

1 Primary pollinator exclusion has divergent consequences for pollen dispersal
2 and mating in different populations of a bird-pollinated tree

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15 **Key words: Pollinator exclusion, pollen dispersal, multiple paternity, bird pollination,**
16 **honeyeater, paternity analysis**

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18 Short title: Mating patterns in a bird-pollinated tree

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20 Word count: 6040 (not including reference list)

ABSTRACT

Pollination by nectarivorous birds is predicted to result in different patterns of pollen dispersal and plant mating compared to pollination by insects. We tested the prediction that paternal genetic diversity, outcrossing rate and realised pollen dispersal will be reduced when the primary pollinator group is excluded from bird-pollinated plants. Pollinator exclusion experiments in conjunction with paternity analysis of progeny were applied to *Eucalyptus caesia* Benth. (Myrtaceae), a predominantly honeyeater-pollinated tree that is visited by native insects and the introduced *Apis mellifera* (Apidae). Microsatellite genotyping at 14 loci of all adult *E. caesia* at two populations (n = 580, 315), followed by paternity analysis of 705 progeny, revealed contrasting results between populations. Honeyeater exclusion did not significantly impact pollen dispersal or plant mating at Mount Caroline. In contrast, at the Chiddarcooping site, the exclusion of honeyeaters led to lower outcrossing rates, a threefold reduction in the average number of sires per fruit, a decrease in intermediate-distance mating, and an increase in near-neighbour mating. The results from Chiddarcooping suggest that bird pollination may increase paternal diversity, potentially leading to higher fitness of progeny and favouring the evolution of this strategy. However, further experimentation involving additional trees and study sites is required to test this hypothesis. Alternatively, insects may be effective pollinators in some populations of bird-adapted plants, but ineffective in others.

INTRODUCTION

Mating patterns and genetic structure play a pivotal role in the evolution of plant populations (Barrett & Harder 1996). Animal pollinator groups differ in their patterns of flower visitation, thereby influencing pollination and pollen dispersal (Harder & Barrett 1996). The majority of flowering plants are insect-pollinated (Ollerton *et al.* 2011), but bird pollination is a geographically widespread phenomenon spanning numerous plant families (Carpenter 1978; Stiles 1978; Cronk & Ojeda 2008; Fleming & Muchhala 2008). Pollination by nectar feeding birds is predicted to result in higher levels of multiple paternity, wider pollen dispersal and higher rates of outcrossing compared to pollination by insects (Krauss *et al.* 2009; Mitchell *et al.* 2013; Bezemer *et al.* 2016; Krauss *et al.* 2017). The genetic consequences of bird pollination may be favourable in certain eco-evolutionary contexts, potentially contributing to the repeated shifts to this strategy in landscapes where native insect pollinators are readily available (Whittall & Hodges 2007; Thomson & Wilson 2008; Tripp & Manos 2008; Toon *et al.* 2014).

Differences in the dynamics of pollen removal and deposition by birds and insects may influence the extent of polyandry within fruits (Castellanos *et al.* 2003; Krauss *et al.* 2009). Polyandry or multiple mating occurs when pollen from different sires fertilizes the ovules of a single fruit or plant, and is a common feature of land plants (Pannell & Labouche 2013). High levels of multiple paternity and low correlation of paternity (i.e. proportion of siblings sharing a father) in seed crops increases the potential for sibling competition and/or maternal selection (Karron & Marshall 1990; Marshall & Folsom 1991), potentially leading to greater average fitness of offspring. For example, rates of seedling emergence are faster, and the proportion of germination and survivorship of progeny significantly higher, in progeny arrays with lower correlated paternity (Karron & Marshall 1990; Breed *et al.* 2014; Nora *et al.* 2016). Paternal

diversity in bird-pollinated plants is on average approximately twice that of plants that are exclusively pollinated by insects (Krauss *et al.* 2017).

In addition to high multiple paternity, bird pollination may increase or maintain allelic diversity by facilitating both wide pollen dispersal (Southerton *et al.* 2004; Byrne *et al.* 2007; Breed *et al.* 2015) and potentially a departure from the predominantly near-neighbor mating that characterizes insect-pollinated species (Hopper & Moran 1981; Harder & Barrett 1996; Sampson 1998; Krauss 2000; Krauss *et al.* 2009; Ritchie & Krauss 2012; Bezemer *et al.* 2016). Departures from predominantly near-neighbor mating in bird-pollinated plants is thought to arise through a combination of high mobility, ability to carry large pollen loads, limited grooming, pollen carryover, and aggressive interactions within and among bird species leading to disruption of optimal foraging behavior (Hopper 1981; Wooller *et al.* 1983; Mac Nally *et al.* 2005; Phillips *et al.* 2014; Krauss *et al.* 2017). The influence of birds on pollen dispersal distributions and the frequency of multiple mating may be underestimated because many bird-pollinated plants are also visited by mammals and insects (Stiles 1978; Ford *et al.* 1979; Paton & Turner 1985; Fleming & Muchhala 2008).

The few studies that have investigated the effect of bird exclusion on pollination using molecular methods have revealed reduced outcrossing rates when only insects pollinate honeyeater-adapted plants (England *et al.* 2001; Schmidt-Adam *et al.* 2009). To our knowledge no comparable studies examining the molecular genetic consequences of bird exclusion in hummingbird or sunbird pollinated plants have been conducted. Studies that move beyond merely estimating rates of self and cross-pollination when comparing pollinator effectiveness and test for differences in patterns of pollen dispersal, correlation of paternity or multiple paternity may yield insights into the relative contributions of different animals to pollination

within a plant species (Barrett 2003; Barrett & Harder 2017; Rhodes *et al.* 2017; Ma *et al.* 2018). For example, in the annual forb *Oenothera harringtonii* (Onagraceae), levels of multiple paternity in progeny resulting from hawkmoth pollination was nearly double that of progeny resulting from solitary bee-pollination, presumably due to differences in pollinator morphology and behaviour (Rhodes *et al.* 2017). Such divergent consequences for mating due to pollination by functionally diverse animal groups may also feature in bird-pollinated plants.

In Australasia, honeyeaters (Meliphagidae) are important pollinators of numerous plant species (Ford *et al.* 1979; Higgins *et al.* 2001). Like hummingbirds and sunbirds, many honeyeaters aggressively defend nectar resources, likely leading to frequent departure from near-neighbor pollen dispersal (Stiles 1978; Hopper & Moran 1981; Mac Nally *et al.* 2005; Phillips *et al.* 2014). The most abundant insect pollinator in our study region, south-west Australia, is the introduced social honeybee *Apis mellifera* (Paton 1996; Phillips *et al.* 2010). Non-native honeybees may disrupt the pollination systems of native plants with floral traits typically associated with bird pollination (Vaughton 1996; England *et al.* 2001; Celebrezze & Paton 2004). Our study species, *Eucalyptus caesia* Benth. (Myrtaceae), is a predominantly honeyeater-pollinated tree endemic on granite outcrops in south-western Australia (Hopper 1981; Hopper *et al.* 1982) – a biodiversity hotspot with an exceptionally diverse vertebrate-pollinated flora (Hopper & Gioia 2004; Phillips *et al.* 2010). High levels of multiple paternity and a departure from predominant near-neighbor mating has been demonstrated in some honeyeater-pollinated plants (Krauss *et al.* 2009; Bezemer *et al.* 2016) presumably due to pollination by birds rather than insects (Hopper 1981; Krauss *et al.* 2017).

Studies that combine exclusion experiments with genotyping enable direct comparisons of multiple pollinator groups in terms of their relative contribution to pollen dispersal and mating

patterns. We conducted pollinator exclusion experiments combined with microsatellite genotyping and paternity analysis to test the effects on plant mating when the primary pollinator group is excluded from inflorescences. We tested the hypothesis that outcrossing rate, paternal diversity and realised pollen dispersal distances are reduced when birds are excluded from inflorescences of predominantly honeyeater-pollinated plants (Bezemer *et al.* 2016; Krauss *et al.* 2017).

METHODS

Study system and field sites

Eucalyptus caesia is an autumn to winter-flowering, long-lived mallee (small, multi-stemmed tree) or tree endemic on granite outcrops in the Wheatbelt region of south-western Australia (Hopper *et al.* 1982). The mixed mating system of *E. caesia* is predominantly outcrossing, but rates of self-pollination show pronounced variation between individuals (Bezemer *et al.* 2016; Bezemer 2018). Flowering is sporadic among and within individuals and does not occur consistently among populations in any given year (SDH, unpublished data). The large pink or red monoecious flowers are relatively long-lived, produce large amounts of dilute nectar and attract nectarivorous birds, particularly honeyeaters (Figure 1; Hopper 1981). Decades of field observations by our team indicate that foraging by native insects on *E. caesia* is rare during the peak flowering period but may be important late in the flowering season. On the other hand, introduced honeybees (*Apis mellifera*) are common visitors to flowers in several populations based on multiple seasons of field observations (authors' personal observations; Figure 1).

The study sites each represent one of the two subspecies of *E. caesia* – *E. caesia* subsp. *caesia* at Mt Caroline Nature Reserve (census size 580 plants) and *E. caesia* subsp. *magna* at Chiddarcooping Nature Reserve (315 plants). Mt Caroline is a small nature reserve of 352 hectares that is surrounded by agricultural land. In comparison Chiddarcooping covers an area of 5400 hectares and is surrounded by relatively large tracts of native vegetation (see maps in Figure 2). These two populations are among the largest known of *E. caesia* and have been comprehensively mapped and genotyped (Bezemer *et al.* 2019). Most populations of *E. caesia* are characterized by strong genetic sub-structuring, with genetic differentiation of sub-populations over a few hundred meters (Bezemer *et al.* 2019). High levels of differentiation are also evident across the species' landscape distribution on isolated granite rocks over ca. 300 km (Byrne & Hopper 2008; Bezemer *et al.* 2019).

Eucalyptus caesia subsp. *caesia* is distinguished from *E. caesia* subsp. *magna* by greater plant height, smaller and more numerous flowers, and smaller leaves and fruit (Brooker & Hopper 1982). It also has fewer stamens per flower and only produces nectar overnight in the field and in cultivation. In contrast, *E. caesia* subsp. *magna* is distinguished by smaller plants with larger and fewer flowers per plant, and larger leaves and fruit. It has more stamens per flower and produces nectar during daylight as well as at night. Further, subsp. *magna* produces more nectar ($298.4 \pm \text{s.e. } 103.2 \mu\text{l}$ per bagged flower over 24 hours) compared to subsp. *caesia* ($109.6 \pm \text{s.e. } 34.3 \mu\text{l}$), and diurnal nectar production appears to be continuous in subsp. *magna* but depleted by late morning in subsp. *caesia* (Hopper and Wyatt, unpublished data). *Eucalyptus caesia* subsp. *caesia* has a wide distribution across the species range while *E. caesia* subsp. *magna* is restricted to the north-east of the species' range. Genotyping at 18 locations of *E.*

caesia by Bezemer *et al.* (2019) supports the hypothesis that the subspecies are genetically distinct (Hopper *et al.* 1984; Byrne & Hopper 2008).

A variety of honeyeater species occur in the Wheatbelt, many of which are likely to be involved in the pollination of *E. caesia* based on observations of foraging on *E. caesia* flowers (Hopper 1981; authors field observations). Brown Honeyeaters (*Lichmera indistincta*), White-eared Honeyeaters (*Lichenostomus leucotis*), Singing Honeyeaters (*Lichenostomus virescens*) and Spiny-cheeked Honeyeaters (*Acanthagenys rufogularis*) have been observed at both sites (Hopper 1981). Chiddarcooping Nature Reserve also has White-fronted Honeyeaters (*Purnella albifrons*), Brown-headed Honeyeaters (*Melithreptus brevirostris*) and Red Wattlebirds (*Anthochaera carunculata*) and had a disjunct northernmost population of Purple-gaped Honeyeaters (*Lichenostomus cratitius*) that disappeared following wildfire. Many introduced honeybees were observed foraging on *E. caesia* at Mt Caroline, whereas few introduced honeybees were seen at Chiddarcooping during the flowering season in which the experiment was conducted (NB and DGR, personal observations). Based on field observations over more than 30 years, honeyeaters are typically abundant at Mt Caroline and Chiddarcooping and are frequent visitors to *E. caesia* flowers, suggesting that honeyeaters are important pollinators at our study sites (SDH, unpublished data). Following observations of nectar feeding on wild plants, the potential role of nocturnal moths, thynnine wasps and native bees in pollination of *E. caesia* requires investigation (Supporting Information Figure S1).

Pollinator exclusion experiment

In the autumn – winter months of 2016, three treatments were applied to unopened flower buds on 21 trees at Mt Caroline and 24 trees at Chiddarcooping. Maternal trees were selected based on accessibility, number of healthy buds, and location, with the intention of including as many widely distributed trees as possible (Figure 2). All treatments were applied to each individual tree to control for individual variation in outcrossing rates resulting from maternal genotype or location effects. For the honeyeater-exclusion (H-E) treatment, 1 cm-gauge wire mesh cages were applied to exclude birds, but allow insects, to access flowers. For the open-pollination (O-P) treatment, which allowed flower access by all potential pollinators including birds and insects, flower buds were tagged as for other treatments. A total exclusion treatment was also applied, where fine-mesh bags were used to exclude all pollinators. However, total exclusion data were not included in our analysis due to extensive flower/fruit abortion leading to insufficient sample sizes for this treatment. At Mt Caroline the rate of fruiting success in the total exclusion treatment (15 % of flower buds developed into fruits) was half that of the open-pollination and honeyeater-exclusion treatments. We cannot discount the possibility that reduced fruiting success was at least in part due to potentially higher microclimate temperatures inside the polyethylene mesh exclusion bags (Gitz *et al.* 2015).

The total number of flower buds included in the experiment at Mt Caroline was 366, 333, and 317 for the O-P, H-E and total exclusion treatments respectively. Numbers for Chiddarcooping were 220, 239 and 104 for the O-P, H-E and total exclusion treatments respectively. High levels of flower and fruit abortion is a natural phenomenon of *E. caesia* at both study sites and reduced the number of trees and inflorescences that could be included in the analysis. Experimental fruit from the open-pollination and honeyeater-exclusion treatments were

collected upon maturity, approximately one year after deployment of exclusion cages, and dried in individual paper bags until seed release. At the end of the experiment, the number of trees with fruit representing both treatments were 6 at Mt Caroline and 3 at Chiddarcooping.

For mating parameter estimates made in MLTR final sample sizes for the two treatments combined were 240 progeny from 6 trees at Mt Caroline and 200 progeny from 3 trees at Chiddarcooping. For paternity analysis in CERVUS, the total number of progeny analysed were 195 from the same 6 trees at Mt Caroline and 118 from the same 3 trees at Chiddarcooping. In addition, open-pollinated progeny sampled from 14 trees at Mt Caroline and 7 trees at Chiddarcooping were included in the mating and paternity analyses to allow a stronger assessment of mating patterns resulting from natural/un-manipulated pollination.

Microsatellite genotyping

Seeds were germinated on moist filter paper, and DNA extracted from shoot material using a modified CTAB method (Doyle & Doyle 1987). Microsatellite genotyping was conducted as described by Bezemer *et al.* (2016) and Bezemer (2018). Samples were genotyped at 14 microsatellite loci including EGM30, EGM47, EGM14, EGM12, EGM34, EMBRA10, EMBRA7, EMBRA20, EMBRA18, EMCRC11, EV22, EV23, EV28 and ES140 (Brondani *et al.* 1998; Glaubitz *et al.* 2001; Steane *et al.* 2001; Bradbury *et al.* 2013; Nevill *et al.* 2013). Two loci (EGM12 and EGM34) were monomorphic in a sample of adults from Chiddarcooping and thus were discarded from analysis for this population.

Paternity analysis and mating system

Paternity assignment by categorical allocation was implemented in CERVUS V 3.0 (Kalinowski *et al.* 2007), as described by Bezemer *et al.* (2016). Previous comprehensive microsatellite sampling of study populations by our team allowed for all local reproductive plants to be included as candidate sires. Paternity analysis for progeny from each study site was first conducted with only local plants included as candidate sires. For progeny that failed to meet the 80 % confidence threshold, analysis was repeated with adults from comprehensively sampled neighboring populations (refer to Bezemer *et al.* 2019) included as candidate sires. To allow detection of self-pollination, maternal genotypes were included in the pool of potential sires. The confidence levels of paternity assignments and the frequency distribution of logarithm of odds (LOD) scores were plotted on histograms for both study sites (Supporting Information Figure S2). If multiple sires with equal LOD scores were identified for a single seed, we considered the true identity of the sire to be ambiguous. Progeny with ambiguous paternity were not included in subsequent analyses or results.

In *E. caesia*, the long life-span (9 – 10 days) of flowers (Hopper and Wyatt, unpublished data) and high number of seeds per fruit (mean of 36 with 0 – 65 per fruit at Mt Caroline; mean of 103 with 12 – 160 per fruit at Chiddarcooping – Hopper *et al.* 1982), together with a capacity for high levels of multiple paternity (Bezemer *et al.* 2016), means that the progeny within fruit are likely to be the products of multiple independent pollination events. We randomly sampled up to 10 seeds per fruit, and so the chances that the sampled progeny are the products of a single pollinator visit, in which case the paternity of sample points would not be independent, are low. Indeed, one estimate of the mean number of sires per fruit is $4.6 \pm \text{s.e. } 0.3$ at the Boyagin stand of *E. caesia* (Bezemer *et al.* 2016). Therefore, progeny were treated as independent sample

points for subsequent pollen dispersal distance analyses. However, the paternal background of progeny within fruit from the same tree may depend to some extent on maternal effects such as genotype, location and plant size (Barrett & Harder 1996; De Nettancourt 2001; Barrett & Harder 2017). Thus, progeny from fruit from the same tree were pooled to give an average value for each maternal tree when testing for significant differences in the number of sires per fruit.

The results of the paternity assignment were used to directly calculate linear spatial distances between mates, outcrossing rates (outcrossed progeny/total progeny), and levels of multiple paternity. In plants with multiple flowers multiple mating may occur either when pollen from different donors is distributed among flowers, or when pollen from multiple donors is deposited on a single stigma. We measured multiple paternity as the average number of sires within fruit per maternal tree. In addition, multilocus outcrossing rate (t_m), biparental inbreeding (t_m-t_s) and correlation of paternity (r_p) were estimated at the tree level using MLTR (Ritland 2002). To test for differences in paternity measures and mating system estimates between treatments, paired t-tests were applied in the software R, assuming equal or unequal variance as appropriate (R Core Team 2014). For these tests, progeny from each maternal tree were pooled into two groups representing the open pollination and honeyeater exclusion treatments.

In addition to the above analyses, we tested for significant effects of treatment on outcrossing rate and number of sires at the fruit level. Here, sample sizes were $n = 24$ fruits for Mt Caroline and $n = 11$ fruits for Chiddarcooping. We applied mixed effects models and Analysis of Variance (ANOVA) using the nlme package in R, with maternal plant included as a random effect. The model was fitted using a restricted maximum likelihood method. The response variables were normalized against the number of seeds genotyped per fruit. To test the significance of the random term (maternal plant) in the model, mixed model likelihood ratio tests

were applied using the ANOVA function to compare the mixed effect models with a second set of null models that excluded the random effect (Molenberghs & Verbeke 2007). A significantly lower AIC (Akaike Information Criterion) value in the mixed effects model compared to the null model indicates a significant influence of the random term on the results of the mixed model. Likelihood ratio tests are considered to be generally appropriate to infer the importance of random effects in ecological studies (Bolker *et al.* 2009).

Frequency histograms were generated to visualise the number of observed mating events at different distance classes. To test for significantly different distributions of mating event distances for the two treatments within each study population, two-sample Kolmogorov-Smirnov tests to compare D , the maximum difference between two cumulative distributions, were implemented in R. Where the outcome of the Kolmogorov-Smirnov test was significant, two-Proportions Z-test to compare two observed proportions were applied to test for significant differences in the observed proportions of mating events between treatments within each distance class.

RESULTS

Mating patterns varied substantially among maternal trees, with outcrossing rates ranging from 0 – 100 % in open-pollinated plants across both study sites (Table 1). At Mount Caroline, there were no significant differences between the open-pollination and honeyeater-exclusion treatments in terms of mean (\pm s.e.) realised outcrossing rate (0.44 ± 0.13 for O-P vs 0.39 ± 0.14 for H-E; $P = 0.789$), mean (\pm s.e.) number of sires per fruit (5.3 ± 1.8 for O-P vs 4.3 ± 0.8 for H-

E; $P = 0.617$), or correlation of paternity ($0.17 \pm \text{s.e. } 0.07$ for O-P vs $0.22 \pm \text{s.e. } 0.11$ for H-E; $P = 0.906$; Table 2). In contrast, at Chiddarcooping mean outcrossing rates ($\pm \text{s.e.}$) were significantly lower when birds were excluded from flowers (0.87 ± 0.08 for O-P vs 0.35 ± 0.09 for H-E; $P = 0.013$). The level of observed heterozygosity ($\pm \text{s.e.}$) was significantly higher for the open-pollination treatment ($H_O = 0.41 \pm 0.02$ for O-P vs 0.19 ± 0.01 for H-E; $P < 0.001$). The mean number of sires ($\pm \text{s.e.}$) was approximately three times higher for the open-pollinated compared with the exclusion treatment, though this difference was non-significant (number of sires 13.0 ± 2.6 for O-P vs 4.3 ± 0.3 for H-E; $P = 0.06$).

Average outcrossing rates as estimated in MLTR were similar to those calculated from paternity assignment results. However, the negative correlation of paternity (r_p) value for progeny resulting from honeyeater-exclusion at Chiddarcooping contradicted the levels of paternal diversity calculated directly from the results of the paternity assignment, suggesting a bias in the MLTR estimate of r_p (Fernández-Manjarrés *et al.* 2006). We suspect that this bias has arisen due to ambiguity of paternal genotypes stemming from low marker polymorphism (Hardy *et al.* 2004). Successive iterations using either expectation maximization (EM) or Newton-Raphson (NR) methods resulted in variation of r_p estimates among bootstraps but the values remained close to zero, suggesting insufficient marker diversity at the Chiddarcooping site (K Ritland, personal communication).

Similar results were obtained when outcrossing rate and number of sires were modelled against treatment at the fruit level rather than the tree level (refer to Supporting Information Table S3). At Mt Caroline, there was no significant effect of treatment on outcrossing rate (F value = 1.84, $P = 0.19$) or number of sires (F value = 1.41, $P = 0.25$) but the inclusion of maternal plant as a random effect significantly influenced both models ($P \leq 0.01$; Table S4). In

contrast, at Chiddarcooping, treatment significantly effected outcrossing rate (F value = 13, P = 0.009) and number of sires (F value = 30.4, P < 0.001) and maternal plant was a significant random effect for outcrossing rate (P = 0.04) but not for number of sires (P = 0.25).

At Mount Caroline, the pollen dispersal distribution was strongly leptokurtic (kurtosis ≥ 6 for all treatments) with some relatively long-distance mating events. The overall distribution of mating event distances did not differ significantly between treatments ($D = 0.09$, $P = 0.790$; Figure 3). Two instances of realised pollen dispersal from a neighboring stand (with these assignments meeting 80 % and 95 % confidence thresholds), over a distance of 5 km, were documented – one each from the two treatments and therefore one or both of these mating events resulted from insect pollination (see data for Maternal 23, Figure 4). The extent of mating among sub-populations varied among maternal plants (Figure 4). Pollen dispersal distributions for open-pollination mating events were similar at Mt Caroline regardless of whether 6 or 14 maternal trees were included in the analysis.

In contrast to Mt Caroline, mating resulting from open pollination had a left-skewed peaked distribution at Chiddarcooping (kurtosis = ~ 6 whether three or seven maternal trees are included), with the highest proportion of mating events (49 %) occurring within the 1 – 40 m distance class (Figure 3). The inclusion of additional maternal trees resulted in detection of mating events occurring at ≥ 80 m (as a result of the location of additional maternal trees in the large northern sub-population). The distribution of distances between mating pairs in the honeyeater exclusion and open pollination treatments were significantly different at Chiddarcooping ($D = 0.53$, $P < 0.001$). There were significantly less frequent self-pollination (0.17 for O-P vs 0.70 for H-E; $p < 0.001$) and more frequent mating occurring between 1 and 40

m (0.49 for O-P vs 0.05 for H-E; $p < 0.001$) and 41 – 80 m (0.19 for O-P vs 0.0 for H-E; $p < 0.01$) when honeyeaters had access to inflorescences. All three maternal trees included in the exclusion experiment mated with plants located in the large northern sub-population (Figure 5).

Mean pollen dispersal distances were not significantly different between treatments. At Mount Caroline the mean pollen dispersal distance (\pm s.e.) was not significantly different when honeyeaters were excluded (145.6 ± 66.0 m for 104 mating events resulting from H-E vs 104.2 ± 50.1 m for 96 mating events resulting from O-P; $P = 0.662$), with a mean distance of 98.3 ± 26.0 m across all 215 open-pollinated progeny. Mean pollen dispersal (\pm s.e.) within Mount Caroline not including self-pollination events was 123.5 ± 35.7 m for O-P and 148 ± 44 m for the H-E treatment. Including self-pollination, the median pollen dispersal values at Mt Caroline were nil.

Similar to Mt Caroline, mean pollen dispersal distance (\pm s.e.) at Chiddarcooping was not significantly different when honeyeaters were excluded (232.5 ± 54.1 m for 57 mating events resulting from H-E vs 137.2 ± 31.9 m for 75 mating events resulting from O-P; $P = 0.113$). Here, high means were driven by a small number of long-distance mating events, similar to what was observed at Mt Caroline. Across the 129 sampled open-pollinated progeny the mean pollen dispersal distance at Chiddarcooping was $144 \pm$ s.e. 22.1 m (median value 39.9 m). Mean pollen dispersal distances (\pm s.e.) not including self-pollination events was 166 ± 37.7 m for O-P and 779.6 ± 87.8 m for H-E, reflecting the high rate of selfing (70 % of mating events) in insect-pollinated progeny. Nonetheless, 25 % of insect-mediated dispersal events occurred between plants ≥ 480 m apart. Median values at Chiddarcooping were nil for honeyeater-exclusion and 34.6 m for open-pollination treatments.

DISCUSSION

The relative contribution of different functional pollinator groups to pollen dispersal and mating patterns has remained largely unexplored within plant species. *Eucalyptus caesia* is a predominantly bird-pollinated tree that is also visited by native insects and the introduced honeybee. We tested the hypothesis that mating parameters will be negatively impacted when the primary functional pollinator group is excluded from inflorescences of a predominantly honeyeater-pollinated plant. Surprisingly, our application of pollinator exclusion experiments and paternity analysis yielded contrasting results between two study populations of *E. caesia*. At Chiddarcooping, honeyeater exclusion resulted in lower outcrossing rates and lower observed heterozygosity among progeny, a threefold reduction in the number of sires per fruit, and a higher incidence of near-neighbour mating, supporting our hypothesis. Conversely, at Mount Caroline, no significant differences between open pollination and honeyeater exclusion treatments were found.

Outcrossing, multiple paternity and correlation of paternity

A key result of our study is that data from our two study sites provide conflicting evidence in relation to the hypothesis that bird pollination facilitates mating with a diverse array of sires (Hopper & Moran 1981; Sampson 1998; Krauss *et al.* 2017). At Chiddarcooping, the average number of sires per fruit was three times higher in progeny resulting from open pollination compared to honeyeater exclusion treatments. Also at this site, higher levels of observed heterozygosity among progeny resulting from open pollination confirm that bird visitation facilitates mating with a wide array of genetically diverse sires. These results are

concordant with studies on other plant families that have focused on the rates of self-pollination via geitonogamy versus outcrossing following bird exclusion from inflorescences. Honeyeater exclusion at Chiddarcooping resulted in significantly lower outcrossing rates, with an effect size of a 52 % difference between treatments – a notably larger effect compared to bird exclusion experiments in *Grevillea macleayana* (< 10 % effect size; England *et al.* 2001) and *Metrosideros exelsa* (31 % effect size; Schmidt-Adam *et al.* 2009). Outcrossing rates, however, can vary substantially among plants, and within eucalypt species may range from nil to complete outcrossing (Hingston & Potts 2005; Patterson *et al.* 2005; Bradbury & Krauss 2013; Bezemer *et al.* 2016). Whether the impact of bird exclusion on outcrossing rates varies among individuals at Chiddarcooping requires further experimentation with additional maternal trees.

In contrast with Chiddarcooping, honeyeater exclusion had no effect on mating parameters or pollen dispersal at Mt Caroline. Consequently, data from this study site do not support our hypothesis. Although further studies are needed, it may be that experimental results from Mt Caroline are largely driven by a large population of *A. mellifera* (Horskins & Turner 1999; Gross 2001). *Apis mellifera* have the ability to regulate hive temperatures (Stabentheiner *et al.* 2003) and warm themselves using thoracic flight muscles (Kovac *et al.* 2010), allowing them to forage on many species of winter-flowering bird-pollinated plants in south-west Australia. Foraging by native insects on winter-flowering bird-adapted plants, particularly in inland regions, is hypothesized to be minimal due to cold winter temperatures that may limit activity of many native insect pollinators (Hopper 1981). Further, winter is likely to be outside the flying season of many native hymenopterans (Brown *et al.* 1997) – although in south west Australia winter flying fungus gnats and some bee species are important pollinators of winter-flowering orchids and pea plants (Phillips *et al.* 2013; Scaccabarozzi *et al.* 2018), and native bees forage on

the winter-flowering *Banksia menziesii* (Ramsey 1988). *Apis mellifera* may be removing large amounts of pollen that may have otherwise been moved by primary native pollinators (Aizen *et al.* 2014) and therefore may be the dominant pollinator in both the open-pollination and honeyeater-exclusion treatments at Mt Caroline. Exclusion experiments in additional populations that vary in abundance of *A. mellifera*, or experiments where visits by both insects and birds are independently manipulated, are required to test the hypothesis that introduced insects may be driving mating patterns in some populations of bird-pollinated plants (Gross 2001; Yates & Ladd 2004).

The species composition of pollinator assemblages differs geographically, which may be an important factor driving our results. In contrast to the large *A. mellifera* population at Mt Caroline, our field observations indicate that Chiddarcooping has very few introduced *A. mellifera*. This study site is therefore likely to be more representative of a ‘natural’ pollinator community. However, thynnine wasps and native bees have only been recorded on flowers of *E. caesia* on warm days at the end of the winter flowering season of *E. caesia* subsp. *magna* (SDH, personal observations). Nonetheless the potential role of insect groups that are tolerant of cool temperatures (e.g. large moths, syrphid flies and some native bees) as pollinators warrants further investigation (Houston 2000; Scaccabarozzi *et al.* 2018).

In eucalypts an open staminal ring surrounding an exposed stigma allows a range of different pollinator groups including birds, insects and mammals to feed on nectar or pollen and potentially contact the stigma (House 1997; Southerton *et al.* 2004). Further, all of the honeyeater species that visit *E. caesia* are generalists that forage on both nectar and insects (Pyke 1980; Hopper 1981). Similar experiments in other relatively morphologically unspecialised plants – such as species that are pollinated by short-billed hummingbirds and insects (Dalsgaard

et al. 2009; Lehmann *et al.* 2019) – may yield similar results to our study. In contrast, plants that are solely pollinated by nectar feeding birds with longer and/or curved bills, such as some hummingbirds (Martén-Rodríguez & Fenster 2008), sunbirds (Van der Niet *et al.* 2015) and honeyeaters (Johnson *et al.* 2010; Ayre *et al.* 2019), may yield different results to our study because the floral architecture prevents or impedes stigma contact by most insect groups (Ne'eman *et al.* 2010). Paternity studies in a group of related plants with contrasting pollination strategies (e.g. *Aloe*; Botes *et al.* 2009), or of multiple unrelated plant species in regions characterised by a hyper-diverse bird-pollinated flora (e.g. the South West Australian Floristic Region; Hopper & Gioia 2004), may provide suitable systems to test for differences or similarities to our study species in other bird-pollinated plants.

Variance in mating parameters was high at Mt Caroline, but seemingly low at Chiddarcooping. We hypothesize that these differences in variance are driven by the spatial distribution of the maternal trees that were able to be included in the analyses – those at Mt Caroline were scattered across the population in isolated patches of trees. In contrast, at the end of the experiment, trees with fruit representing both treatments at Chiddarcooping were all located within the same small stand to the south of the main stand (refer to Figure 5). The number and distribution of maternal plants may have influenced the results at this site. Indeed, when outcrossing rate and number of sires were modelled at the fruit level, maternal plant was an important random effect influencing the outcome of the analyses. As indicated by our study and others, the absolute value of mating parameter estimates, especially outcrossing rate, varies between individual plants. However, we hypothesis that the relative difference (i.e., the effect size) between treatments will be more or less consistent across individuals. A repeat experiment involving additional or different maternal plants is required to test this hypothesis. However,

even with few numbers of maternal trees, statistical confidence in differences between mating parameters between treatments may be achieved where the effect size is large or the variance in combined error rates is small (Ioannidis 2005; Lemoine *et al.* 2016), as was the case for our study populations.

Pollen dispersal distances

We are aware of only a single published paper that involved quantification of differences in realised pollen dispersal distances using a combined pollinator exclusion and genotyping experiment (Rhodes *et al.* 2017). In the annual forb *Oenothera harringtonii* there was no significant difference in average pollen dispersal distance or the distribution of realised mating events when pollinated by either hawkmoths or solitary bees (Rhodes *et al.* 2017). In contrast, we found that honeyeater exclusion at Chiddarcooping resulted in a more spatially restricted pollen dispersal distribution, even though a surprisingly large proportion of insect-mediated pollen dispersal events occurred over relatively large distances (refer to Figure 3). Over the course of our experiment we observed high levels of bud abortion at both study sites and many plants did not flower at all. This naturally patchy spatial distribution of inflorescences may be beneficial for pollen dispersal as pollinators need to travel further among flowering plants (Sampson 1998; Phillips *et al.* 2014). Such a scenario would support the findings of Rhodes *et al.* (2017) that density, distribution or abundance of flowering plants influences realised pollen

dispersal. As with the mating system parameters, there was no significant effect of honeyeater exclusion on pollen dispersal distance or the distribution of mating events at Mt Caroline.

Patterns of pollen dispersal at both study sites were congruent with population spatial genetic structuring in *E. caesia* (Bezemer *et al.* 2019). At Mt Caroline, most realised pollen dispersal events were restricted to within 80 metres but 14 % of all mating events were between mates that were beyond the area to which positive spatial structure occurs, confirming predictions of infrequent admixture among spatially disjunct sub-populations. In contrast, at Chiddarcooping strong positive spatial autocorrelation occurs to < 100 m and a relatively large proportion of mating events (35 % of total) surpassed this distance. At this study site, the pollen dispersal distribution is more restricted when only three maternal trees (rather than seven) are included because the maternal trees are located in a relatively small and isolated sub-population (refer to Figure 2).

Instances of mating among widely spaced individuals (> 480 m apart) was documented at both study sites. We documented two pollen dispersal events of 5 km from a small neighbouring stand to Mt Caroline, one or both that were the result of insect pollination. We suspect relatively large-bodied *A. mellifera* rather than small native insects to be responsible for these mating events because *A. mellifera* are known to move pollen over areas spanning up to several kilometers (Southwick & Buchmann 1995; Pasquet *et al.* 2008; Pahl *et al.* 2011) and because of their high abundance at this site. However, we note that body size of bees is not a consistent predictor of relative contribution to long-distance pollen flow in other tree species (Castilla *et al.* 2017) and native wasps and bees may move pollen over surprisingly large distances, especially in undisturbed landscapes (Nason *et al.* 1996; Jha & Dick 2010; Menz *et al.* 2013). Indeed, small

native insects are responsible for long distance dispersal of pollen dispersal in *Eucalyptus nitens* (Barbour *et al.* 2005) and possibly other eucalypts (Byrne *et al.* 2008; Jones *et al.* 2008).

Candidate sires could not be identified for 31 progeny (16 of which resulted from open pollination and 15 from honeyeater exclusion treatments) at Chiddarcooping. Unassigned progeny – particularly those in open-pollinated arrays – could indicate pollen migration from an external source such as other subpopulations within Chiddarcooping Nature Reserve or from neighbouring stands of *E. caesia* that are located 5 – 10 km away. Indeed honeyeaters are capable of moving 10 – 15 km over a few days (Saunders & De Rebeira 1991; Higgins *et al.* 2001). However, no non-local alleles were detected among the unassigned progeny suggesting that failure to assign paternity could simply reflect insufficient power to differentiate between candidate sires due to relatively low levels of polymorphism and overall lower allelic diversity at Chiddarcooping compared to other populations of *E. caesia* (Bezemer *et al.* 2019).

The results of our experiment support mating system studies at the Boyagin population of *E. caesia* that suggest that mating is largely restricted to plants within spatially isolated stands (Bezemer *et al.* 2016; Bezemer 2018). Pollen migration among populations of other bird-pollinated plants in south-west Australia also appears to be primarily restricted to neighboring populations a few kilometers apart, though in some cases pollen movement over this distance is frequent (Byrne *et al.* 2007; Tapper *et al.* 2014; Nistelberger *et al.* 2015). Spatially restricted pollen dispersal also characterizes historically fragmented populations of the predominantly hummingbird- and bat-pollinated *Encholirium horridum* (Hmeljevski *et al.* 2017). It is possible that the restricted pollen dispersal found in these studies largely relates to the territorial behavior of the bird pollinator species involved (Stiles 1978; Ford *et al.* 1979; Krauss *et al.* 2017). Pollination by nectarivorous birds that move long distances to forage on large nectar sources

(e.g. parrots) or forage on scattered small nectar sources (i.e. trapline foragers) may result in a higher proportion of long distance mating events than observed in our study populations if nectar sources are sporadically located (Franceschinelli & Bawa 2000).

Variability of mating patterns among plants and populations

Our study supports existing evidence that pollen dispersal and mating patterns are highly variable within and among plant populations (Whitehead *et al.* 2018). Populations of *E. caesia* at Boyagin (see Bezemer *et al.* 2016), Mount Caroline and Chiddarcooping have strikingly different patterns of mating and pollen dispersal. Differences in relative visitation rates of the main pollinator functional groups and differences in population spatial structure and are likely to be important drivers of the strikingly divergent mating patterns among populations of *E. caesia*. Alternatively, the differential 24 hour nectar production of the two subspecies of *E. caesia* may favour greater attention from birds v/s insects (Castellanos *et al.* 2002), or birds may behave differently on flowers of subspecies *magna* v/s subspecies *caesia* due to differences in flower size and dispersion (Parachnowitsch & Kessler 2010). It is also possible that insects may be less likely to contact the reproductive structures of the larger *E. caesia* subsp. *magna* flowers, which could explain the more pronounced difference between treatments at Chiddarcooping. Experiments in additional populations of each subspecies are required to test these hypotheses.

Similar to other studies in eucalypts we found pronounced variation in mating parameters within and among individual trees (Hingston & Potts 2005; Bradbury & Krauss 2013). For instance, outcrossing rates in individuals of *E. caesia* range from nil to completely outcrossed (Bezemer *et al.* 2016; present study). A combination of ecological factors and genetic factors are

likely to be important here. For example, flowering phenology, patterns of pollinator activity, individual plant sizes and density/proximity of conspecifics may influence rates of self- versus cross-pollination or levels of multiple paternity (Murawski & Hamrick 1991; Charpentier 2001; Hingston & Potts 2005; Rhodes *et al.* 2017). Genetic factors including the stringency of self-incompatibility (SI) mechanisms directly impact mating patterns and variation in levels of SI have been documented in eucalypts (Patterson *et al.* 2005; McGowen *et al.* 2010).

Concluding remarks

Few studies have examined the molecular genetic consequences of the exclusion of the primary pollinator group. Our results extend the findings of England *et al.* (2001) and Rhodes *et al.* (2017), showing that not only outcrossing rate, but also pollen dispersal and key mating parameters including multiple paternity may be adversely impacted when highly mobile, large-bodied pollinators such as honeyeaters are excluded from the inflorescences of plants adapted for bird pollination (Hopper 1981; Bezemer *et al.* 2016; Krauss *et al.* 2017). In some populations, honeyeater visitation increases the likelihood that flowers are pollinated by a wide array of genetically diverse sires, which has positive consequences for offspring fitness (Breed *et al.* 2014; Nora *et al.* 2016). However, if populations of historically fragmented plants have undergone genetic purging in response to long periods of isolation and inbreeding then the potentially beneficial effects of wide outcrossing associated with bird pollination may be inconsequential (James 2000; Byrne & Hopper 2008; Hopper 2009; Bezemer *et al.* 2019).

Pollination by the introduced *A. mellifera* may set plant species on a new evolutionary trajectory of potentially greater rates of self-pollination and spatially restricted mating with genetic relatives. We hypothesize that large numbers of honeybees at the Mt Caroline stand of *E. caesia* are reducing the opportunity for pollination by birds, either by removing most of the pollen that may have otherwise been moved by birds (Botes *et al.* 2009) or by physically disrupting native pollinator foraging bouts (Aizen *et al.* 2014). For example, at the Boyagin stand of *E. caesia*, honeyeaters have been observed removing *A. mellifera* from inflorescences to forage (DGR, personal observation). Alternatively, insects including *A. mellifera* may be effective pollinators in some populations of plants adapted to bird pollinators, but ineffective in others depending on their abundance and levels of activity during the flowering season. A third possibility is that pollination by insects including *A. mellifera* and honeyeaters may result in similar patterns of mating and pollen dispersal. Experiments in other bird-pollinated plants are needed to assess the molecular genetic consequences of loss or replacement of native bird pollinators following the introduction of non-native pollinators.

ACKNOWLEDGEMENTS

This project was funded by an Australian Research Council grant to SDH, SLK and RD Phillips (DP140103357), including a discovery Outstanding Researcher Award to SDH, and by a Holsworth Wildlife Research Endowment – Equity Trustees Charitable Foundation, Wiley Fundamental Ecology Award, Ecological Society of Australia Student Research Award, and UWA and Graduate Women (WA) Research Scholarship to NB, who was supported by an Australian Postgraduate Award and a UWA Top-up Scholarship at the University of Western Australia. SDH was also supported by grants from the Great Southern Development Commission

and Jack family trust. A licence to take flora for scientific purposes within Department of Biodiversity, Conservation and Attractions lands (SW018638) and approval was obtained from the Animal Ethics Committee, University of Western Australia (RA/3/100/1344).

DATA ACCESSIBILITY

Microsatellite genotypes and GPS locations of all samples will be uploaded to the Dryad online repository.

AUTHOR CONTRIBUTIONS

Conception of this research was contributed to by all authors. NB and DGR deployed and maintained the field experiments following a preliminary field excursion with SDH. NB did the laboratory and statistical analysis and drafted the manuscript. All authors provided critical revisions on drafts of the manuscript and approved the final submission.

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Table 1 Outcrossing rate and number of different sires calculated from CERVUS paternity assignment results for *Eucalyptus caesia* progeny resulting from open-pollination and honeyeater-exclusion treatments at Mount Caroline and Chiddarcooping Nature Reserves, Western Australia.

Mount Caroline Nature Reserve						
Tree	Open-pollination (O-P, n progeny = 92)			Honeyeater-exclusion (H-E, n progeny = 103)		
	n progeny (n progeny per fruit)	proportion outcross	number sires	n progeny (n progeny per fruit)	proportion outcross	number sires
Ec13	14 (6, 8)	0.57	5	16 (8, 8)	1.00	5
Ec18	17 (8, 9)	0.71	3	14 (5, 9)	0.36	3
Ec19	10 (3, 7)	0.20	3	14 (7, 7)	0.21	4
Ec23	17 (7, 10)	0.88	14	20 (10, 10)	0.55	8
Ec24	19 (9, 10)	0.16	4	20 (10, 10)	0.10	3
Ec30	15 (6, 9)	0.13	3	19 (9, 10)	0.11	3
Mean ± s.e.	15.3 ± 1.3	0.44 ± 0.13	5.3 ± 1.8	17.2 ± 1.2	0.39 ± 0.14	4.3 ± 0.8
Ec15	19 (9, 10)	0.37	5	-	-	-
Ec22	18 (8, 10)	0.45	7	-	-	-
Ec26	15 (9, 6)	0.0	1	-	-	-
Ec27	18 (9, 9)	0.28	6	-	-	-
Ec29	18 (10, 8)	0.28	5	-	-	-
Ec31	16 (6, 10)	0.06	2	-	-	-
Ec800	6 (6)	0.83	2	-	-	-
Ec822	9 (9)	0.45	5	-	-	-
Total mean ± s.e. (n = 211)	15.1 ± 1.1	0.38 ± 0.07	4.6 ± 0.8	-	-	-
Chiddarcooping Nature Reserve						
Tree	Open-pollination (O-P, n progeny = 61)			Honeyeater-exclusion (H-E, n progeny = 57)		
	n progeny (n progeny per fruit)	proportion outcross	number sires	n progeny (n progeny per fruit)	proportion outcross	number sires
CNR1	17 (17)	1.0	12	17 (17)	0.47	4
CNR4	10 (6, 4)	0.90	10	10 (10)	0.40	5
CNR20	34 (17, 9, 8)	0.71	18	30 (13, 8, 9)	0.27	4
Mean ± s.e.	20.3 ± 7.1	0.87 ± 0.08	13.3 ± 2.4	19 ± 5.9	0.35 ± 0.09	4.3 ± 0.3
CNR11	14 (5, 9)	0.64	9	-	-	-
CNR65	18 (9, 9)	1.0	14	-	-	-
CNR97	8 (5, 3)	1.0	8	-	-	-
CNR239	9 (5, 4)	1.0	7	-	-	-
Total mean ± s.e. (n = 110)	16 ± 3.3	0.90 ± 0.06	11.1 ± 1.4	-	-	-

Table 2 Mating system estimates and observed heterozygosity (H_o) for *Eucalyptus caesia* progeny resulting from open-pollination and honeyeater-exclusion treatments at Mount Caroline and Chiddarcooping Nature Reserves. Six trees at Mount Caroline and three trees at Chiddarcooping were part of a paired experiment, where both open-pollination and honeyeater-exclusion treatments were applied to each tree. Estimates calculated in MLTR include multi-locus outcrossing rate (t_m), biparental inbreeding (t_m-t_s) and correlation of paternity (r_p).

Study site	Treatment	n		MLTR estimates (value \pm standard error)			GenAlEx H_o
		trees	progeny	t_m	t_m-t_s	r_p	
Mount Caroline	Open-pollination	14	270	0.38 ± 0.01	0.13 ± 0.01	0.53 ± 0.03	0.30 ± 0.01
	Open-pollination	6	120	0.38 ± 0.04	0.08 ± 0.01	0.17 ± 0.07	0.30 ± 0.01
	Honeyeater-exclusion	6	120	0.30 ± 0.05	0.09 ± 0.01	0.22 ± 0.11	0.28 ± 0.01
Chiddarcooping	Open-pollination	8	218	0.68 ± 0.01	0.03 ± 0.00	0.15 ± 0.00	0.37 ± 0.01
	Open-pollination	3	103	0.84 ± 0.01	0.03 ± 0.00	0.06 ± 0.00	0.41 ± 0.02
	Honeyeater-exclusion	3	97	0.25 ± 0.03	0.09 ± 0.01	-0.31 ± 0.05	0.19 ± 0.01



Figure 1 A White-eared Honeyeater on *Eucalyptus caesia* (top left), a bird-exclusion wire mesh cage (top right; photograph by DGR), and introduced *Apis mellifera* that were observed visiting flowers contained within honeyeater-excluding cages at Mount Caroline Nature Reserve (bottom left and right). Photographs by NB unless otherwise indicated.

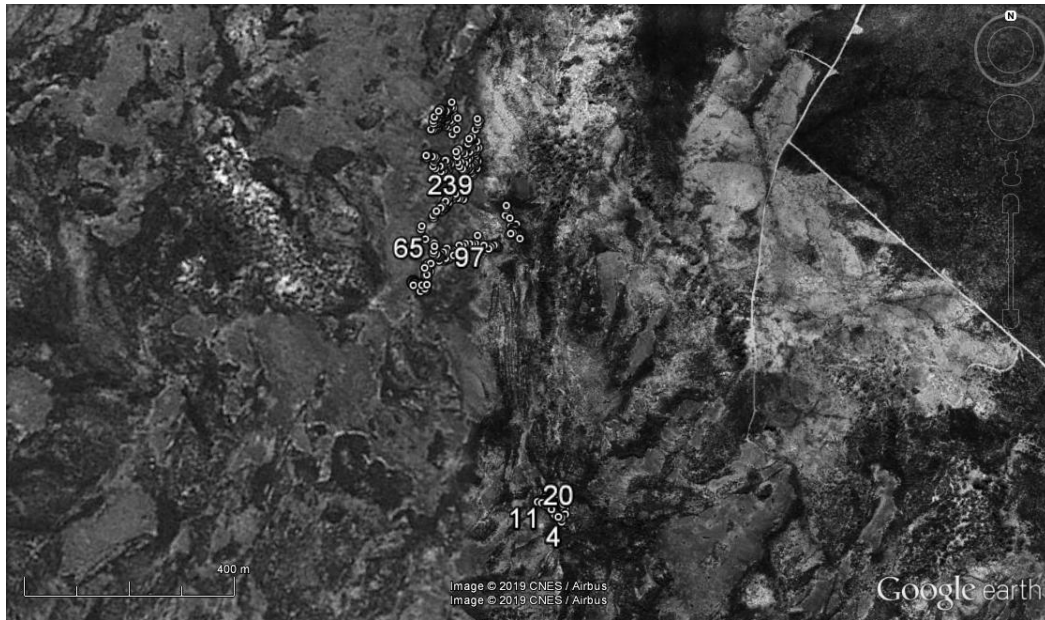
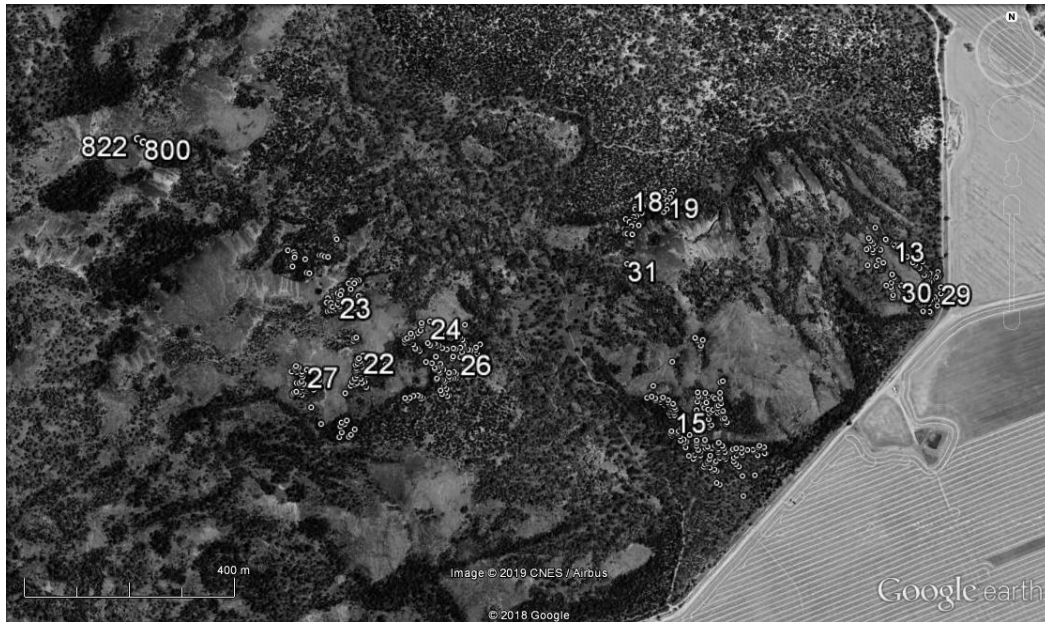


Figure 2 Distribution of comprehensively sampled population of *Eucalyptus caesia* at Mount Caroline (31°47'29"S, 117°38'14" E) and Chiddarcooping (30°53'41"S, 118°39'11"E) Nature Reserves overlaid on Google Earth imagery, and location of maternal plants (denoted by numbers) included in a paternity and mating system study.

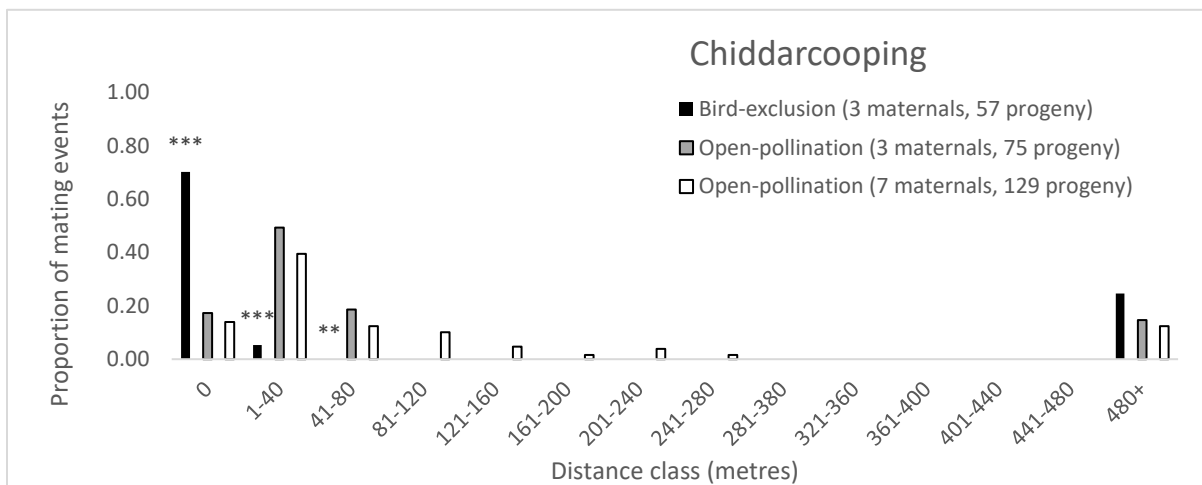
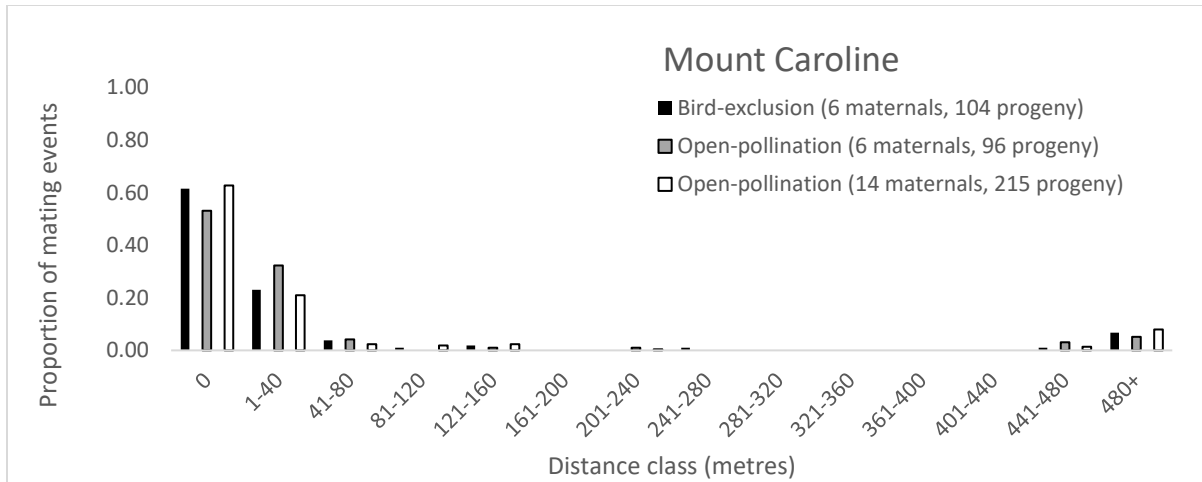
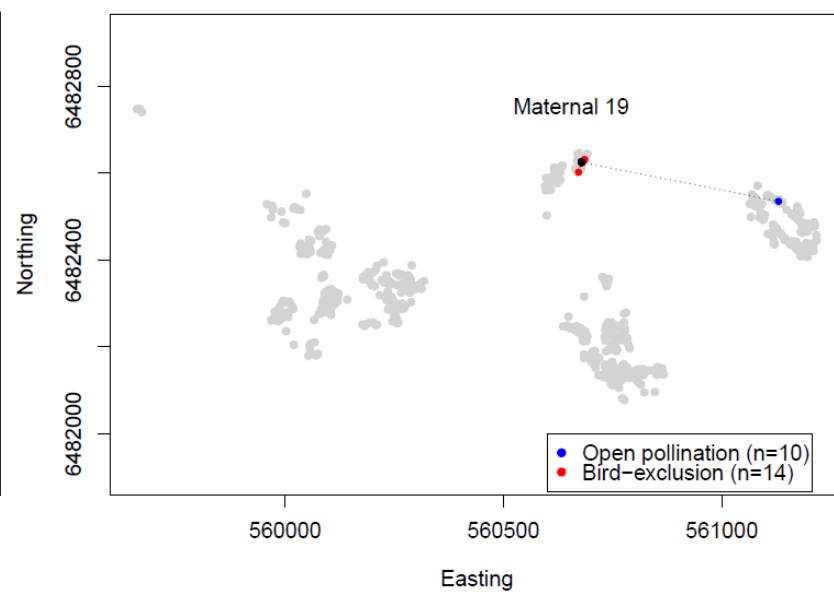
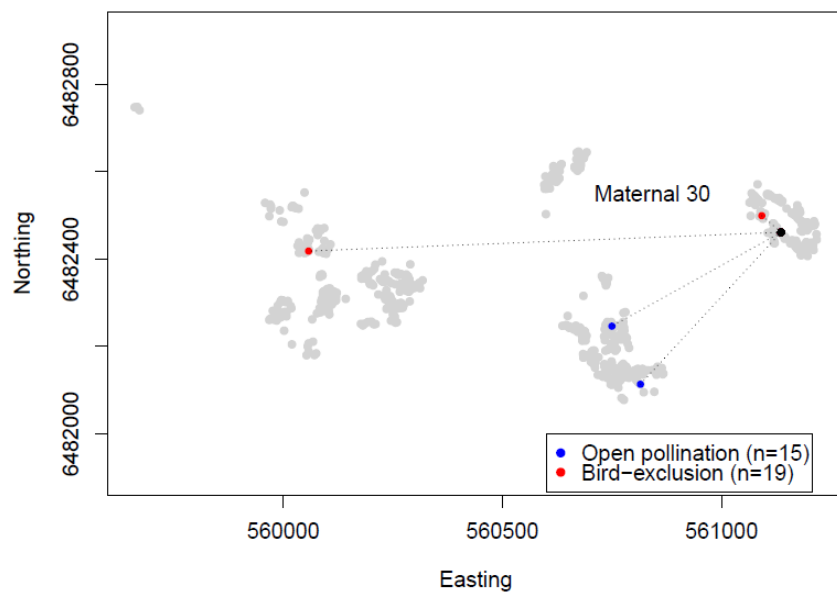
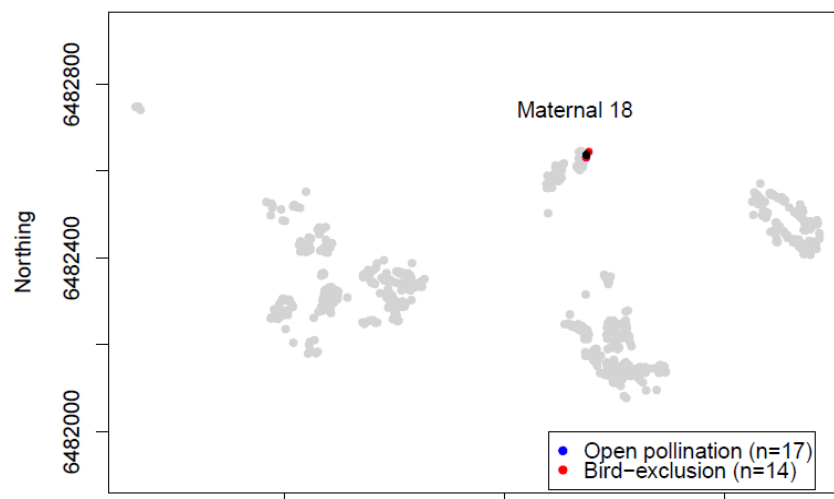
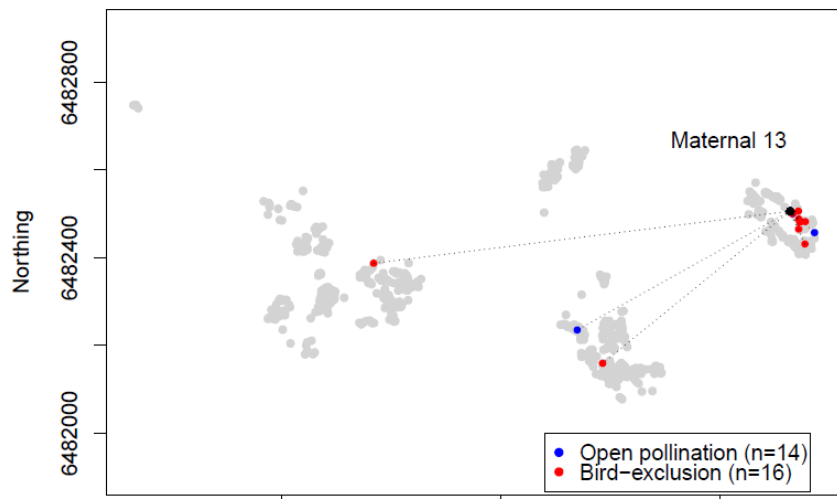


Figure 3 Frequency histogram of the distribution of mating events based on results of a paternity assignment of *Eucalyptus caesia* progeny resulting from open-pollination and honeyeater-exclusion treatments at Mount Caroline and Chiddarcooping Nature Reserves, Western Australia. Six trees at Mount Caroline and three trees at Chiddarcooping were part of a paired experiment, where both open-pollination and honeyeater-exclusion treatments were applied to each tree. Significant differences in the proportion of mating events between treatments in the paired experiments at each distance class are indicated ($p = 0.001$ ‘***’ and 0.01 ‘**’). Data from additional open-pollinated trees at each site are included.



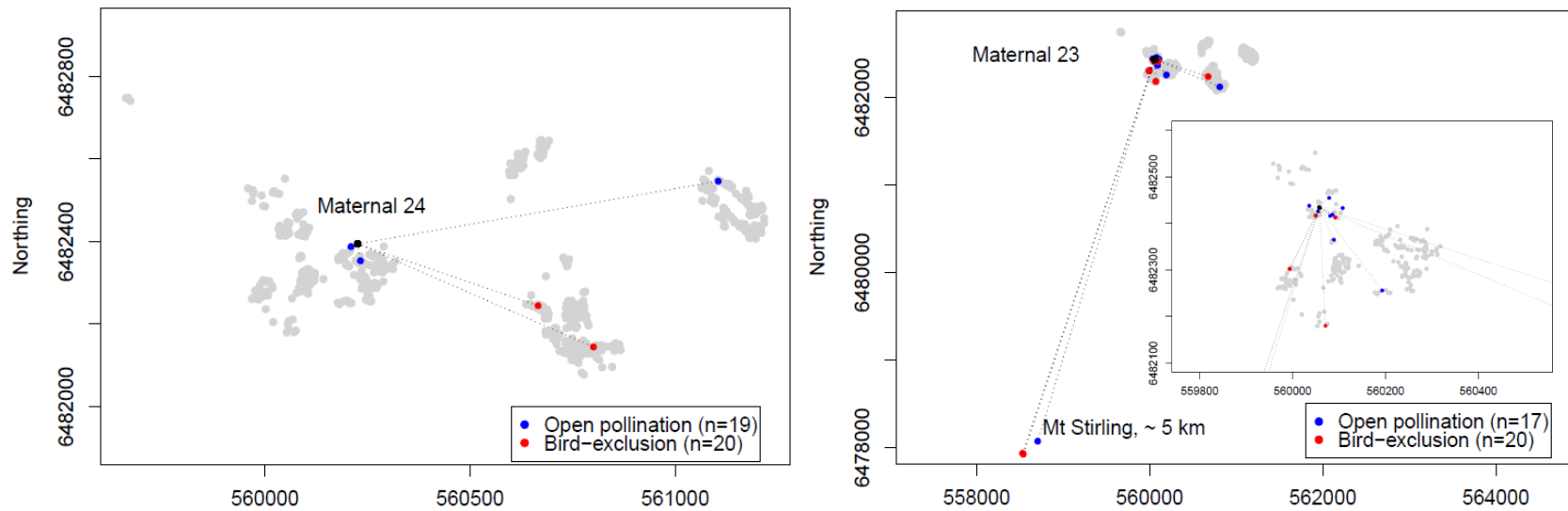


Figure 4 Comparison of pollen dispersal distances in *Eucalyptus caesia* under open-pollination and bird-exclusion treatments for six maternal plants at Mount Caroline Nature Reserve (n = number of progeny). Grey dots indicate comprehensively sampled *E. caesia*, included as candidate sires for paternity assignment.

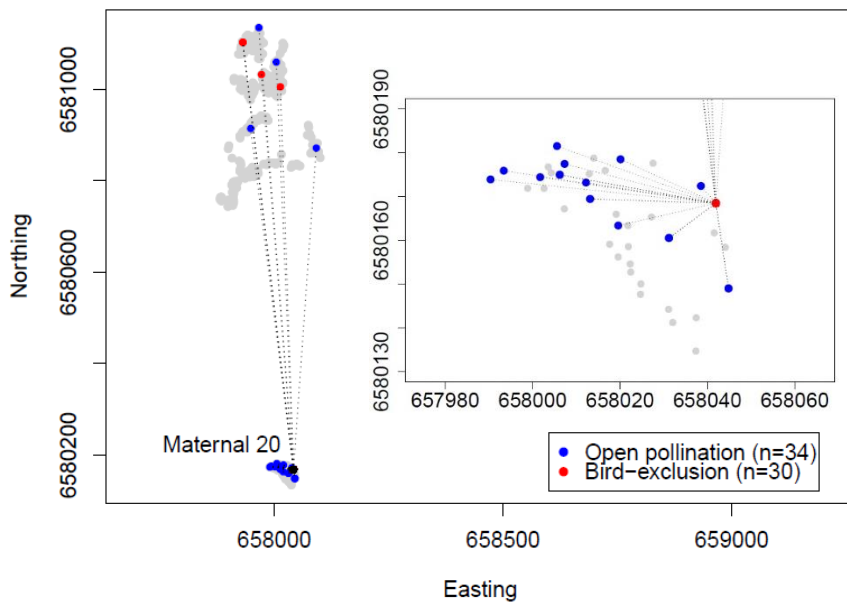
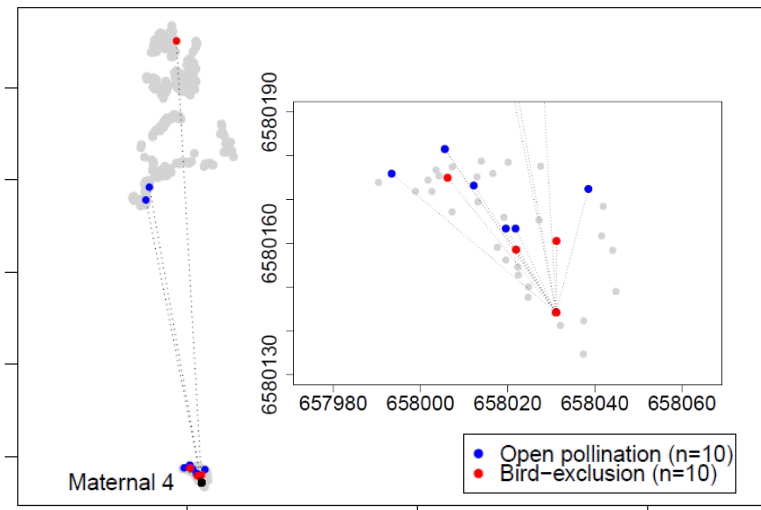
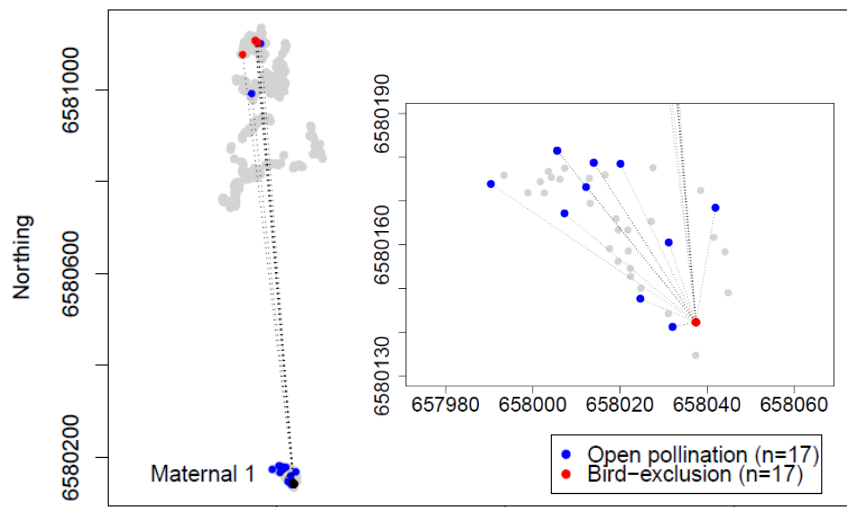


Figure 5 Comparison of pollen dispersal distances in *Eucalyptus caesia* under open-pollination and bird-exclusion treatments for three maternal plants at Chiddarcooping Nature Reserve (n = number of progeny). Grey dots indicate comprehensively sampled *E. caesia*, included as candidate sires for paternity assignment.

