Effectiveness of native nectar-feeding birds and the introduced *Apis mellifera* as pollinators of the kangaroo paw, *Anigozanthos manglesii* (Haemodoraceae)

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Running title: Birds, bees and *Kangaroo Paw* pollination

Summary text (table of contents): Plants pollinated by vertebrates are often visited by native and introduced insect species foraging for pollen and nectar, with potentially negative consequences for plant fitness. European Honeybees are the most common visitor to the Red and Green Kangaroo Paw but primarily steal nectar and pollen, with native nectar-feeding birds critical for high reproductive success. For many plant species, the relative importance of bird-pollinators and exotic insect species remains largely unknown.

Abstract:

Plants pollinated by vertebrates are often visited by native and exotic insects foraging for pollen and nectar. We compared flower visitation rates, foraging behaviour, and the contribution to reproduction of nectar-feeding birds and the introduced honeybee *Apis mellifera* in four populations of the bird-pollinated *Anigozanthos manglesii* (Haemodoraceae). The behaviour of floral visitors was quantified with direct observations, motion-triggered and hand-held cameras. Pollinator access to flowers was manipulated by enclosure in netting to either exclude all visitors or to exclude vertebrate visitors only. *Apis mellifera* was the only insect observed visiting flowers, and the most frequent flower visitor, but primarily acted as a pollen thief. While birds visited *A. manglesii* plants only once per week on average, they were 3.5 times more likely to contact the anther and/or stigma as foraging honeybees. Exclusion of birds resulted in 67% fewer fruits and 81% fewer seeds than flowers left open and unmanipulated. Unnetted flowers that are open to bird and insect pollinators showed pollen-limitation and a large variation in reproductive output within and between sites. While honeybees have been shown to pollinate other Australian plants, they are highly inefficient pollinators compared to birds for *A. manglesii*.

Key words: Pollinator exclusion, bird-pollination, *Apis mellifera*, pollen limitation, pollen-thief

Introduction:

Worldwide, approximately 500 of 13,500 vascular plant genera have members pollinated by birds (Sekercioglu 2006; Whelan *et al.* 2015). While some plants are pollinated by one or a few bird species, most nectarivorous birds, including those with highly specialised beak morphology, feed on a range of plant species (Johnson 2010; Abrahamczyk *et al.* 2014). A diverse range of bird families are involved, but hummingbirds, sunbirds and honeyeaters are the groups of pollinators richest in species (Krauss *et al.* 2017). Unlike some other pollen vectors, birds often display aggressive territorial behaviour (Stiles 1975; Hopper 1980a; Temeles and Kress 2010) and are likely to move further within and between populations than many of their insect counterparts (Stiles 1975; Phillips *et al.* 2014; Krauss *et al.* 2017). Bird-pollination is more prevalent in the tropics and in the Southern Hemisphere (Krauss *et al.* 2017), and a feature of plant communities on old, climatically buffered, infertile landscapes (OCBILS) (Hopper 2009; Hopper *et al.* 2016). One explanation for this trend is the James Effect (Hopper 2009) - the selection for adaptations that conserve heterozygosity in small populations. Indeed, there is evidence that, compared with insects, birds facilitate comparatively wide outcrossing, high paternal diversity, and connect fragmented plant populations (Breed *et al.* 2014; Krauss *et al.* 2017), most likely through carrying large, genetically diverse pollen loads over greater distances.

In many cases, bird-pollinated flowers are also visited by other pollinator groups, particularly non-flying mammals and bees (Goldingay 1990; Anderson *et al.* 2016). However, the morphological features that promote bird-pollination may show a mismatch with the morphology and foraging behaviour of insect visitors, meaning that they are comparatively ineffective at removing and/or depositing pollen (Castellanos *et al.* 2004; Hargreaves *et al.* 2009; Stang *et al.* 2009; Hargreaves *et al.* 2012). Insect visitors may act as nectar and/or pollen ‘thieves’, visiting flowers but not contacting stigmas, or contacting the stigmas but only depositing poor-quality pollen (heterospecific, self), or no

pollen at all (Hargreaves *et al.* 2009). They may also act as ‘robbers’ if they damage the flowers while committing floral larceny (Iouye 1980, Maloof and Inouye 2000). Most animals that thief pollen or nectar are legitimate pollinators of other plant species, suggesting that in many cases the pollination efficacy of a particular animal taxon for a given plant is driven primarily by floral traits, rather than the foraging behaviour of the pollinator (Hargreaves *et al.* 2009).

One of the most common insect visitors to flowering plants is *Apis mellifera*, the European Honeybee (Han *et al.* 2012). Although its native range encompasses Europe, Africa and the Middle East, it is now found across all continents except Antarctica (Han *et al.* 2012). In Australia, the European honeybee was introduced in the 1820’s, with feral and managed colonies now spread across the continent (Pyke 1999). They visit flowers of at least 1000 Australian plant species spanning over 200 genera (Paton 1996). While they are able to successfully pollinate some native Australian species (e.g. Vaughton 1992; Paton 1993; Gilpin *et al.* 2016), they are also known to disrupt the foraging of native insects (e.g. Paini 2004; Gross 2001) and vertebrate pollinators (e.g. Paton 1993; Vaughton 1996). There is some evidence that the presence of *A. mellifera* reduces seed production and/or rates of pollination for some predominantly bird-pollinated plants (Taylor and Whelan 1988; Vaughton 1996). The efficacy of *A. mellifera* at transferring pollen can depend on whether they are foraging on nectar or collecting pollen, with pollen foraging leading to more frequent contact with the stigma in primarily vertebrate pollinated plants (e.g. Paton and Turner 1985; Ramsey 1988; Vaughton 1992; Paton 1993b).

Plants with floral traits indicative of vertebrate pollination may be reliant on the presence of vertebrate pollinators to maintain reproduction (Ratto *et al.* 2018). While bird pollination is often associated with vivid red tubular flowers, little detectable floral scent, and copious amounts of diluted nectar (Johnson and Wester 2016), flowers in a diverse range of other colours and shapes are visited by nectar-foraging birds (Cronk and Ojeda 2008; Shrestha *et al.* 2013). A recent meta-analysis of 126

studies (Ratto *et al.* 2018) identified that the exclusion of all vertebrate pollinators from plants resulted in an average 63% reduction in fruit and seed production, with exclusion of birds resulting in an average of 43% fewer fruits and seeds. Many species of vertebrates that visit flowers (birds, bats, scansorial mammals, primates and lizards) are in decline, with an average of 2.5 of these species per year moving one category closer to extinction on the IUCN Red List of Threatened Species (Regan *et al.* 2015). While there are few studies assessing the long-term impacts of reduced vertebrate pollinator abundance, there is some evidence for an association between a reduction in bird-pollinators and plant population decline (Carpenter 1976; Rathcke 2008; Anderson 2011; Sakai *et al.* 2014).

The Southwest Australian Floristic Region (SWAFR) has the highest incidence of bird-pollination in the world, with 15% of flowering plant species pollinated by birds and/or mammals including 40% of threatened flora (Keighery 1980; Hopper and Gioia 2004; Hopper 2009). It is not known if this apparent increased extinction threat is caused by a tendency for rare species to evolve bird pollination or is driven by the characteristics of bird-pollinated species (Phillips *et al.* 2010). However, an overall reduction in the abundance and diversity of nectar feeding birds and small-mammals has been observed throughout the SWAFR over the last ninety years, including the local extinction of species (Recher and Servety 1991; Garavanta 1997; How and Dell 2000; Davis *et al.* 2013). Here, we aim to determine the contribution of nectar feeding birds and *A. mellifera* to the pollination of *Anigozanthos manglesii* (Haemodoraceae), a herbaceous wildflower endemic to south-western Australia. *Anigozanthos manglesii* has long, tubular flowers that are foraged on by nectar feeding honeyeaters (Hopper and Burbidge 1978) and has no known native insect pollinators. While there are few records of visitation by *A. mellifera* (Hopper 1993), their impact on the pollination of *A. manglesii* is unknown, and their high abundance in the study region and generalist foraging behaviour may mean that their visitation rates are underestimated. We hypothesise that, for *A. manglesii* 1) nectar feeding

birds will be more frequent visitors to flowers than insects, 2) *Apis mellifera* will be the most common insect visitor, 3) the mismatch between floral structure and pollinator body size will cause honeybees to primarily act as nectar robbers and thieves, 4) as a result of this mismatch, flowers that are excluded from nectar-feeding birds will set less seed and fruit than flowers open to all potential pollinators.

Methods:

Study species and sites

Anigozanthos manglesii is a perennial herb characterised by large showy, red and green inflorescences (Figure 1), on stems between 30-110cm tall (Hopper 1993). The species is Western Australia's Floral Emblem. There are two subspecies recognised, the northern *A. manglesii* subsp. *quadrans*, and the southern *A. manglesii* subsp. *manglesii*. An individual inflorescence produces between 5 to 30 red and green tubular flowers that open in stages over successive days from the base upwards, with higher seed set and greater germination success seen in basal fruits of wild pollinated flowers (Tieu *et al.* 2001). The flowers of both subspecies have six anthers between 5-12mm, a long stigma of up to 4cm, and produce up to 250µl of nectar per day (Hopper and Burbidge 1978). While flowers are typically red and green, rare individuals with combinations of purple, pink, yellow, orange and green flowers are also found across the distribution of the species. *Anigozanthos manglesii* is preferentially outcrossing (Hopper 1980b), with lower seed set and germination success after self-pollination or bi-parental inbreeding. After hand pollination, outcrossing results in an average of 100 seeds per fruit and a maximum of 300 seeds per fruit (Ayre *et al.* 2019).

Anigozanthos manglesii flowers are pollinated by nectar-feeding birds (Hopper and Burbidge 1978; Hopper 1993), with documented visits by Western Spinebill (*Acanthorhynchus superciliosus*), Red Wattlebird (*Anthochaera carunculata*), Brown Honeyeater (*Lichmera indistincta*), Singing Honeyeater (*Lichenostomus virescens*), Tawny-crowned Honeyeater (*Phylidonyris melanops*), White-

129 cheeked Honeyeater (*Phylidonyris nigra*), and New-Holland Honeyeater (*Phylidonyris*
130 *novaehollandiae*). The Red-capped Parrot (*Purpureicephalus spiurius*) and Silvereye (*Zosterops*
131 *lateralis*) are also known to visit *A. manglesii* (Brown *et al.* 1997), but are likely to rob nectar or
132 consume flowers (Hopper 1993). In parts of its geographic range, *A. manglesii* is potentially
133 pollinated by small non-flying mammals, such as the pygmy possum and honey possum, but this has
134 not been confirmed (Brown 1988; Hopper 1993). Insects have not been recorded pollinating *A.*
135 *manglesii* flowers, though bush crickets (*Zaprochilinae* sp.) have been seen feeding on pollen grains
136 of open and unopened flowers (Bailey and Lebel 1998), while ants (species unreported) and *A.*
137 *mellifera* have been observed occasionally thieving nectar (Hopper 1993).

138 Pollinator surveys and experimentation were conducted in four populations of *A. manglesii* subsp.
139 *manglesii*. There were two study populations in Kings Park bushland (KP1 at 31.966°E, 115.830°S
140 and KP2 at 31.962°E, 115.829°S), an urban reserve of 300ha within the Perth metropolitan area, and
141 two in Korung National Park (KN1 at 32.081°E, 116.091°S and KN2 at 31.962°E, 115.829°S), a
142 6000ha National Park south-east of Perth. The overstory of Kings Park bushland is dominated by the
143 bird-pollinated *Banksia menziesii* and *B. attenuata* and the wind-pollinated *Allocasuarina fraseriana*
144 (Recher 1997), with scattered trees of the predominantly insect-pollinated marri (*Corymbia*
145 *calophylla*), tuart (*Eucalyptus gomphocephala*) and jarrah (*Eucalyptus marginata*) (Yates *et al.* 2005).
146 Korung National Park is an open jarrah forest, with widespread *Banksia sessilis*, a species commonly
147 visited by nectar feeding honeyeaters (Collins 1985). In Kings Park bushland, the community of
148 nectar feeding birds consists of Brown Honeyeaters (*L. indistincta*), Red Wattlebirds (*A.*
149 *carunculata*), Western Wattlebirds (*Anthochaera chrysoptera*), Singing Honeyeaters (*L. virescens*),
150 Western Spinebills (*A. superciliosus*), White-cheeked Honeyeaters (*P. nigra*), New Holland
151 Honeyeaters (*Phylidonyris novaehollandiae*), Silvereyes (*Z. lateralis*) and the introduced Rainbow
152 Lorikeet (*Trichoglossus moluccanus*) (Recher and Serventy 1991, Recher 1997; Davis and Wilcox

2013, Atlas of Living Australia 2019). Within Korung National Park, there are scattered recorded sightings of Gilbert's Honeyeater (*Melithreptus chloropsis*) and the above nectar feeding birds (Atlas of Living Australia 2018). Although historically abundant, mammal pollinators are now thought to be locally extinct at both sites.

Flower visitors

To determine the abundance and pollination effectiveness of animal visitors to *A. manglesii*, we observed visitors to flowers in all four populations in both 2015 and 2016. Observations of birds were undertaken between dawn and dusk for twelve days in Spring 2015 and Spring 2016, during the peak flowering time of *A. manglesii*. Direct observations were only made in clear weather, whilst motion triggered cameras recorded data in all weather conditions. In both 2015 and 2016, patches of *A. manglesii* at each site were observed between dawn to dusk, with 45x 20-30-minute surveys undertaken, and any bird visits recorded.

In 2016, ten camera traps (Reconyx HC 500) were placed out for four weeks across September and October at the site KP1. Cameras were attached to wooden stakes with cable ties, positioned between 50cm to 1m away from the flower, level with the respective flowering height of each plant. They were set at high sensitivity, rapid-fire picture interval, and to take ten photos over 9 seconds each time the camera was triggered. Each camera was active for 660 hours. For each series of images with animal visitation, the animal species was identified, the number of flowers probed and instances of contact with anthers and/or stigma recorded. Visitation rates are estimates, due to known imperfections with camera traps accurately detecting all bird foraging events (e.g. Krauss *et al.* 2018).

In this study camera traps were unable to detect insect visitation. Instead, direct observations of visiting insects were undertaken during days when bird-observations were conducted, as well as during weekly visits to field sites to ensure the integrity of the enclosures used to manipulate pollinator visitation (refer below). In addition, 40 honeybee foraging events were videoed (NIKON

D3300) over ten hours in 2017. The number of flowers probed and contact with anthers, nectaries and/or stigma were logged.

Exclusion experiment

In both 2015 and 2016, the contribution of bird and insect visitors to pollination was assessed by manipulating access to plants with wire or mesh constructs (Figure 1). Each year, across each of the four populations, three treatments were applied, with nine replicate plants per treatment (different plants in each year): 1) total exclusion, with plants enclosed in a fine mesh (<1mm) to exclude all insect and vertebrate foragers, 2) vertebrate excluded plants, with plants enclosed in a wire mesh (1cm) to allow insect visitors but exclude vertebrates (*A. mellifera* were observed moving in and out of cages to visit flowers) and 3) no exclusion, with plants left open for both vertebrate and insect visitors.

Following flowering, for each plant the percentage of flowers forming mature fruit (capsules) was scored (fruit set). Fruit were collected just prior to dehiscence, between late December and early January. Fruits were stored in paper envelopes for a minimum of eight weeks, before fruit size (length and width) was measured, the number of seeds within each fruit counted, and x-rayed (Faxitron MX-20 X-ray cabinet, Tucson, AZ, U.S.A.) as an indicator of viability. Seeds with a visible endosperm were considered viable, and those without a visible endosperm as inviable (e.g. Kamra 1964; Gagliardi and Marcos-Filho 2011). Viable seeds were grouped by fruit and weighed, and the average individual seed mass calculated for every fruit. Ten viable seeds, from three fruits per plant, were heat shocked at 100°C for 3 hours in an oven to break dormancy (Tieu *et al.* 2001), before being germinated on filter paper in a 15°C incubator, with 12hrs of light and 12hrs of darkness. Seeds were considered germinated when the radical was a third of the length of the seed. Germination success was assessed daily over 30 days and was estimated as the percentage of seeds germinating for a given plant.

Data Analysis

The location of flowering plants within each population was mapped each year using a differential Global Positioning System (dGPS), and the density of each population determined by dividing the number of flowering plants by the size of the population. As all data sets violated normality assumptions and could not be transformed to fit a normal distribution, differences within and between years, sites and treatments were determined using the non-parametric Kruskal-Wallis rank sum test, followed by a post-hoc Dunn test (R Development Core Team 2016; Dinno 2017).

Results:

Flower Visitors:

A. Birds and mammals

During 100 hours of direct observation in Kings Park, the only vertebrate visitors seen foraging on *A. manglesii* was a pair of Singing Honeyeater (*L. virescens*). In Korung National Park nine birds were seen foraging over eighty hours of direct observation: six Brown Honeyeaters (*L. indistincta*), a Singing Honeyeater (*L. virescens*), a Western Spinebill (*A. superciliosus*) and a White-cheeked Honeyeater (*P. nigra*).

Camera traps in Kings Park recorded 22 visits by Brown Honeyeater (*L. indistincta*), one by a Singing Honeyeater (*L. virescens*) and two by a Silvereye (*Z. lateralis*) (Figure 2). The timing of visits ranged from 4.45am to 2.38pm, with 75% of visits occurring between 8am and 11am. Of the 22 Brown Honeyeaters (*L. indistincta*) captured by camera traps, 96% foraged on flowers. Multiple flowers on the same plant were probed during 45% of recorded foraging visits, and an individual flower was probed twice on 16% of recorded visits (mean $\pm$ SE; 2.13 flowers probed $\pm$ 0.20). Anthers and stigmas were contacted during 47.1% and 45.1% of probes respectively (Figure 3). Only one visit from a Singing Honeyeater (*L. virescens*) was recorded, and no flowers were probed (Figure 2C). Two Silvereye (*Z. lateralis*) foraging events were captured, during both the bird created slits in

two flowers through which to consume nectar, and no contact was made with either the anther or stigma (Figure 2D). Despite the proven efficacy of Reconyx HC 500 camera traps at recording visitation by nocturnal nectarivorous vertebrates in the study region (Krauss *et al.* 2018), none were detected in this study.

B. Insects

Four species of bush cricket (*Hemisaga denticulata*, an *Elephantodeta nobilis* nymph, *Kawanaplila natree* and a juvenile *Phasomodes ranatriormis*) were all observed either consuming pollen from unopened and opened flowers or found on flowers that showed signs of recent pollen predation. Ants (species unknown) were observed consuming nectar, but without contacting the plant's reproductive structures.

Apis mellifera were observed foraging on flowers throughout the day (~8am to 6:30pm), with flowering plants visited an average ($\pm$ SE) of 4.25 ± 1.07 times per hour. Across the forty flower visits by *A. mellifera* recorded by video, 97.5% of visits involved foraging on pollen (Figure 2F), with only one attempt at nectar foraging recorded (Figure 2E). When foraging for pollen, *A. mellifera* contacted the stigma 12.8% of the time (Figure 3). An average of 1.9 ± 0.06 flowers within the focal area (1.5m² patch of flowering plants) were visited per foraging event, with 8.8% of flowers visited twice and 2.9% visited three times. *Apis mellifera* were observed gathering from all anthers on a flower and removing large quantities of pollen every visit.

Pollinator Exclusion:

A. Fruit Set

The percentage of flowers setting fruit varied significantly amongst all treatments. A higher percentage of open treatment flowers produced fruits ($70\% \pm 3\%$) than plants from which vertebrates were excluded ($26 \pm 2\%$; $\chi^2 = 142.69$, $P < 0.001$) or all pollinators were excluded ($18 \pm 2\%$; $\chi^2 = 115.01$, $P < 0.001$). Plants from which vertebrates were excluded produced more fruits than those

from which all pollinators were excluded ($\chi^2 = 52.393$, $P < 0.001$). Comparing within sites and years (Figure 4), total pollinator excluded flowers and vertebrate excluded flowers had levels of fruit set that were not significantly different in all cases except in KP2 in 2016. There, total exclusion flowers had lower levels of fruit set than vertebrate exclusion ($17 \pm 6\%$ cf. $47 \pm 6\%$; $\chi^2 = 46.77$, $P < 0.001$).

B. Seed Set

Flowers that were open to all potential pollinators produced more seeds per fruit (Figure 5) than flowers from the treatments with exclusion of vertebrate pollinators and total pollinator exclusion ($\chi^2 = 488.39$, $P < 0.001$). There was one exception, KP1 in 2015, where open pollinated flowers produced an average of 22.6 ± 2.3 seeds per fruit, which was not significantly different to the 9.4 ± 1.2 seeds per fruit in vertebrate excluded flowers ($\chi^2 = 25.81$, $P = 0.29$).

In most sites and years there was no significant difference in the numbers of seeds per fruit between the vertebrate exclusion and complete pollinator exclusion treatments (Figure 5). However, in KP2 in 2016 vertebrate exclusion resulted in 8.3 ± 0.7 seeds/fruit, compared to 2.7 ± 3.0 seeds/fruit produced in the complete pollinator exclusion treatment ($\chi^2 = 135.81$, $P < 0.001$).

There was great variation across sites and years in numbers of seeds per fruit produced following open pollination. In 2015, plants in Korung National Park produced a greater number of seeds per fruit than plants in Kings Park ($\chi^2 = 167.89$, $P < 0.001$), with 64.0 ± 4.2 seeds per fruit compared to 29.1 ± 1.9 in Kings Park. The reverse of this pattern was seen in 2016, with Korung National Park plants producing fewer seeds per fruit than plants in Kings Park (34.4 ± 2.8 cf. 46.2 ± 2.8 seeds per fruit, respectively a 53.7% decrease, $\chi^2 = 217.32$, $P < 0.01$, and 63.1% increase, $\chi^2 = 344.47$, $P < 0.01$, in production from the preceding year). The total number of seeds per fruit following total exclusion of pollinators was equivalent across sites and years ($\chi^2 = 567.03$, $P > 0.05$).

C. Fruit volume

There was no consistent effect of year, site, plant, or exclusion treatment on fruit volume (Supplementary Figure 1), with no overall site, treatment or year having a greater fruit volume than any other ($P > 0.05$). Fruit volume and number of seeds per fruit were found to be weakly correlated ($r^2 = 0.38$, $P < 0.001$), with fruit volume increasing with increasing numbers of seeds per fruit.

D. Seed mass

There was no effect of site, year, or exclusion treatment on seed mass ($\chi^2 = 585.0$, $P = 0.52$). Seed mass and the number of seeds per fruit were not correlated ($r^2 = -0.087$, $P = 0.068$). Seed mass ranged from 0.03 mg to 1.8 mg per seed, highlighting great variation in seed mass within each treatment (Supplementary figure 2).

E. Seed viability

There was no overall difference in seed viability between treatments, sites or years ($\chi^2 = 3.69$, $P = 0.16$; Supplementary figure 3). Within sites and exclusion treatments there is some variation between years (Supplementary Table 1). In KP2, seed viability was greater in 2016 ($99 \pm 0\%$) compared to 2015 ($96 \pm 1\%$; $\chi^2 = 33.0$, $P < 0.0001$), while in KN1 seed viability increased from $82 \pm 4\%$ in 2015 to $86 \pm 0\%$ in 2016 ($\chi^2 = 16.05$, $P = 0.01$). There was no difference between years in KP1 ($\chi^2 = 75$, $P = 0.42$) and KN2 ($\chi^2 = 1.39$, $P = 0.33$).

F. Germination

There was no difference in germination success (Figure 6) between seeds collected from flowers that had all pollinators excluded (mean of $72 \pm 4\%$) and those that had vertebrate pollinators excluded (mean of $77 \pm 2\%$) within any site or year ($\chi^2 = 34.41$, $P > 0.05$). Germination success of flowers open to all pollinators varied between sites and years. KN2 flowers that had vertebrates excluded had particularly high germination success in 2015, with $88 \pm 6\%$ germination success compared to $63 \pm 8\%$ of seeds from open flowers ($\chi^2 = 5.95$, $P < 0.01$). In 2016, this decreased to $71 \pm 5\%$ ($\chi^2 = 10.22$,

P < 0.01). No site, treatment or year had consistently faster germination (time to fifty percent germination) than another (Supplementary Figure 4).

G. Population density

The density of flowering plants varied between sites and years, being highest in KN2 in 2015, with 1.78 plants per m², and lowest in KP2 in 2016, with only 0.018 plants per m².

Discussion:

Exclusion of bird pollinators had a negative impact on the reproductive output of *Anigozanthos manglesii*. Despite possessing floral traits that suggest regular pollination by birds (Hopper 1993), *Apis mellifera* was the most frequent visitor to *A. manglesii* flowers across all populations and years. Exclusion of bird visitors resulted in 67% fewer fruits and 80% fewer seeds than flowers left open to all floral visitors. The floral structure of *A. manglesii* appears to compromise the effectiveness of *A. mellifera* as a potential pollinator, with seed production in plants from which vertebrates were excluded equal to those from which all pollinators were excluded. This is partially due to the size and behaviour of honeybees, with the frequency of honeybee stigma contact one-third of that observed for native nectarivorous birds. Consequently, effective pollination of *A. manglesii* is primarily reliant on nectar-feeding birds.

Flower visitation

A. Introduced European Honeybee

Apis mellifera was the most common flower visitor, the primary insect pollinator, and almost exclusively foraged on pollen instead of nectar. While there is a previous record of *A. mellifera* foraging on *A. manglesii*, it was considered to be an occasional occurrence focused on nectar foraging, and unlikely to result in pollination due to the distance between nectaries and stigmas (Hopper 1993). In contrast, we found that when *A. mellifera* foraged on *A. manglesii* they focused on pollen collection, with honeybees visibly removing most of the pollen from anthers in a foraging

bout. In some other Australian plant species, pollination success appears to be influenced by the resource *A. mellifera* is foraging on. Higher levels of pollination occur if pollen is being collected rather than nectar (Ramsey 1988; Vaughton 1992). When foraging on pollen of *A. manglesii*, *A. mellifera* only contacted the stigma in 14% of flower visits. If contact was made, it typically occurred in the middle of a foraging event, increasing the chances of pollen from the same plant being deposited. Given the significantly lower fruit and seed production after exclusion of vertebrate pollinators, it appears that *A. mellifera* are ineffective pollinators of *A. manglesii*.

While there are several Australian plant species that are successfully pollinated by *A. mellifera* (Vaughton 1992; Paton 1993; Gross 2001; Yates *et al.* 2005; Gilpin *et al.* 2016), many species appear to have fruit and/or seed production disrupted (e.g. Paton 1988, 1993; Taylor and Whelan 1988; Vaughton 1996; Higham and McQuillan 2000; Celebrezze 2002; Celebrezze and Paton 2004). This may be caused by *A. mellifera* displacing native pollinators. For example, an increase in the numbers of *A. mellifera* foraging on *Callistemon* spp. reduced the density of New Holland Honeyeaters and their flower visitation rate (Patton 1993). Flowers may also be less attractive to bird pollinators due to a reduction in nectar (Patton 1993), while intense honeybee foraging can also essentially emasculate flowers, therefore limiting a plant's male reproduction (Hargreaves *et al.* 2009).

Here, lower fruit and seed set after vertebrate exclusion appears to be driven primarily by the mismatch between the floral structure of *A. manglesii* and the body size and foraging techniques of *A. mellifera*. Honeybee foraging on small flowers or flowers with stigmas, anthers and nectaries in close proximity is predicted to lead to higher rates of pollination than occurs after foraging on larger flowers (Paton 1993). In *Anigozanthos pulcherrimus*, honeybees appear to be successful pollinators (Brown 1988), likely due to the floral structure of *A. pulcherrimus*, which, unlike *A. manglesii*, have a more deeply enclosed perianth tube but considerably shorter flowers. Aside from occasional nectar robbing through slits in the base of the flower, this structure causes *A. mellifera* to unavoidably

contact the anthers, and in most cases the stigma, when foraging on nectar or pollen of *A. pulcherrimus* (Brown 1988). Introduced honeybees are known to visit other *Anigozanthos* species in Australia (Brown 1988, Phillips *et al.* 2014), but native honeybees have not been reported visiting introduced populations of *A. flavidus* or *A. rufus* in South Africa (Le Roux *et al.* 2010).

B. Native insects

Native insects were not observed to be contributing to the pollination of *A. manglesii* in our study populations. While ants were observed collecting nectar from *A. manglesii* flowers, for most plants' ants are primarily nectar and pollen thieves. Pollen does not readily adhere to ant bodies, and their small size means they rarely contact the anthers and stigmas when searching for nectar (Abrol 2012). Ants may also secrete antibacterial and antifungal antibiotics that are known to disrupt the development and germination of pollen grains (Beattie *et al.* 1984). If ants do spread viable *A. manglesii* pollen, this is likely to only occur within flowers on the same plant, perhaps enhancing levels of self-pollination (Abrol 2012). While there is limited evidence of pollination by crickets in plants in general (though see Micheneau *et al.* 2010), bush crickets are known to be pollen robbers of *A. manglesii*. Two of the bush cricket species we recorded, *Phasmodes rantriformis* and *Kawanaphila nartee*, have been observed to consumptively emasculate *A. manglesii* flowers by stripping all pollen from the anthers one at a time (Hopper 1993), and feeding on immature perianths (Bailey and Lebel 1998). To our knowledge, this was the first time the bush crickets *Hemisaga denticulata* and *Elephantodeta nobilis* were recorded visiting *A. manglesii*. During our sole sighting of *Hemisaga denticulata* it was feeding on immature perianths, while *Elephantodeta nobilis* was not observed feeding on *A. manglesii*, and is thought to primarily use plants as camouflage (Wardhaugh 2015). The collective evidence suggests that during their infrequent visits to *A. manglesii*, native insects are primarily acting as nectar and pollen thieves.

C. Nectar feeding birds

The frequency of bird visitation to *A. manglesii* in Kings Park was low, with on average one visit every ten hours detected by motion-triggered cameras, and only one visit every 100 hours observed directly by an investigator. This is a much lower visitation rate than expected, with the only other published study of *A. manglesii* pollination recording an average of 20.4 plants visited each hour by Red Wattlebirds (*A. carunculata*) (Hopper and Burbidge 1978). One possible reason for this difference is likely to be the density of *A. manglesii* plants at the study populations. Flowering plant density is known to impact visitation rates in *Anigozanthos*, with the number and diversity of nectar feeding birds visiting *A. flavidus* increasing with *A. flavidus* population size (Phillips *et al.* 2014). The population of *A. manglesii* at Gingin cemetery studied by Hopper and Burbidge (1978) was frequently slashed and burnt to promote a higher density of flowers, with ca. 480 plants per hectare reported. In our unmanipulated, long unburnt study sites the population density was as low as 182 plants per hectare, representing a 63% reduction compared with Gingin cemetery densities. The low density and number of flowers open on an individual *A. manglesii* inflorescence may mean that honeyeaters opt for other more profitable nectar sources.

The presence of co-flowering *Banksia* species at all sites may reduce rates of honeyeater foraging on *A. manglesii*. If there are not enough pollinators in an area to utilise all available floral resources, then the pollinator visitation rate decreases per plant (Veddeler *et al.* 2006; Holzschuh *et al.* 2006; Jha and Vandermeer 2009; Schmid *et al.* 2016), and plants need to compete for a limited number of shared potential pollinators. Following optimal foraging theory (Pyke *et al.* 1997), pollinators may focus on plants rich in nectar or pollen within the plant community. Species that produce a lower number of flowers, or less nectar per plant, may have lower visitation rates (Schuett and Vamosi 2010; Schmid *et al.* 2016; Grab *et al.* 2017). In Korung National Park, the main competitor for the attraction of nectar feeding birds is *B. sessilis*, which produces an average of 43.5µl of nectar per flower, and 47 inflorescences per plant (Collins and Briffa 1982). This represents four times the nectar content

available compared with an *A. manglesii* plant, where there is a maximum of 250µl of nectar per flower recorded and an average of two flowers per plant open at any given time. In Kings Park, *B. attenuata* overlaps with *A. manglesii* flowering in October and November and produces an estimated 300µl of nectar per plant (Collins and Briffa 1982) while *B. menziesii* flowering overlaps in August, producing c.a. 120 to 323mg of nectar per plant (Newland 1982). These banksias provide an alternative nectar-rich foraging options for honeyeaters, which may partially explain low visitation rates to *A. manglesii*.

There may also be fewer bird pollinators present in our study sites, particularly Kings Park, due to habitat fragmentation, degradation and urbanisation. Urbanisation causes changes in vegetation structure and food resources and introduces new predators and competitors (Evans *et al.* 2009). For remnant dependent species, habitat fragmentation adds barriers for species' dispersal and connectivity and often sees the introduction of weed species (Fernández-Juricic 2000; Stenhouse 2004; Evans *et al.* 2009). While bird-pollinators may be able to overcome barriers and disperse between fragmented reserves (Ritchie *et al.* 2019), the local abundance of pollinators may be reduced, potentially leading to flowers experiencing lower rates of visitation. The abundance and diversity of birds in the inner-city Kings Park has been monitored since 1928 (Recher and Serventy 1991). Between 1928 and 1986, nine species went locally extinct, 14 species decreased in abundance, and 11 increased in abundance. The population changes included a decrease in Western Spinebills (*A. superciliosus*) and an increase in Singing Honeyeaters (*L. virescens*) and Brown Honeyeaters (*L. indistincta*) (Recher and Serventy 1991). Indeed, we observed no Western Spinebills (*A. superciliosus*) in our Kings Park study sites in either 2015 or 2016. While many vertebrate pollinators tend to be considered generalist foragers (Collins and Briffa 1982; Saffer *et al.* 2000; Menz *et al.* 2011), there are locally important relationships between plants and specific pollinator taxa, which can vary between populations and years. Brown Honeyeaters (*L. indistincta*) were the main pollinator of *A. manglesii* in our Kings Park

sites, while in Korung National Park plants were visited by a greater diversity of bird pollinators. While local plant-pollinator relationships will be influenced by differences in the geographic ranges among pollinator species (Sánchez-Lafuente 2002, Ollerton *et al.* 2006), they are also impacted by differing responses of species to habitat fragmentation and landscape alteration (Davis *et al.* 2013).

Fruit and seed production without floral visitors

When all pollinators were excluded from *A. manglesii* flowers, there was limited fruit and seed production, with an overall average of 18.2% of flowers developing into fruits, and fruits producing an average of only 5 seeds. Although preferentially outcrossing, *A. manglesii* will occasionally form fruits after self-pollination, producing low amounts of seeds (Ayre *et al.* 2019). As the pollination exclusion cages were placed over multiple flowering stems, there is also the possibility that multiple individuals were present within the cages. As the stems of *A. manglesii* move around in the wind, low quantities of pollen could also have been transferred between individuals despite the absence of pollinators. This may explain the few instances of fruits that contain more than 10/seeds per fruit in the total exclusion treatment (Figure 5).

Pollen limitation

Despite a higher reproductive output from flowers open to bird pollination, there still appears to be pollen limitation occurring, with an average of only 43 ± 1.6 seeds per fruit compared with 280-300 seeds per fruit following experimental addition of outcross pollen (Ayre *et al.* 2019). While low levels of fruit set after foraging by *A. mellifera* can be partially explained by infrequent contact with the stigma, the quality and quantity of pollen deposited will impact on both fruit and seed production. Pollen limitation, where reproductive success is limited by pollen quantity or quality, is common across plant species and populations (Ashman *et al.* 2004; Knight *et al.* 2005; Burd *et al.* 2009). In *A. manglesii*, pollen limitation could arise from low quantities of pollen transfer either from low bird visitation rates, or pollen removal by *A. mellifera*. While data from the present study are insufficient

to test the latter hypothesis, in the closely related bird-pollinated *Anigozanthos pulcherrimus* comparisons between sites with and without *A. mellifera* found no significant difference in seed production (Brown 1988). The quantity of pollen being deposited by *A. mellifera* is also likely to be low as most pollen collected is groomed from the body and deposited in corbiculae where it is not available for pollination (Hargreaves *et al.* 2009). Even with multiple visits to the same flower, it is unlikely that there is sufficient pollen deposited to prevent pollen limitation. Floral structure also appears to impact on pollination success from bird visitation. Instead of a closed tube, with internal anthers and a stigma, mature *A. manglesii* flowers split open allowing birds to forage on nectar without contacting the anthers or stigma, up to 55% of the time.

Sub-optimal seed set could also be driven by both honeyeater and honeybee foraging behaviour leading to the deposition of low-quality pollen. *Anigozanthos manglesii* is preferentially outcrossing, with selfed flowers producing fruits only 10% of the time. Pollination by nearest neighbours results in an average of 50 seeds per fruit, compared to 280-300 seeds per fruit after outcrossing over greater distances (Ayre *et al.* 2019). Multiple probes by birds on flowers on the same plant will increase the amount of self-pollen deposited. Birds foraging on groups of neighbouring plants (Pyke *et al.* 1997), will facilitate bi-parental inbreeding and lower *A. manglesii* reproductive output (Ayre *et al.* 2019). Likewise, *A. mellifera* move predominantly between flowers on the same plant or flowers of neighbouring plants, resulting in lower seed set due to pre- and post-zygotic barriers. Honeyeaters are likely to forage on multiple plant species within our study sites, increasing the chance of application of heterospecific pollen. This may clog the stigma and reduce conspecific pollen germination (Brown and Mitchell 2001) but does not negatively impact all species (Eakin-Busher *et al.* 2016).

Despite lower than optimal seed set, and low visitation rates, *A. manglesii* appear to have been historically dependent on nectar feeding birds for their pollination. Even with the introduction of *A. mellifera*, the absence of nectar-feeding birds dramatically lowers reproductive output. More work is

needed to document the importance of vertebrate pollinators for plant reproduction, the consequences of their declining numbers on plant populations, and whether exotic pollinators lead to reduced plant reproduction, or can compensate to some extent for declining native pollinators.

Conflicts of Interests

The authors declare no conflicts of interest.

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Figure captions:

Figure 1: A) 1mm netting providing total pollinator exclusion and b) 1cm chicken wire excluding vertebrate pollinators from *Anigozanthos manglesii*. Photos B. Ayre.

Figure 2: Camera trap images and video stills of nectar-feeding birds and *Apis mellifera* visiting *Anigozanthos manglesii*. From L-R, Top to bottom: A) Brown Honeyeater feeding on nectar while contacting anther and stigma, B) A Brown Honeyeater stealing nectar C) Singing Honeyeater perched on *A. manglesii* stem, D) Silveryeye creating slits in the base of *A. manglesii* flowers to enable nectar foraging, E) *A. mellifera* foraging for nectar and F) *A. mellifera* foraging for pollen.

Figure 3: The percentage of time honeyeaters and the introduced *Apis mellifera* contacted the anther, stigma and nectaries while foraging on *Anigozanthos manglesii*. Error bars indicate standard errors.

Figure 4: Tukey's boxplots showing the percentage of flowers setting fruit per plant following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2= Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Figure 5: Tukey's boxplots showing seeds per fruit after pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold KN1 = Korung National Park Site 1; KN2= Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Figure 6: Tukey's boxplots showing germination success (the percentage of seeds germinating) after pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2 = Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Supplementary Figure 1: Tukey's boxplots showing fruit volume following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2 = Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Supplementary Figure 2: Tukey's boxplots showing seed mass (mg) following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2 = Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Supplementary Figure 3: Tukey's boxplots showing seed viability following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion =

Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2 = Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Supplementary Figure 4: Tukey's boxplots showing time to 50% germination following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years. Total Exclusion = Flowers excluded from all pollinators, Bird-Exclusion = Flowers excluded from all bird pollinators and No exclusion = Flowers that were left open and unmanipulated. Squares represent the mean, thick horizontal lines the median. The box represents the upper, median and lower quartiles and the whiskers indicate the 1.5× interquartile range. Black dots represent outliers. Letters indicate significance groupings with a $p < 0.05$ threshold. KN1 = Korung National Park Site 1; KN2 = Korung National Park Site 2; KP1 = Kings Park Site 1; KP2 = Kings Park Site 2.

Supplementary Table 1: Measures of reproductive output following pollinator exclusion treatments on *Anigozanthos manglesii* flowers across four sites and two years.

Means and standard deviations reported.

Site	Treatment	Year	Total Seeds	Total Fruits	Flowers that produced fruit	Seeds per fruit	Fruit Volume (cm ³)	Seed mass (mg)	Viability	Percent germinated	T50
Kings Park 1	Total Exclusion	2015	116	17	11.72 ± 4%	6.50 ± 2.70	0.26 ± 0.08	0.87 ± 0.06	84 ± 6%	82 ± 10%	19.60 ± 0.75
		2016	215	46	24.21 ± 6%	4.67 ± 0.63	0.34 ± 0.02	0.71 ± 0.02	84 ± 6%	73 ± 9%	19.00 ± 0.78
	Bird Exclusion	2015	75	20	13.33 ± 3%	9.40 ± 1.21	0.51 ± 0.13	0.81 ± 0.06	95 ± 4%	87 ± 7%	17.9 ± 1.33
		2016	263	53	19.92 ± 6%	4.96 ± 0.98	0.24 ± 0.01	0.77 ± 0.04	95 ± 4%	74 ± 6%	15.69 ± 1.24
	Open	2015	1194	26	61.29 ± 8%	22.58 ± 2.28	0.58 ± 0.03	0.85 ± 0.03	82 ± 4%	83 ± 7%	16.87 ± 0.74
		2016	7432	124	62.94 ± 7%	59.94 ± 5.37	0.45 ± 0.03	0.67 ± 0.04	86 ± 3%	87 ± 5%	19.07 ± 0.73
Kings Park 2	Total Exclusion	2015	10	2	1.39 ± 2%	5.00 ± 1.0	0.24 ± 0.01	0.86 ± 0.01	88 ± 12%	75 ± 25%	17.50 ± 0.5
		2016	62	23	11.33 ± 6%	2.70 ± 3.04	0.32 ± 0.02	0.83 ± 0.08	100 ± 0.0	66 ± 9%	17.29 ± 0.81
	Bird Exclusion	2015	314	42	25.3 ± 8%	7.48 ± 2.25	0.33 ± 0.03	0.66 ± 0.04	90 ± 3%	83 ± 6%	18.92 ± 0.45
		2016	1334	159	43.88 ± 6%	8.39 ± 0.65	0.30 ± 0.02	0.77 ± 0.04	100 ± 0.0	85 ± 4%	17.00 ± 0.24
	Open	2015	3412	113	71.52 ± 9%	30.19 ± 2.5	0.41 ± 0.02	0.82 ± 0.01	96 ± 1%	90 ± 2%	18.73 ± 1.0
		2016	8501	221	72.46 ± 5%	38.47 ± 3.04	0.82 ± 0.04	0.74 ± 0.03	99 ± 0.0	82 ± 5%	18.57 ± 0.42
Korung National Park 1	Total Exclusion	2015	54	10	11.76 ± 3%	5.4 ± 2.30	0.23 ± 0.07	0.82 ± 0.13	83 ± 15%	78 ± 14%	18.50 ± 0.87
		2016	154	25	30.12 ± 9%	6.16 ± 1.34	0.36 ± 0.03	1.1 ± 0.03	75 ± 19%	74 ± 6%	18.75 ± 0.48
	Bird Exclusion	2015	159	24	18.90 ± 5%	6.63 ± 2.65	0.14 ± 0.01	0.93 ± 0.06	80 ± 7%	63 ± 8%	19.82 ± 1.08
		2016	355	28	28.00 ± 4%	12.68 ± 4.35	0.36 ± 0.04	0.93 ± 0.06	82 ± 8%	75 ± 6%	17.08 ± 0.49
	Open	2015	3797	57	68.67 ± 8%	66.61 ± 6.09	0.43 ± 0.06	0.97 ± 0.03	93 ± 2%	88 ± 6%	16.53 ± 0.47
		2016	3295	70	67.96 ± 7%	47.07 ± 5.05	0.45 ± 0.03	0.95 ± 0.06	100 ± 0.3%	71 ± 5%	17.29 ± 0.31
Korung National Park 2	Total Exclusion	2015	89	17	18.28 ± 7%	5.24 ± 1.11	0.13 ± 0.01	1.04 ± 0.08	94 ± 5%	59 ± 14%	19.75 ± 2.39
		2016	148	30	25.66 ± 5%	5.10 ± 1.02	0.33 ± 0.03	1.03 ± 0.08	75 ± 7%	72 ± 8%	16.00 ± 1.32
	Bird Exclusion	2015	201	16	13.11 ± 6%	12.58 ± 5.61	0.33 ± 0.05	1.0 ± 0.11	85 ± 8%	81 ± 12%	17.29 ± 0.92
		2016	253	48	29.19 ± 5%	5.38 ± 1.02	0.30 ± 0.03	1.09 ± 0.2	83 ± 2%	72 ± 7%	18.62 ± 0.63
	Open	2015	2933	52	80.00 ± 5%	57.49 ± 5.66	0.56 ± 0.05	0.85 ± 0.4	94 ± 1%	91 ± 5%	16.00 ± 0.28
		2016	2229	95	77.97 ± 8%	24.68 ± 2.78	0.42 ± 0.02	0.93 ± 0.4	94 ± 5%	78 ± 7%	17.71 ± 0.82

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Figure 4

Figure 5

Figure 6.