, 1986; Indsto *et al.*, 2006), where it was shown that *Diuris* and some Faboideae share pollinators and have similar floral coloration according to a bee visual model. While *Diuris* encompasses a range of floral shapes and colorations, this yellow and brown Faboideae-like flower type has evolved at least twice within the genus (Indsto *et al.*, 2009), suggesting that these traits could be adaptations to the mimicry of Faboideae (see argument of Johnson *et al.*, 2003). However, to determine if this is truly a pollination strategy based on mimicry, or convergent evolution of floral signals that are attractive to bee pollinators, requires comparison with the floral traits of the broader plant community (de Jager *et al.*, 2016), observations of pollinator behaviour on model and mimic, and data on reproductive success of the orchid in relation to the abundance of the model (Roy and Widmer, 1999; Peter and Johnson, 2008). Furthermore, a compelling line of evidence for the existence of mimicry would be if the pollinator is deceived into engaging in the same specific behaviours with the putative mimic that it typically exhibits only with the model species. Such behavioural evidence confirms that the orchid is functioning as a mimic, regardless of whether

some of its floral traits originally evolved through selection for mimicry, or independently to exploit the foraging behaviour of the bee.

We tested the mimicry hypothesis in *Diuris brumalis*, an orchid species that co-occurs with a range of Faboideae species that exhibit similar yellow–brown colour patterns. Having identified candidate model species based on the diet of the bee species involved in pollination of *Di. brumalis*, we tested the following predictions: (1) that the colour and morphology overlap between models and mimic, but not with the remainder of the floral community; (2) that the flowering phenology of the proposed mimic overlaps with the models; (3) that the pollinator exhibits with the mimic a deceived behaviour normally only associated with the model; (4) that the fitness of the mimic is greater in the presence of the models; and (5) that the fitness of the mimic increases with the number of flowers of the model species. Furthermore, to investigate if this mimicry system operates with more than one model species, observations of pollinator behaviour were undertaken in habitats that differed in the pea plant species present.

## MATERIAL AND METHODS

### Study species

*Diuris* is a primarily Australian genus comprising approx. 100 species, with centres of diversity in south-western and south-eastern Australia (Jones, 2006). *Diuris* are terrestrial geophytes, with a solitary scape produced per plant in any given year (Jones, 2006). Most species of *Diuris* appear to be capable of clonal reproduction through vegetative multiplication of daughter tubers (Dixon *et al.*, 1989). *Diuris brumalis* produces yellow–brown nectarless flowers during July and August, with between three and 15 flowers per inflorescence (Brown *et al.*, 2013). A vector is required for pollination, and the flowers are self-compatible (Supplementary Data Appendix S1). Pollination within a given flowering season is primarily pollen-limited, with most or all flowers on a scape forming fruit after pollination by hand (Elliott and Ladd, 2002; Appendix S1). *Diuris brumalis* occurs in a range of habitats, which differ in their community of winter-flowering Faboideae species. Unlike *Di. brumalis*, these Faboideae produce floral nectar (Appendix S1).

### Study sites

Data were collected from *Di. brumalis* populations in the Darling Range, near Perth, Western Australia, during 2016 and 2017 (Fig. 1). The populations were selected across two different habitat types (Fig. 1; Table S1): Jarrah forest (hereafter referred to as ‘forest’; 15 sites) and heathland surrounding granite outcrops (hereafter ‘outcrop’; three sites). No other species of *Diuris* was observed flowering at any site during the study period.

*Diuris brumalis* frequently co-occurs with several species of Faboideae (Fabaceae; Marshall, 1995). Six species of flowering Faboideae, commonly referred to as pea plants, were identified at the study sites (Fig. 2A–F), namely *Daviesia decurrens*, *Da. horrida*, *Da. rhombifolia*, *Hovea chorizemifolia*, *H. pungens*

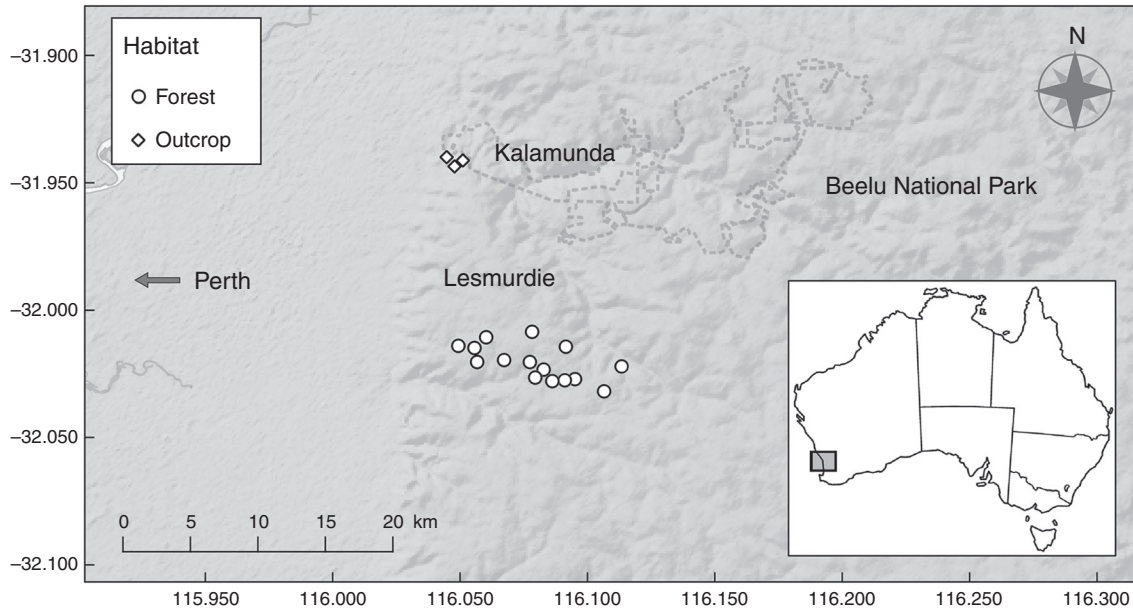


FIG. 1. Distribution of the 18 *Diuris brumalis* study sites in the Darling Range, Western Australia. Fifteen sites were in Jarrah forest and three in granite outcrops.

and *Bossiaea aquifolium* (Table S1). While *Da. decurrens*, *Da. rhombifolia*, *H. chorizemifolia*, *H. pungens* and *B. aquifolium* were present at forest sites and only *Da. horrida* and *H. pungens* were present at outcrop sites. Voucher specimens of all studied species were collected and accessioned at Herbarium of Western Australia in Perth (Table S2).

#### Observation of pollinators on *Diuris brumalis*

To identify the pollinators of *Di. brumalis* and quantify their behaviour, observations of pollinator visitation to orchid flowers were undertaken at three sites (F1, F2 and O3) between 13 July to 15 August 2016 and 12 July to 13 August 2017. A total of 191, 15-min observation periods were conducted (for a total of 2865 min of observation), with insect behaviour recorded using an EOS M video camera (Canon, Tokyo, Japan) for subsequent viewing in slow motion. Observations were conducted between 1000 and 1530 h when temperatures were above 17 °C (temperature ranged between 8 and 19 °C, as measured with a Smartsensor AR827, set 20 cm above the ground). Arrays of orchid flowers were designed to replicate the colony-forming habit of *Di. brumalis* and comprised multiple inflorescences that had been cut and placed in glass vials (two inflorescences per vial, each with 4–6 flowers). For each observation period, three vials were spaced 10–20 cm apart to create a conspicuous floral display, with vials placed 1–2 m from flowering individuals of *Da. decurrens*, *Da. rhombifolia* or *Da. horrida*. While artificial arrays of flowers were used as the basis for pollinator observations, naturally occurring *Di. brumalis* were common at each of these three sites where observations were undertaken.

For each individual insect visiting a flower of *Diuris* and pea plants, the behaviour was recorded and categorized as follows: (1) the insect approached the flower, (2) the type of behaviour exhibited upon approaching the flower: zig-zag flight = moving side to side in flight as they approached the flowering plant; direct flight = flying in a straight line as they approached the flower;

aligned = body of visitor aligned along the midpoint of the labellum/keel during attempts to forage; patrolling = appearing to inspect multiple flowers around the plant; searching = the bee approaches a flower closely (<5 cm) but then chooses to alight on a different flower, (3) the insect was carrying pollinia of *Di. brumalis*, (4) if the insect landed on the flower, (5) the length of time spent on the flower (if >1 s), (6) if the insect attempted to forage on the flower, either attempting to manipulate the labellum/keel by opening the wings, or attempting to feed on nectar (Fig. 2G), (7) if the insect removed or deposited pollen of *Di. brumalis* or of pea plants (based on filament contact with the insect) and (8) if the insect visited additional *Di. brumalis* or pea plant flowers (Tables S3, S4). Behaviour was only recorded for the first flower visited, as due to the very rapid movement of pollinators, it was often impossible to accurately quantify visits to subsequent flowers.

To determine whether pollinator behaviour differs in response to flowers of *Diuris* compared with *Da. decurrens* and *Da. rhombifolia*, we compared the proportion of floral visitors exhibiting food-foraging behaviours between pea plants and *Diuris* using a Generalized Linear Model with a Bernoulli distribution of the response variable. Plant species was the fixed effect and was treated as a categorical variable. Specifically, we tested (1) if between *Diuris* and pea plants (*Da. decurrens* and *Da. rhombifolia* in the forest habitat) there is a difference in the proportion of bees landing on the flower, and (2) if among the bees landing on the flower, there a difference in the proportion of bees that exhibit foraging behaviour, either by manipulating the labellum/keel or attempting to forage on nectar.

#### Observation of pollinators on co-flowering plants

To determine if *Di. brumalis* shares pollinators with co-flowering pea plants, pollinator observations were undertaken at two forest sites (F1, F2) and one outcrop site from 13 July to 6 September in 2016 and from 11 July to 9 September 2017.



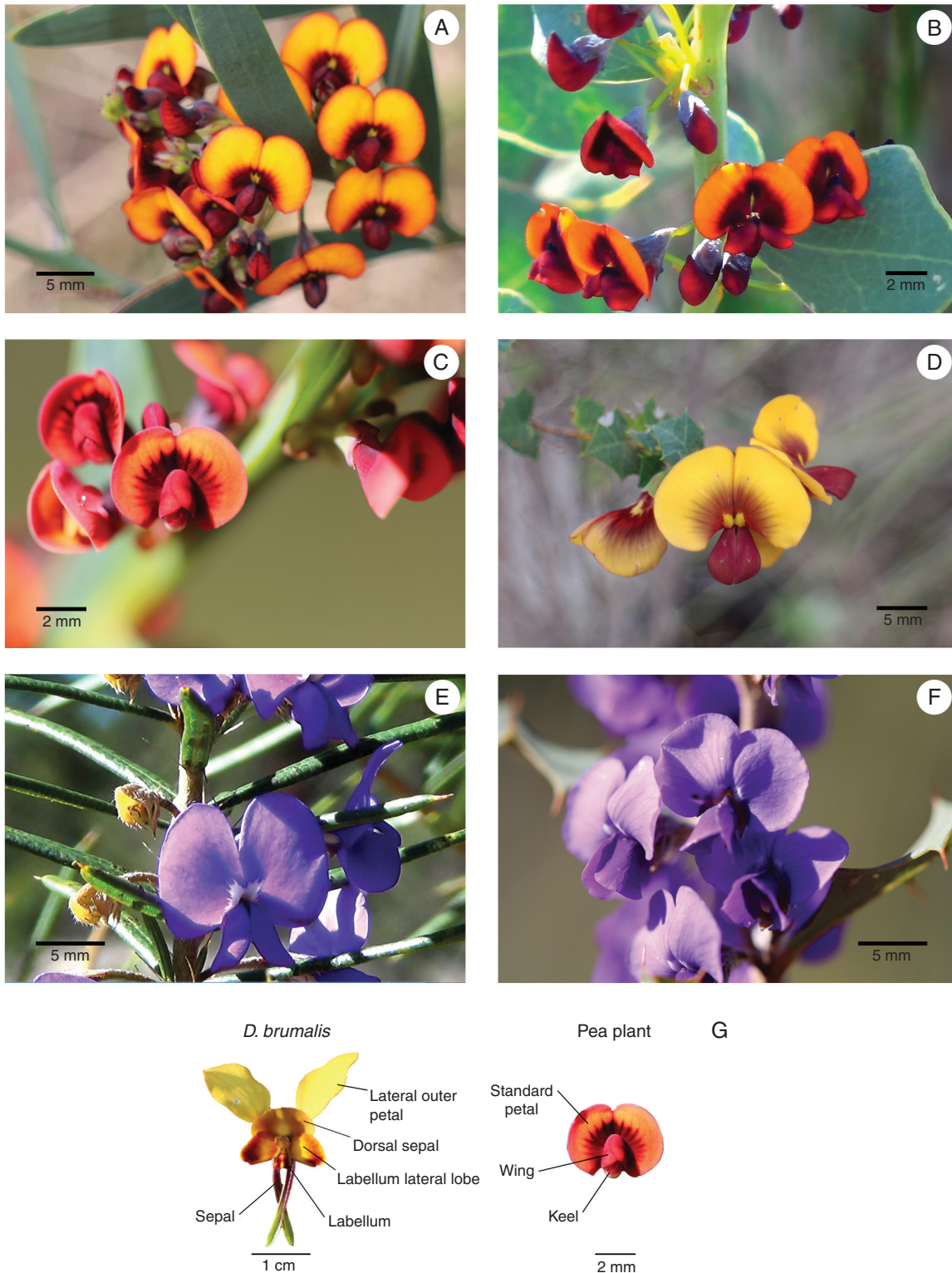


FIG. 2. Faboideae co-occurring with *Diuris brumalis*: A, *Daviesia horrida*; B, *Da. rhombifolia*; C, *Da. decurrens*; D, *Bossiaea aquifolium*; E, *Hovea pungens*; F, *Hovea chorizemifolia*; G, pea-like floral morphology of *Di. brumalis* formed by two lateral petals, the dorsal sepal, labellum lateral lobe, labellum and two basal sepals (Hoffman and Brown, 2011).

Observations were made between 1100 and 1500 h daily, with the same video camera set up as described above. A total of 32 observation periods were undertaken for *B. aquifolium*, *Da.*

*decurrens*, *Da. horrida*, *Da. rhombifolia*, *H. chorizemifolia* and *H. pungens* individuals, each of 15 min, yielding a total of 480 min of observation for each plant species. The pollinator

behaviours recorded corresponded to those used for visitors to the *Diuris* (see above), to enable a formal behavioural comparison. To test if bees that visited *Di. brumalis* also visited members of the plant community other than pea plants, additional 10-min observation periods were undertaken for other dominant co-flowering species: *Acacia pulchella*, *Adenanthos barbiger*, *Calothamnus sanguineus*, *Hakea lissocarpha*, *Hibbertia hypericoides* and *Hypocalymna robustum* (from four to five observation periods per species, for a total of 270 min).

#### Identification of pollinators

Pollinators observed on *Di. brumalis* and pea plant flowers (particularly individuals carrying the distinctive white pollinia of *Di. brumalis*) were collected for identification. All collected insect pollinators were sent to the Western Australian Museum as voucher specimens (Table S5). Native bee pollinators observed were sexed and identified according to [Batley and Houston \(2012\)](#) based on behavioural (patrolling – males; collecting pollen on the abdomen – females) and morphological features including differences in antennae length (generally longer in males), body size (larger in females), abdomen width (larger in females) and number of hairs on the head (more abundant on males).

#### Quantification of pollen loads of floral visitors

As a complementary approach to resolving the food plants of the floral visitors, pollen was identified from the bodies of bees caught (Table S5) visiting *Di. brumalis* and pea plants. Pollen observed on the tibiae or abdomen of pollinators during identification was removed by washing the insect with distilled water, acetolysed following the methods of [Erdtman \(1960\)](#) and mounted on glass microscope slides. All pollen samples were identified 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 *Di. brumalis*, *B. aquifolium*, *Da. decurrens*, *Da. horrida*, *Da. rhombifolia*, *H. chorizemifolia*, *H. pungens* and other commonly co-flowering plant taxa.

#### Morphological evaluation of floral traits and spectral reflectance

To test if *Di. brumalis* shows greater overlap in floral morphology with the candidate model species than the remainder of the plant community, a morphological evaluation of the floral traits of the dominant co-flowering plant species, including functional pollinators traits, was conducted at three forest sites (F1, F2 and F3). Morphological evaluation was conducted on *Di. brumalis* and 20 co-flowering species from 11 families in accordance with the descriptions in [Marchant et al. \(1987\)](#). The traits included were corolla symmetry (zygomorphic, actinomorphic), corolla shape (rotate, papilionaceous, bilabiate, ligulate), flower width and length (in mm), flower orientation (pendant, upright, horizontal), plant height (in cm), petal projections as a platform for pollinators (presence or absence), anther position (exposed or not exposed) and inflorescence

type (umbel, raceme, spike, panicle, solitary) (Table S6). Morphological similarity of floral traits was evaluated using non-metric multi-dimensional scaling (NMDS) following the methodology of [Jolles \(2015\)](#).

To test if the floral colour of *Di. brumalis* flowers is likely to be distinguishable by bees from the co-flowering pea plants species (*Da. decurrens*, *Da. horrida*, *Da. rhombifolia*, *H. chorizemifolia*, *H. pungens* and *B. aquifolium*), we took spectral reflectance measurements and analysed them using the [Chittka \(1992\)](#) model of bee vision. In addition, spectral reflectance was also measured for two yellow-flowered species that occurred at all sites, *Hibbertia hypericoides* (Dilleniaceae) and *Acacia pulchella* (Fabaceae), to test if other yellow-flowered species could also be part of the same guild as the pea plants. Spectral reflectance was measured on two flowers per plant for six randomly selected individuals of each species using a spectrometer (Jaz, DH-2000 UV-VIS-NIR Light source) with an integration time of 50 milliseconds. For *Di. brumalis*, measurements of spectral reflectance were taken from the outer lateral petals, the central dorsal sepal, the labellum and labellum lateral lobes; for pea plant species measurements were taken from the standard and wing petals (Fig. 2G) and for *Hibbertia hypericoides* and *Acacia pulchella* from the most conspicuous part of the floral display (the corolla and stamens, respectively). Spectral reflectance was analysed using the colour hexagon model, which is based on the sensitivities of photoreceptors of the bee *Apis mellifera* ([Chittka, 1992](#); [Chittka and Kevan, 2005](#)). For quantifying similarity of spectral reflectance, the distance between colour loci coordinates was measured as the Euclidean distance.

#### Comparative flowering phenology of *Diuris brumalis* and candidate model species

To test the prediction that mimics overlap in flowering period with their proposed models, the extent of flowering across the study period was quantified for *Di. brumalis* and the co-occurring pea plants (*Da. decurrens*, *Da. horrida*, *Da. rhombifolia*, *H. chorizemifolia*, *H. pungens* and *B. aquifolium*). For each species, weekly counts of open flowers were undertaken in 30 × 30-m quadrats at intact forest sites (F1, F2 and F3) from 28 June to 11 October 2017. For pea plants, due to the high number of flowers, we scored the total number of flowers per quadrat as (binned category): (1) 1–100, (2) 101–200, (3) 301–400, (4) 401–500 and so on, up to 2000 for a total of 19 categories (1–19). However, in the case of *H. chorizemifolia* and *Di. brumalis*, due to the paucity of flowers per inflorescence, the exact number of flowers on each plant was counted. The average number of flowers (or binned category) was calculated as the measure of flowering during any given week.

#### Reproductive success of the mimic in relation to the abundance of the model

To test if the fitness of *Di. brumalis* (Table S7) increased with the number of flowers of the putative model species, the proportion of flowers with pollen removal and the proportion of flowers with fruit formation was quantified at 18 populations.



In 2016 (15 sites) and 2017 (18 sites), we delimited a single 30 × 30-m quadrat per site, and at the peak flowering period for *Di. brumalis* we recorded: (1) the number of pea plants of each species within the quadrat; (2) the estimated number of flowers for each pea plant species; and (3) how many *Di. brumalis* plants and flowers were present (counted at the end of the flowering season in August). Variable (2) was estimated by counting the number of flowers on ten stems per pea plant to enable calculation of a mean, then multiplying this value by the total number of stems on the plant. Following evidence that the pollinators of *Di. brumalis* fed almost entirely on *Daviesia*, this variable was modified to be the estimated number of flowers of *Daviesia*. In both years, at the end of the flowering period of *Di. brumalis* we collected data on the number of flowers without pollinia and the number of fruits produced. The proportion of flowers with pollinia removal was used as a proxy for male fitness, while the proportion of flowers setting fruit was used as a proxy for female fitness.

Pollinia removed and fruit set were analysed by Generalized Linear Mixed Models (GLMMs) using the package glmmTMB in R Studio (Version 3.3.2). First, we tested if pollinia removal and fruit-set were greater at sites where *Daviesia* was present. Secondly, we tested if pollinia removal and fruit-set increased when there were more *Daviesia* flowers. In these latter models we included the abundance of *Daviesia* flowers, habitat and year all as fixed effects. In each model, site was treated as a random effect, as pollinia removal and fruit-set was quantified at the same sites in two field seasons. Because pollinia removal and fruit-set were analysed as proportions of the total number of flowers, they were assumed to be binomially distributed. However, when using a binomial distribution, the models for pollinia removed showed overdispersion (overdispersion parameters: 5.833 for the model of presence–absence and 3.897 for the model testing the effect of *Daviesia* flower abundance, habitat and year; see Zuur et al., 2013 for calculations), necessitating a switch to a betabinomial distribution. Evaluation of which model was most strongly supported by the data was undertaken using the corrected Akaike's information criterion (AICc) index, which dropped 120 and 57 points respectively for the two models by switching to the betabinomial distribution. Models testing the effect of the covariates (see above) on fruit-set were not over-dispersed. Therefore, fruit-set was confirmed to be a binomially distributed response variable. The abundance of *Daviesia* flowers was log-transformed to improve

the fit of the fruit-set model. The improvement of the model following a log transformation was confirmed by the AICc index, which dropped 6.5 points, and verified using the 'anova' R function ( $\chi^2_{33,4} = 6.371$ ,  $P < 0.001$ ). For all models we undertook the checks suggested by Zuur et al. (2013) to confirm that the underlying assumptions of the model are not violated.

## RESULTS

### Pollinators of *Diuris brumalis*

During baiting experiments a total of 132 insects were observed visiting *Di. brumalis*. Of these, 102 visits were by *Trichocolletes* spp. (Colletidae) and 25 by *Apis mellifera* (Apidae). Other visitors included Syrphidae (three) and *Leioproctus* sp. (two; Colletidae). Only *Trichocolletes* spp. and *Apis mellifera* were observed with orchid pollinia attached, in each case to the frontal region of the head (Fig. 3). However, only in the case of *Trichocolletes* was deposition of pollinia observed, with parts of the pollinia deposited in visits to subsequent flowers.

A total of 32 insects were caught for identification during baiting experiments and observations of pea plants (Table S5). In 2016 a total of 14 *Trichocolletes capillosus*, two *T. leucogenys* and one *T. dives* were caught, while seven *T. leucogenys* were caught in 2017. A total of 14 *Trichocolletes* were observed carrying pollen of *Di. brumalis*, eight while visiting *Di. brumalis*, and six while foraging on *Daviesia* spp. (see example in Supplementary video). Of the eight individuals observed carrying pollinia while visiting *Di. brumalis*, two arrived at the plant already carrying pollinia, and six removed pollinia while being observed. As the six individuals removing pollinia were all captured for identification, the remaining bees must have all sourced pollinia from wild *Di. brumalis*, independent of our artificial arrays. The identification of captured visitors and/or pollinators shows that the *Trichocolletes* spp. individuals caught on *Di. brumalis* and on pea plants with orchid pollinia included both females (four) and males (six). On two occasions *Apis mellifera* were collected with attached *Di. brumalis* pollinia. Of the *Trichocolletes* collected during the study, ten carried on their hind legs pollen of the same colour as seen in pea plants (yellow–orange). *Trichocolletes capillosus* was recorded in 2016 in the habitat forest, whereas *T. leucogenys*

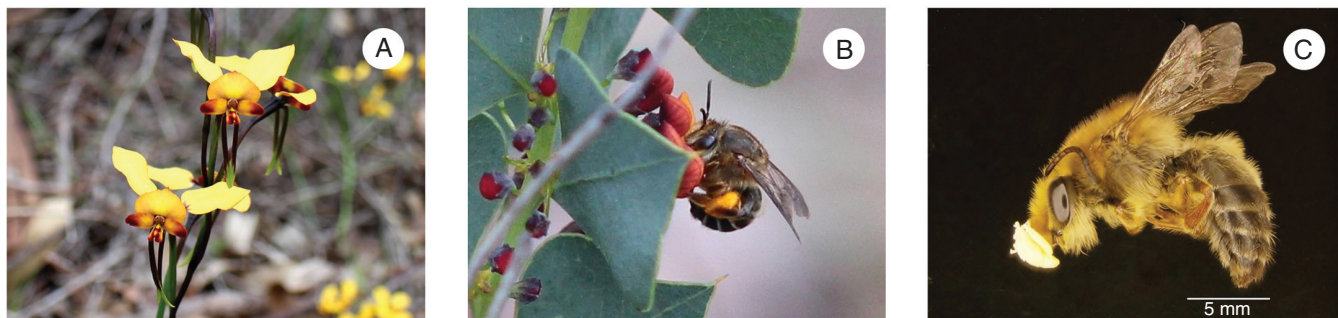


FIG. 3. Pollinators of *Diuris brumalis* and *Daviesia* spp.: A, inflorescence of *Diuris brumalis* (Orchidaceae); B, female of *Trichocolletes leucogenys* with pea plant pollen (orange in colour) on the abdomen and posterior legs, feeding on *Daviesia rhombifolia* by positioning the abdomen over the keel of the flower; C, male of *Trichocolletes capillosus* carrying *Diuris brumalis* pollinia on the head.

was recorded in both 2016 and 2017 in the habitats forest and outcrop (Table S5).

#### Pollinators of co-occurring pea plants

Based on observations of contact with the reproductive structures, *Da. decurrens* and *Da. rhombifolia* (occurring at only the forest sites) were pollinated by both *T. capillosus* and *T. leucogenys*, while *Da. horrida* (occurring only at the outcrop sites) was pollinated only by *T. leucogenys*. *Apis mellifera* was also observed to pollinate all three species as well as *H. pungens* and *B. aquifolium* (Table S4). No *Trichocolletes* species were observed visiting other pea plants or other plant species in the community.

#### Quantification of pollinator behaviour

Of the 102 *Trichocolletes* spp. visiting *Di. brumalis*, 74.6 % alighted on the flower. In each case the insect aligned along the labellum with its head facing the column. Those individuals that flew around the flowers without landing (25.6 %) were mostly ‘patrolling’ and could be visually distinguished as males by longer antennae and smaller body size, suggesting mate-searching behaviour (Barrows, 1976). Occasionally, males behaving in this fashion were observed mating with females that had been located while foraging on *Daviesia* spp. Both male and female of *Trichocolletes* spp. landed on the flowers of *Di. brumalis* for 1–2 s. However, we were unable to record the behaviour of *Trichocolletes* that landed for less than 1 s due to the rapidity of visits. Of the bees alighting on the flower,

81.3 % attempted to manipulate the labellum by articulations of the anterior legs and/or pushing of the abdomen onto the labellum, as seen when foraging on nectar and pollen from *Daviesia* spp. (Fig. 4). On 50.8 % of occasions, attempting to manipulate the labellum resulted in pollinia removal, with 29.5 % attributable to females and 21.3 % to males. After the visit, 19.3 % of insects extracting orchid pollinia visited other orchid flowers within the clump, with the remaining 80.7 % of bees going on to forage on *Daviesia* spp. flowers.

The behaviour exhibited by *Trichocolletes* spp. on *Di. brumalis* is characterized by similar behaviour as seen when foraging on the flowers of *Daviesia* spp. in the forest habitat (*Da. decurrens* and *Da. rhombifolia*). However, significantly more visitors landed on the *Daviesia* spp. than on *Di. brumalis* (*Di. brumalis* 74.2 %,  $n = 102$  vs. *Da. rhombifolia* 100 %,  $n = 43$ ,  $P = 0.009$ ; *Di. brumalis* vs. *Da. decurrens* 91.3 %,  $n = 74$ ,  $P = 0.004$ ). Alternatively, among the bees that landed, there was no difference in how frequently the bees attempted to forage on the flower (*Di. brumalis* 81.3 %,  $n = 75$  vs. *Da. rhombifolia* 86 %,  $n = 37$ ,  $P = 0.513$ ; *Di. brumalis* vs. *Da. decurrens* 86.4 %,  $n = 64$ ;  $P = 0.394$ ).

#### Floral similarity of mimics and models

An NMDS plot shows that all pea plants are morphologically similar, and formed a tight cluster, with a pronounced similarity of *Daviesia* spp. that overlap in the plot (Fig. 5). *Diuris brumalis* is more similar to pea plants than the remainder of the co-flowering plant community, but does not overlap with the morphology of peas in the NMDS plot (Fig. 5). Investigation of the species by trait matrix reveals that *Di. brumalis* matches pea plants for all morphological traits except height of plant and flower size. In the case of flower size, *Di. brumalis* is much larger because of the prominently projecting lateral sepals (Fig. 2G).

Analysis of spectral reflectance using the hexagon bee vision model (Chittka, 1992; Chittka and Kevan, 2005) showed that the average colour loci of *Di. brumalis*, all three *Daviesia* spp. and *B. aquifolium* corresponded to the UV region. The colour loci for *Hovea* spp. fell in the UV–blue region (Fig. 6A), and the colour loci for *Acacia pulchella* and *Hibbertia hypericoides* were located in the UV–green and green region, respectively. The mean distance of the colour loci measured on flower parts between *Di. brumalis* and *Da. decurrens*, *Da. rhombifolia*, *Da. horrida* and *B. aquifolium* was 0.12, 0.05, 0.06 and 0.1 respectively (Table S9). Colour loci for individual plants of *Daviesia* spp., distributed in the coordinates range  $y: [-0.39; -0.10]$   $x: [-0.12; -0.40]$ , overlap the visual space of *Di. brumalis* individuals ranging across the positions  $y: [-0.34; -0.09]$   $x: [-0.33; -0.08]$  (Fig. 6B). In *B. aquifolium* the overlap of colour space of individual colour loci with *Di. brumalis* is limited to the dorsal petals, as the keel is in the UV–blue region (Fig. 6B).

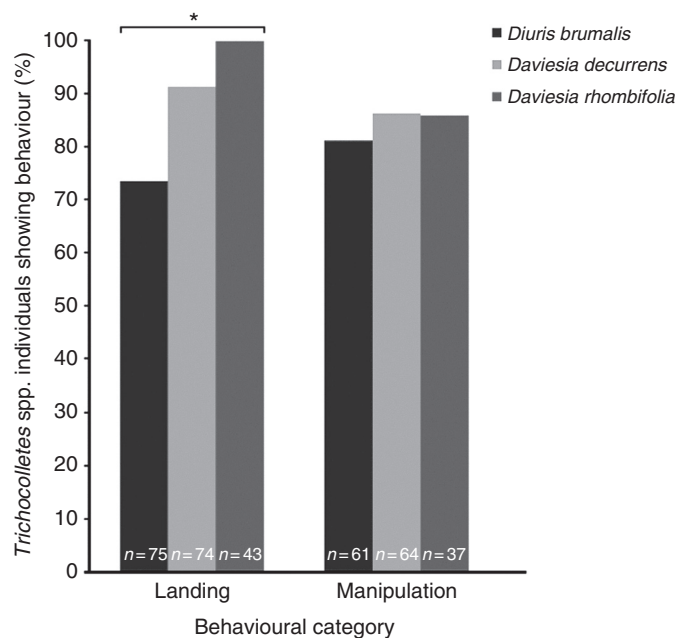


FIG. 4. Comparison of the foraging behaviour of *Trichocolletes* spp. (*Trichocolletes capillosus*, *T. leucogenys*) on *Diuris brumalis* and *Daviesia* spp. (*Daviesia decurrens*, *Da. rhombifolia*, *Da. horrida*). Bars represent the proportion of individuals engaging in: 1, Landing: flying directly to the flower and alighting on keel or labellum; 2, Manipulation: attempted to manipulate the flower as part of foraging behaviour for either nectar or pollen. \* indicates a significant difference.

#### Quantification of the pollen load of floral visitors

Pollen counts (Table S8) showed that the pollen assemblage carried by four *Trichocolletes* specimens consisted of almost 100 % *Daviesia* pollen with traces (<10 pollen grains in the scanned slide) of Myrtaceae spp. and *Grevillea*. One

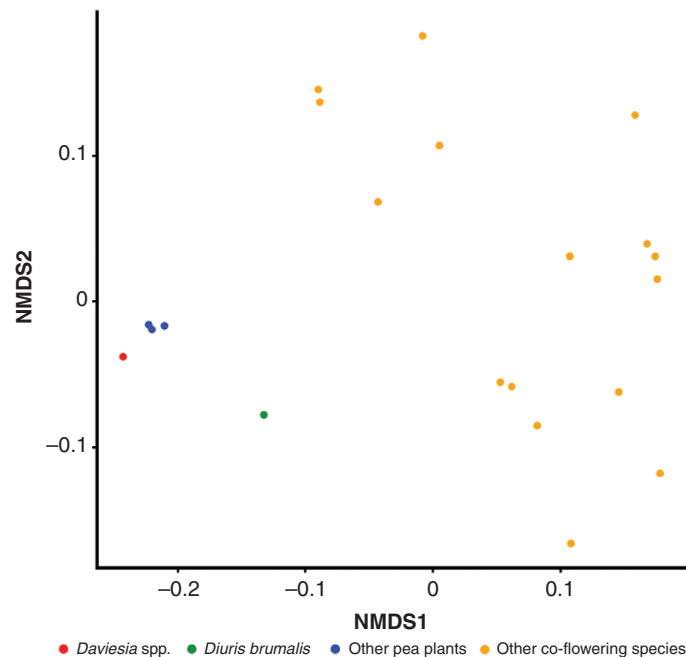


FIG. 5. Non-metric multi-dimensional scaling plot of floral traits for *Diuris brumalis* and co-flowering species in the forest habitat. *Diuris brumalis* and co-occurring pea plants (Faboideae) form a distinct cluster reflecting strong morphological similarity compared to the remainder of the plant community.

specimen of *T. leucogenys* from the outcrop habitat (Table S8, no. 2), which was caught on *Da. horrida*, contained 97.5 % *Daviesia* pollen and traces of pollen of Myrtaceae (2 %) and *Hovea* (0.5 %). The amount of *Daviesia* pollen in samples from *Apis mellifera* specimens caught foraging on *Daviesia* plants was variable (80–98 %), and also they contained pollen of *Banksia*, *Acacia* and Myrtaceae (1–20 %). On the *Apis mellifera* specimen caught foraging on *B. aquifolium* (Table S8, no. 10), *Bossiaea* pollen comprised 97.5 % of the assemblage with 1.5 % *Banksia* pollen, 1 % Myrtaceae pollen and traces of *Acacia* pollen.

#### Flowering phenology of target species

There was pronounced variation in the overlap of the flowering periods of *Di. brumalis* and the co-occurring pea plants (Fig. S1). Among the species that are frequently visited by *Trichocolletes* spp., flowering of *Da. decurrens* and *Da. rhombifolia* peaked 2 and 5 weeks respectively after the peak of *Di. brumalis*. Flowering of *H. chorizemifolia* peaks 2 weeks before *Di. brumalis*, while the peak of *H. pungens* corresponded to the peak of *Di. brumalis*. Peak flowering for *B. aquifolium* occurred near the end of the *Di. brumalis* flowering period.

#### Reproductive success of the mimic in relation to the abundance of the model

Pollinia removed did not show any significant difference between sites where *Daviesia* spp. were present (marginal mean of  $0.119 \pm <0.001$  s.e.) or absent ( $0.1 \pm <0.001$ ,  $P = 0.592$ ).

However, fruit-set was higher in the presence of *Daviesia* spp. ( $0.027 \pm 0.003$ ) than in their absence ( $0.008 \pm 0.001$ ,  $P = 0.049$ ).

The proportion of pollinia removed exhibited a positive relationship (mean =  $0.2982 \pm 0.1237$ ,  $P = 0.016$ ) with the abundance of *Daviesia* flowers (Fig. 7A). Year also had a significant effect on the proportion of pollinia removed from *Di. brumalis* flowers (2017 =  $0.146 \pm 0.013$ ; 2016 =  $0.069 \pm 0.006$ ,  $P = 0.019$ ). The proportion of pollinia removed was marginally higher in the jarrah forest ( $0.123 \pm 0.011$ ) compared to the outcrop habitat ( $0.057 \pm 0.015$ ,  $P = 0.068$ ), although the difference was non-significant. Fruit-set showed a positive relationship with the number of *Daviesia* flowers (log-transformed mean =  $0.21398 \pm 0.08328$ ,  $P = 0.01$ ) (Fig. 7B). Fruit-set was significantly different between years (2016 =  $0.031 \pm 0.006$ ; 2017 =  $0.01 \pm 0.002$ ,  $P < 0.001$ ), but did not differ between the forest ( $0.021 \pm 0.004$ ) and outcrop habitats ( $0.017 \pm 0.006$ ,  $P = 0.692$ ). The relationship between the number of *Daviesia* flowers and both pollen removal and fruit-set was influenced by several sites where *Daviesia* spp. did not occur, and there was very little or no reproductive success in *Di. brumalis*.

## DISCUSSION

#### Pollinator sharing between models and mimic

One of the most fundamental criteria to assess the occurrence of floral mimicry is to establish whether the proposed model and mimic species share the same pollinators (Roy and Widmer, 1999; Johnson and Schiestl, 2016). Data from this study indicate that *Di. brumalis* shares the same pollinators (the bees *Trichocolletes capillosus* and *T. leucogenys*) with *Da. decurrens* and *Da. rhombifolia* in jarrah forest, and *Da. horrida* in heathland with granite outcrops. Additionally, observations



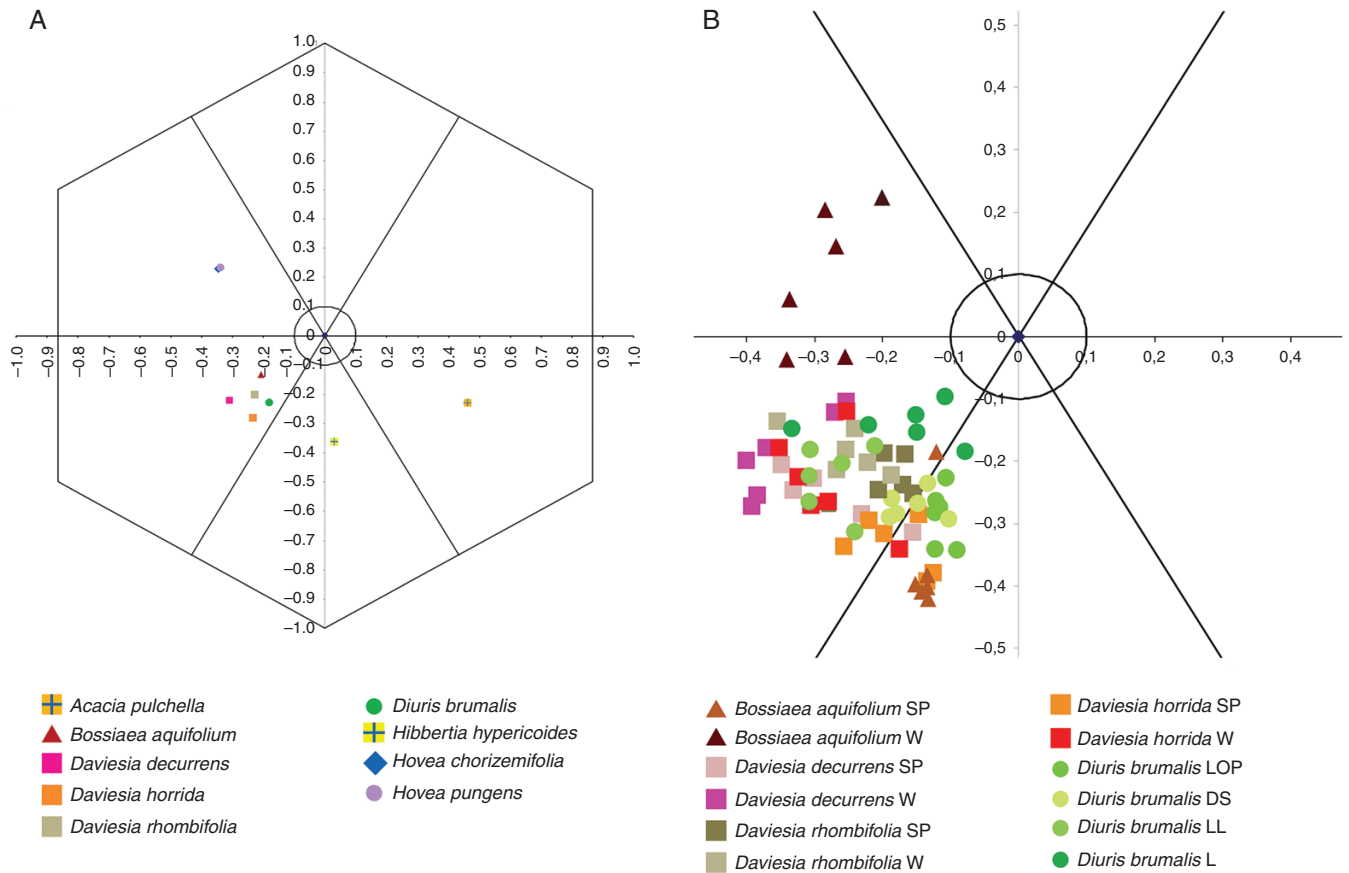


FIG. 6. (A) Mean values of colour loci calculated for floral parts of *Daviesia decurrens*, *Da. horrida*, *Da. rhombifolia*, *Hovea chorizemifolia*, *H. pungens* and *Bossiaea aquifolium*. In addition, colour loci are presented for two commonly occurring yellow-flowered species present at all sites, *Hibbertia hypericoides* (Dilleniaceae) and *Acacia pulchella* (Fabaceae), to test model similarity based on floral colour. (B) Distribution of colour loci most similar to the *Di. brumalis* colour signal. Measurements of spectral reflectance were taken for *Di. brumalis*: LOP = lateral outer petal; DS = dorsal sepal; LL = labellum lateral lobe; L = labellum; for pea plant species (Faboideae): SP = standard petal; W = wing petals. The calculations were made using the Hexagon colour model of bee vision (Chittka, 1992).

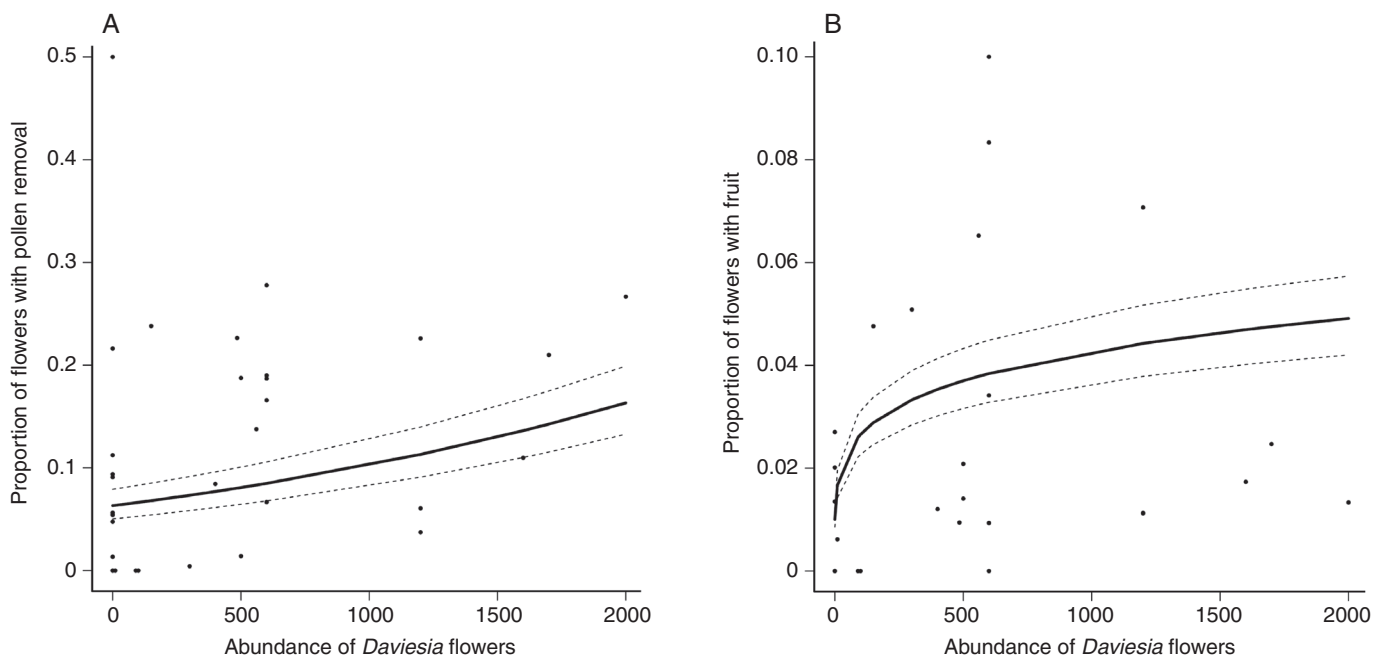


FIG. 7. The proportion of flowers with pollen removal (A) and fruit-set (B) of *Diuris brumalis* as a function of the number of flowers of *Daviesia* spp.

of pollinator foraging, and analysis of pollen collected from the bodies of pollinators, revealed that in the study areas both *T. capillosus* and *T. leucogenys* feed primarily on *Da. decurrens*, *Da. rhombifolia* and *Da. horrida* (Table S8). *Trichocolletes capillosus* individuals were observed and caught only in 2016 in forest sites, while *T. leucogenys* were observed and caught in both 2016 and 2017 in forest and outcrop sites. Previous observations suggest that depending on seasonal conditions, the numbers of *Trichocolletes* that emerge at particular sites can vary from year to year, and in some years none may emerge (T. Houston, Western Australian Museum, pers. comm.). *Apis mellifera* and *T. dives* are potential pollinators as they have been observed to extract orchid pollinia, but they were not observed to deposit pollen on *Di. brumalis*. However, given their ability to remove and carry pollen, they may be responsible for occasional pollination events.

#### Behavioural evidence for mimicry

Exhibiting specific behaviours typically associated with the model species provides strong evidence that the proposed mimic has deceived the operator. In the present study, *T. capillosus* and *T. leucogenys* exhibit very similar foraging behaviour on *Di. brumalis* and *Daviesia* spp. (Fig. 4) [Supplementary data, Video], suggesting that the orchid is sufficiently similar to the model to deceive pollinators. For all three *Daviesia* species observed, *Trichocolletes* spp. show abdomen bending around the keel when attempting to collect nectar/pollen on *Daviesia*. Furthermore, female bees part the keel using their hind legs to collect pollen from the anthers. Our observations of *T. capillosus* and *T. leucogenys* individuals on *Di. brumalis* flowers suggested that they attempt to use the same stereotyped foraging behaviour. Both species landed along the midline of the labellum and push their abdomen upon it, unsuccessfully attempting to open it using their anterior legs in a similar fashion to the pollen-collecting behaviour they exhibit on *Daviesia* [Supplementary data, Video]. Crucially, *Trichocolletes* have only been recorded exhibiting this keel-parting behaviour on pea plants, meaning that this behaviour indicates mimicry of Faboideae, not other plant groups. Interestingly, other insects observed visiting *Daviesia* (particularly *Apis mellifera* and *Leioproctus* spp.) that have broader foraging preferences beyond the Faboideae were seen to land on the flowers and probe the keel with the body orientated in different directions, not necessarily along the keel.

When visiting *Di. brumalis* flowers, some male *T. capillosus* and *T. leucogenys* individuals appeared to exhibit patrolling behaviour, where the male searches for females in specific landmarks or rendezvous places that can be resourced-based (Haas, 1960; Barrows, 1976; Paxton, 2005). In *Trichocolletes* landmarks are represented by flowering *Daviesia* bushes, where males often approach closely without landing, probably searching for females engaged in foraging behaviour. Males exhibiting this same apparent patrolling behaviour were observed occasionally to mate with females foraging on *Daviesia* plants. Exhibiting this 'patrolling' behaviour provides evidence that male *Trichocolletes* confuse *Di. brumalis* with *Daviesia* food sources, even though courtship or patrolling behaviour is not directly involved in pollination.

#### Physical similarity between the mimic and the models

A multivariate analysis of floral morphological traits indicated that *Di. brumalis* is more similar to species of Faboideae than any other co-flowering species in the studied communities (Fig. 5). While *Di. brumalis* did not overlap with the Fabaceae in the NMDS plot, all of the characters scored were matching between pea plants and *Di. brumalis* except for plant height and overall flower size. Among the pea plants, the spectral reflectance of *Di. brumalis* was most similar to that of the *Daviesia* species on which *Trichocolletes* feed (Fig. 6A, B). The similarity of colour loci between *Di. brumalis* and *Daviesia* appeared particularly pronounced between the standard (model) and dorsal petal (mimic), and between the wing (model) and labellum lateral lobe (mimic), suggesting a level of colour matching between morphologically corresponding floral parts (Figs 2G and 6B). Between *Daviesia* species and *Di. brumalis* the distances between mean colour loci (averaged across floral parts) ranged between 0.05 and 0.10. In some individuals, the coloration of *Da. rhombifolia*, *Da. decurrens* and *Da. horrida* overlapped in colour space with *Di. brumalis*, and was less than the 0.06 units whereby bumble bees and honey bees cannot distinguish colours (Dyer and Chittka, 2004a, b; Giurfa, 2004). However, due to only partial overlap of colour loci of individual plants in *Daviesia* and *Di. brumalis*, colours of model species and mimic are likely to often be distinguishable by pollinators. Furthermore, it is likely that precise colour patterns differ between *Di. brumalis* and the *Daviesia* species. Nonetheless, mimics do not have to be identical, as long as they are perceived as similar by the pollinator (Dalziel and Welbergen, 2016). Indeed, *Diuris* may benefit from an unspecific mimicry of a range of pea plants, rather than appearing identical to a single species, as this may enable them to function effectively with multiple model species.

While the labellum, dorsal sepal and labellum lateral lobes of *Di. brumalis* appear to replicate the keel, dorsal and wing petals of *Daviesia*, the prominently projecting external petals in *Di. brumalis* are a component of floral architecture that is absent in *Daviesia* spp. (Fig 2G). However, it may be possible that some floral parts are involved in the mimicry of pea plants while others are not essential for mimicry and are free to vary. For example, in some genera of sexual deceptive orchids, where the role of floral traits in pollinator attraction is well studied, mimicry of the sex pheromone of the pollinator is often precise (Peakall et al., 2010; Bohman et al., 2018), while colour is not a close match to the female (Gaskett et al., 2016). Similarly, parts of the flower involved in positioning of the pollinator may be under stronger selection than morphologically inactive parts (Rakosy et al., 2017; de Jager and Peakall, 2018). In *Diuris* flowers, selection may operate through a dual mechanism, where floral traits involved in mimicry, such as colour and shape of the labellum and column wings, have evolved to resemble pea plants whereas the projecting outer petals may have evolved exaggerated size to increase long-distance attraction of pollinators. Indeed, there is a large body of supporting evidence suggesting that a greater floral display increases pollinator visitation rates (e.g. Peter and de Jong, 1990; Karron et al., 2004).

### Overlap between mimics and models in flowering phenology

An overlap in flowering phenology between mimic and model is another key requirement of floral mimicry (Roy and Widmer, 1999; Johnson and Schiestl, 2016). Here, we have shown that the flowering periods of *Di. brumalis* and *Daviesia* spp. overlap, but that the flowering peak of *Di. brumalis* precedes the peak of the model species (2 weeks before *Da. decurrens* and 5 weeks before *Da. rhombifolia*) (Fig. S1). In *Trichocolletes*, males often emerge several days prior to females (observations by T. Houston, Western Australian Museum, unpubl. res.), meaning that *Diuris* may take advantage of early emerging males that are searching for females and nectar on pea plants. This interpretation was supported in the present study, where most observations at the start of the flowering period were of males, but the number of females increased as the *Daviesia* came into flower. The exploitation of naïve pollinators appears to be a common characteristic of food-deception systems. Species that use generalized food deception often flower when naïve pollinators emerge (Pellissier et al., 2010) and are yet to learn that the orchid flowers are rewardless (Internicola and Harder, 2012). Alternatively, pollinators can exhibit an innate sensory bias to certain colours and shapes and, following emergence, automatically searching for food sources with these traits (Çakmak and Wells, 1995; Lunau and Maier, 1995). In the case of *Di. brumalis*, pollinators may attempt to forage on the mimetic *Diuris* through either naivety or an innate preference for pea-like flowers, even though flowering individuals of the model pea plants may be scarce at the time of emergence.

### Does fitness of the mimic increase in the presence of the model?

Adaptive resemblance between mimic and model species is achieved when pollinators are not able to distinguish between them, and this ‘misclassification’ behaviour enhances the fitness of the mimic (Endler, 1981; Skelhorn and Ruxton, 2010). As such, it is expected that in mimicry systems the fitness of the mimic should be greater when the model is more abundant (Anderson and Johnson, 2006). However, in practice it is difficult to separate the effects on fitness of reduced pollinator learning in the presence of the more model flowers, and of greater pollinator abundance in the presence of more model flowers. This challenge applies to *Di. brumalis*, as the *Trichocolletes* species foraged primarily on the model flowers, making pollinator abundance likely to be highly correlated with abundance of the model. For example, fruit-set was lowest at sites where *Trichocolletes* were not observed and *Daviesia* were almost absent. Furthermore, fruit-set increased with the number of *Daviesia* flowers, with this relationship likely to be influenced by sites where there were few or no *Daviesia*, and thus very low reproductive success of *Di. brumalis*. As expected under pollinator learning, rates of pollinia removal increased when there were greater numbers of *Daviesia* flowers, although this could also potentially be attributable to greater numbers of pollinator at these sites. To resolve this issue, it would be of interest to compare the response of *Trichocolletes* to experimentally presented *Diuris* flowers in areas with and without natural populations of *Diuris*.

Interestingly, even at sites where *Trichocolletes* were not observed and *Daviesia* were largely absent, occasional cases of pollen removal and deposition occurred. These events may be partly attributable to the introduced honey bee *Apis*

*mellifera*, which was frequently observed foraging on co-occurring flowering plants in both habitats, including sites where *Trichocolletes* was not observed. However, forest sites without *Daviesia* exhibited a level of fruit-set approaching zero, despite some level of pollen removal, suggesting that honey bees may fail to complete pollination through pollen deposition. At present, there is very little information on the potential negative or positive effects of *Apis mellifera* on pollination of Australian orchids (e.g. Adams and Lawson, 1993; Phillips et al., 2009), although given the occasional visitation witnessed here, *Diuris* may represent an interesting study genus to tackle this issue.

### Is there evidence for guild mimicry in *Diuris brumalis*?

While pollination via mimicry of flowering plants usually involves a particular model species, there is evidence that some plants mimic a guild of plant species rather than a specific model (Jersáková et al., 2016). Plant guilds are recognized by both sharing a particular pollinator (or group of related pollinators) and having very similar floral traits (Manning and Goldblatt, 1996), which are likely to represent adaptations to the particular pollinator(s) (Johnson, 2010). Based on some sharing of pollinators and their striking resemblance, *Diuris* have been hypothesized to mimic a guild of pea plants (Beardsell et al., 1986; Dafni and Bernhardt, 1990; Indsto et al., 2006). The present study shows that while *Daviesia* spp. share pollinators and may form the basis of a guild, this does not extend to all pea plant species in the community. However, based on behavioural observations and floral traits, we provide evidence that mimicry functions with different *Daviesia* species in different habitats (*Da. decurrens*, *Da. rhombifolia* in jarrah forest; *Da. horrida* in the outcrop heath). As such, through the use of more than one model species the *Diuris*–*Daviesia* mimicry system may meet some of the conditions for guild mimicry.

While the guild mimicry hypothesis has received support from observational studies in orchids (e.g. Jersakova et al., 2016 and the present study), at present experimental tests are lacking. A complementary approach to conducting field observations in different habitats would be to move experimental arrays of orchids between pea plant communities, thereby testing if any given population of *Di. brumalis* can attract pollinators in the presence of other pea plant species. In addition, it would be of interest to investigate the breadth of phenotypes that can achieve mimicry through the use of models or manipulated *Diuris* flowers. Alternatively, experiments with bees conditioned on different species of pea plant could be used understand the full range of models that *Di. brumalis* can mimic. However, the outcomes of such experiments would also be partly affected by whether the bees learn to associate rewards with particular pea plants, or if the attraction is innate. If the attraction is innate it is possible that *Di. brumalis* may be attractive to pollinators regardless of the pollinators’ prior experience with food plants.

## CONCLUSIONS

Here we present evidence that *Di. brumalis* achieves pollination by mimicking the flowers of multiple co-flowering species of *Daviesia*. In addition to meeting the criteria for sharing pollinators and flowering times, pollinators exhibited pea plant-specific



foraging behaviour on the *Diuris*, providing strong evidence that the mimic had successfully deceived the pollinator. This evidence was further supported by data on morphology and colour, showing that not only are *Diuris* and *Daviesia* spp. very similar compared to the remainder of the co-flowering community, but that based on bee vision models, in many cases the colour of *Diuris* and the proposed model species will not be readily distinguishable to pollinators. Fruit-set and pollen removal of *Di. brumalis* was more frequent in the presence of *Daviesia*, although evidence suggests that this is probably through some combination of both learning and greater pollinator abundance at sites where the model is present. The diversity of species related to *Di. brumalis* with pea-like floral traits (*Diuris corymbosa* complex) suggests that this may be an effective system for understanding diversification in lineages that use floral Batesian mimicry.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Fig. S1: phenology of *Diuris brumalis* and co-occurring Faboideae. Table S1: habitat assigned with description. Table S2: plant species vouchered at the WA Herbarium. Table S3: observations of floral visitors to *Diuris brumalis*. Table S4: observations of floral visitors to Faboideae. Table S5: insects caught on *Diuris brumalis* and co-occurring Faboideae. Table S6: Floral traits of *Diuris brumalis* and the 20 most abundant co-flowering species. Table S7: populations and reproductive data of *Diuris brumalis*. Table S8: composition of pollen loads. Table S9: means and standard deviation of colour loci of *Diuris brumalis* and pea plants. Appendix S1: floral biology of *Diuris brumalis* and co-occurring Faboideae. Video: *Trichocolletes* behaviour on *Daviesia decurrens* (model) and *Diuris brumalis* (mimic). Key behaviours illustrated: ‘patrolling’, courtship behaviour by males looking for females, keel (model) or labellum (mimic) ‘manipulation’, ‘foraging’ behaviour by females, including searching for sources without landing. Video is presented in slow motion.

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