1 **Unexpectedly low paternal diversity is associated with infrequent pollinator visitation for a**

2 **bird-pollinated plant[1]**

3

4 *Oecologia Highlighted Student Paper: We examined the mating system of a bird-pollinated herb to test the prediction that*

5 *bird pollination leads to high paternal diversity. Contrary to expectations, we found very low paternal diversity.*

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[1] All authors conceived of the concepts and ideas. JHK, RDP and SLK led the writing with significant contributions from all others**.**

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Abstract

The behaviour of pollinators has important consequences for plant mating. Nectar-feeding birds often display behaviour that results in more pollen carryover than insect pollinators, which is predicted to result in frequent outcrossing and high paternal diversity for bird-pollinated plants. We tested this prediction by quantifying mating system parameters and bird visitation in three populations of an understory bird-pollinated herb, *Anigozanthos humilis* (Haemodoraceae). Microsatellite markers were used to genotype 131 adult plants, and 211 seeds from 23 maternal plants, from three populations. While outcrossing rates were high, estimates of paternal diversity were surprisingly low compared with other bird-pollinated plants. Despite nectar-feeding birds being common at the study sites, visits to *A. humilis* flowers were infrequent (62 visits over 21,552 recording hours from motion-triggered cameras, or equivalent to one visit per flower every 10 days), and the majority (76%) were by a single species, the western spinebill *Acanthorhynchus superciliosus* (Meliphagidae). Pollen counts from 30 captured honeyeaters revealed that *A. humilis* comprised just 0.3% of the total pollen load. For 10 western spinebills, *A. humilis* pollen comprised only 4.1% of the pollen load, which equated to an average of 3.9 *A. humilis* pollen grains per bird. Taken together, our findings suggest that low visitation rates and low pollen loads of floral visitors have led to the low paternal diversity observed in this understory bird-pollinated herb. As such, we shed new light on the conditions that can lead to departures from high paternal diversity for plants competing for the pollination services of generalist nectar-feeding birds.

50

Introduction

52

Polyandry is a near ubiquitous feature of land plants, where mating with more than one pollen donor typically leads to multiple individuals siring seeds (Pannell and Labouche 2013). From a female perspective, polyandry may be beneficial if it increases the amount of pollen received, thereby alleviating pollen limitation and increasing the number of offspring (Knight et al. 2005). Further, genetically diverse pollen loads deposited onto stigmas provide an opportunity for fitness benefits from competition among pollen from potential sires within pistils (Wilson 1990; Minaar et al. 2018), and an opportunity for female choice among diverse male gametophytes (Delph and Havens 1998). Multiple donors to the offspring gene pool reduces biparental inbreeding, and increases the likelihood of favourable combinations of diverse genes within progeny, enhancing adaptive ability (Breed et al. 2014; Nora et al. 2016). From a male perspective, fitness benefits from polyandry arise from pollen carryover and increased access to mates (Mitchell et al. 2013). The fitness benefits of multiple paternity favour the evolution of floral traits that increase both pollinator visitation and pollen transfer between plants, and those plant traits that increase female choice and/or opportunities for male-male competition, such as large stigmas or elongated styles (Pannell and Labouche 2013; Barrett and Harder 2017; Christopher et al. 2020).

68

Almost 90% of 400,000 flowering plant species are dependent on animals for pollination (Ollerton 2011), the behaviour of which has profound effects on the frequency of polyandry in wild plant populations (Mitchell et al. 2009a,b). In particular, the tendency for pollinators to move to nearby flowers to reduce energy expenditure when foraging (Pyke et al. 1977; Pyke 1981) suggests that most pollen movement is also among nearby flowers, resulting in geitonogamy and pollen deposition from near neighbours (Linhart 1973; Escaravage and Wagner 2004; Hardy et al. 2004). From the perspective of the plant, pollinator behaviour resulting in longer distance pollen dispersal and pollen

76 carryover can lead to a greater capacity for mating with multiple sires (Ellstrand 1984; Pannell and

77 Labouche 2013; Mitchell et al. 2013). Consequently, fitness benefits associated with higher genetic

78 diversity in offspring are likely to have played a major role in driving floral evolution to exploit pollen

79 vectors that display behaviours that promote mate diversity (Krauss et al. 2017; Ratto et al. 2018).

80

81 The evolution of pollination by birds is a major evolutionary trend in many groups of flowering plants

82 (Wilson et al. 2007; Cronk and Ojeda 2008; Anderson et al. 2016; Abrahamczyk 2019). Globally, it

83 has been estimated that members of at least 65 plant families rely on birds for pollination, with over

84 1,000 bird species predicted to affect pollination in at least some plant species (Cronk and Ojeda

85 2008; Regan et al. 2015). Compared to many insect pollinators, nectarivorous birds exhibit less

86 rigorous grooming techniques (Holmquist et al. 2012), have a capacity to forage over larger areas

87 (Higgins 1999; Hadley and Betts 2009), and some taxonomic groups display more frequent intra and

88 inter-species aggression that can interrupt optimal foraging behaviour (Stiles 1978; Schemske 1980;

89 Hopper and Moran 1981; Cheke and Mann 2008; Phillips et al. 2014; Krauss et al. 2017). Therefore,

90 the behaviour of birds is predicted to increase levels of pollen carryover, pollen dispersal and the

91 diversity of pollen deposited on stigmas relative to other groups of pollinators (Krauss et al. 2017).

92 As such, pollination by birds has the potential to promote frequent outcrossing, accentuated pollen

93 dispersal distances, and high mate diversity among plants (Ford et al. 1979; Krauss et al. 2009;

94 Bezemer et al. 2016, 2019; Krauss et al. 2017; Ayre et al. 2019).

95

96 At present, tests of the predictions of the genetic consequences for pollination by birds are

97 predominately based on studies of woody shrubs and trees. These studies have provided support for

98 the prediction that bird pollinators tend to facilitate extensive pollen carryover (Krauss et al. 2009),

99 which promotes both paternal diversity (Campbell 1998; Krauss et al. 2017; Bezemer et al. 2019) and

100 genetic connectivity between geographically isolated individuals and populations (Byrne et al. 2007;

101 Bezemer et al. 2016; Ritchie et al. 2019; Nakanishi et al. 2020). However, few studies have examined

102 non-woody species (Krauss et al. 2017), which in many cases will occur in the understory, are less

103 floriferous than their woody counterparts, and experience lower pollinator visitation rates (e.g.

104 Collins and Briffa 1982; Kay and Schemske 2003; Turner and Midgely 2016). Despite these

105 potentially important ecological differences, we know of only one mating system study for a bird-

106 pollinated herb species. This study suggested mixed mating with low to moderate levels of

107 outcrossing for sunbird (Nectariinidae) pollinated *Babiana spp.* (Iridaceae), although estimates were

108 deemed unreliable by the authors due to issues with the markers employed (De Waal et al. 2012).

109 These findings do, however, raise the possibility that understory species may exhibit departures from

110 the general hypothesis of high paternal diversity for bird-pollinated plants.

111

112 South western Australia has an exceptionally high number of bird-pollinated plant species (Kieghery

113 1982), many of which are largely unstudied herbaceous or understory species (Phillips et al. 2010).

114 The genus *Anigozanthos* (Haemodoraceae) contains 11 species endemic to south western Australia,

115 all of which exhibit floral traits associated with pollination by nectar-feeding birds (Ford et al. 1979;

116 Hopper 1993). *Anigozanthos humilis* is a relatively common and geographically widespread perennial

117 herb, with nectar-feeding red wattlebirds (*Anthochaera carunculata*; Meliphagidae), western

118 spinebills (*Acanthorhynchus superciliosus*; Meliphagidae) and brown honeyeaters (*Lichmera*

119 *indistincta*) known to visit their flowers (Hopper and Burbidge 1978; van der Kroft et al. 2019). Here,

120 we quantify the mating system and paternal diversity in *A. humilis* to test the hypothesis that

121 pollination by nectar-feeding birds facilitates frequent outcrossing, extensive pollen dispersal and

122 high paternal diversity (Krauss et al. 2017). We complement our genetic data with field data on

123 pollinator visitation rates and pollen loads for a mechanistic understanding of our genetic results.

124

125 **Materials and Methods**

126

127 **Study species and study site**

128   *Anigozanthos humilis* is short-lived, common and widespread perennial herb that occurs in a range

129   of kwongan, woodland and forest habitats (Hopper 1993). *Anigozanthos humilis* is a rhizomatous

130   seeder species, germinating in autumn and flowering from late winter to late spring (Hopper 1993).

131   Scapes are 10 - 30 cm long with a single terminal inflorescence (Fig. 1) (Hopper 1993). Generally,

132   up to six inflorescences are produced by one plant over a single flowering season, and ten flowers

133   produced on average per inflorescence, with only one or two flowers being open and receptive to

134   pollen at any given time (Hopper 1977; Hopper 1993). The flowers range in size from 25 – 50 mm

135   long, have six stamens and an elongated stigma near the front of a tubular perianth, while the nectary

136   lies at the base of the flower, above the basal ovary (Hopper 1993). The flowers range in colour from

137   yellow to red to orange and last up to three days (Hopper 1993). Nectar production averages 10-20

138   μl per flower per day, and contains 10-20% sugar (Hopper 1993). Pollen grains from *A. humilis*

139   remain viable for one day after being released, while the stigma remains receptive for the first few

140   days after the flower opens (Hopper 1993).

141

142   Study populations were located in Ioppolo Nature Reserve (INR), north of Perth, Western Australia

143   (31° 28' 54.20" S, 115° 57' 52.23" E). Ioppolo Nature Reserve contains remnant banksia woodland

144   covering ~ 1200 ha (van der Kroft et al. 2019). The sandy south-western lowlands of the reserve are

145   dominated by *Banksia ilicifolia* (Proteaceae) and *Adenanthos cygnorum* (Proteaceae), transitioning

146   to *Banksia menziesii* and *Banksia attenuata* woodland with increasing elevation (van der Kroft et al.

147   2019). The higher elevations in the north-east of the reserve are predominantly occupied by

148   *Eucalyptus marginata* (Myrtaceae) with some scattered *Banksia grandis* over lateritic soils (Hort and

149   Hort 2010). *Anigozanthos humilis* is found scattered across these sites within the reserve.

150

151   To assess variability in mating system parameters, three *A. humilis* populations (named A, B and C)

152   were chosen within INR. Our study populations were separated by 550-1200 m. Each of these

153   populations co-occurred with at least some mass-flowering plants that are visited by birds for nectar

154 (Ford et al. 1979; Collins and Briffa 1982; Ramsey 1988; Millar et al. 2000). In population A, *A.*

155 *humilis* occurred with spring-flowering *Banksia ilicifolia*, summer flowering *B. attenuata*, winter

156 flowering *B. menziesii*, and mid-winter to early-summer-flowering *A. cygnorum*. In population B, *A.*

157 *humilis* occurred with *A. cygnorum*, *Banksia menziesii,* and *B. attenuata*. In population C, *A. humilis*

158 occurred with early-winter to early-summer-flowering *Eucalyptus marginata*, early-spring to early-

159 summer-flowering *B. grandis*, *B. attenuata*, and *A. cygnorum*. These co-occurring woody shrubs and

160 small tree species often produce large floral displays offering significant quantities of nectar and/or

161 pollen when compared with the smaller rewards offered by *A. humilis* (Collins and Briffa 1982;

162 Hopper 1993; van der Kroft et al. 2019).

163

164 **Sample collection and seed treatment**

165 Within equivalent areas (ca. 2.7 ha) across the three populations (see Supplementary materials S.1),

166 a total of 131 adult flowering *A. humilis* plants were located (N = 30 (A), 56 (B), 45 (C)). All sampled

167 plants were considered candidate sires for paternity analyses, and 70 were also sources of seed (i.e.

168 maternal plants). Universal Transverse Mercator (UTM) coordinates were recorded for all sampled

169 plants to an accuracy of < 1 m using a Trimble Geo7X GeoExplorer differential GPS (Digital

170 Mapping Solutions, Perth, W.A., Australia). A 1 cm^2 leaf tissue sample was taken from the flowering

171 scape of each flowering individual and kept cool in a collection tube before being stored at -80°C

172 prior to DNA extraction. A total of 266 filled fruits were collected from 70 maternal plants across the

173 three populations and dried at room temperature for one month until seeds were released (van der

174 Kroft et al. 2019). Viability of seeds was inferred by the presence of an embryo using an MX-20

175 digital X-ray cabinet, (Faxitron, Tucson AZ, U.S.A.). Seeds with an embryo present were deemed

176 viable and germinated following published protocols (van der Kroft et al. 2019).

177

178 **Microsatellite Genotyping**

179   Genomic DNA was extracted from frozen adult leaf samples following a modified Carlson extraction

180   method as per Anthony et al. (2016). Seedlings were sampled when approximately 1 cm tall, and

181   genomic DNA extracted as per Anthony et al. (2016) with the following modifications: one ceramic

182   bead and 1 ml of Carlson extraction buffer were added to 2 ml specimen collection tubes, seedling

183   tissue was ground in a MP Fastprep-24 5G Homogeniser (MP Biomedicals, San Diego CA, USA) for

184   2 cycles of 20 seconds, no chloroform: isopropyl step was used, and after tubes air-dried for 2 hours

185   DNA pellets were dissolved in 20 µl of 0.1 M Tris-EDTA, instead of 50 µl used for adults.

186

187   Four primer pairs were used to amplify microsatellite loci. Primer pairs were developed by Ayre et

188   al. (2017) for *Anigozanthos manglesii* and optimised for *A. humilis* using DNA extracted from four

189   families each made up of the maternal plant and six offspring. Screened primer pairs were grouped

190   into one multiplex mix using QIAGEN Multiplex PCR kit (QIAGEN, Venio, Limburg, Netherlands).

191   The multiplex master mix contained 5.1 µl of Master Mix, 1.9 µl of Q-Solution, 2 µl of DNA at a

192   concentration of 5-10 ng/µl and 1 µl of forward and reverse primers diluted to 2 mM. Multiplex PCR

193   conditions were as follows: 95°C for 15 min, then 35 cycles of (94°C for 30 sec, 59°C for 1 min 30

194   sec, 72°C for 30 sec), then 60°C for 30 min. DNA amplification was carried out in a Veriti

195   Thermocycler (Life Technologies, Carlsberg, CA, USA). Fragment analysis was carried out on an

196   automated ABI sequencer (3500 Genetic Analyzer, Applied Biosystems) and samples were scored

197   for allelic variation using GENIOUS V.7.1.4 (Kearse et al. 2012).

198

199   **Correcting for null alleles**

200   Null alleles were identified and scored from individual family arrays using an iterative approach with

201   paternity assignment, made possible by hyper variability at these four loci (all loci were heterozygous

202   for almost all individuals). Corrected data adjusted apparent homozygotes to null heterozygotes with

203   a dummy value (500) representing the null allele for 2 of 4 loci (further explanation in results).

204   Similarly, apparently missing data at a locus were scored as null homozygotes. Mendelian inheritance

205 in progeny arrays and parentage assignments were used to confirm these corrections, which were then

206 applied to the full data set (offspring, maternals, candidate paternals), and these corrected data were

207 used for all genetic analyses.

208

209 **Genetic diversity**

210 Parameters of genetic diversity were estimated on null allele corrected data using GenAlEx V6.51b2

211 (Peakall and Smouse 2012). Diversity measures included N_a (Number of alleles), N_e (effective

212 number of alleles), H_E (expected heterozygosity), and H_O (observed heterozygosity) (Peakall and

213 Smouse 2012).

214

215 **Mating system, paternal diversity, paternity assignment and pollen dispersal**

216 Mating system parameters and paternity assignment was estimated for 211 germinants from 23

217 maternal plants (pop. A, $N = 72$ offspring from 9 maternals; B, 76 from 7; C, 63 from 7). Mean family

218 size was 9.2 offspring per maternal plant. Parental inbreeding coefficient (F), multi-locus outcrossing

219 rate (t_m), single locus outcrossing rate (t_s), bi-parental inbreeding rate (t_m-t_s), correlation of paternity

220 (r_p), and effective number of pollen donors per family ($1/r_p$) were all estimated using MLTR (Ritland

221 2002). Standard errors were calculated according to 1000 bootstraps.

222

223 Paternity assignment implementing maximum likelihood was undertaken using CERVUS V3.0.7

224 (Kalinowski et al. 2007). Here, Logarithm of Odds (LOD) scores estimate the likelihood of paternity

225 given the genotypes of the maternal, offspring and each candidate sire. Simulation was used to define

226 critical values of LOD scores and delta, the difference between the most likely and second most likely

227 candidate sire, with one mismatch allowed. Paternity was inferred when the most likely sire exceeded

228 thresholds for the LOD score and delta. Paternity assignments from CERVUS were then contrasted

229 to those manually estimated from full exclusion analysis with no mismatches allowed. Discrepancies

230 were checked by correcting for missed null alleles or when non-maternal and putative sire alleles at

231 a mismatched locus were <2 base pairs different in size. Final paternity assignment was based on

232 these corrected exclusion results.

233

234 In addition, parentage and sibship was inferred jointly using maximum likelihood (ML) methods in

235 COLONY v 2.0.6.5 (Jones and Wang 2010). Parameter settings included medium precision, full

236 likelihood, long length of run, 10 runs, strong sibship prior based on known maternals, polygamy for

237 males and females, inbreeding present, and a mutation rate of 0.0001. Paternal genotypes, inferred

238 from ML configurations of each offspring with known maternal, were used to infer ML full- and half-

239 sibships for each family. This enabled an estimate of paternal diversity without assignment to a

240 candidate from the known potential pool of sires. Paternity assignment results were used to estimate

241 realised pollen dispersal distances. Realised pollen dispersal distances were calculated by measuring

242 the distance (in metres) between maternal plants and assigned sires.

243

244 **Bird abundance and visits to *Anigozanthos humilis* flowers**

245 To estimate honeyeater abundance, 10-minute area searches were undertaken in each of two 1-ha

246 plots in each of the three sites. Area searches were undertaken on four occasions during the flowering

247 period of *A. humilis* (8th - 23rd October 2018). All observations were made during the morning, three

248 to five hours after dawn, using methods adapted from Davis et al. (2014). The start order of the 1-ha

249 area searches was randomised to minimise temporal bias. For each plot, one observer walked through

250 the entirety of the plot noting all nectar feeding birds observed and/or heard, while a second person

251 scribed. For analysis, only honeyeater species observed visiting *A. humilis* plants during the study

252 were included. Tukey's Post-Hoc tests were used to compare *A. humilis* pollinating honeyeater

253 abundance across the three sites with 95% confidence intervals.

254

255 Bird visitors to *A. humilis* flowers were detected using motion-triggered cameras (Reconyx HyperFire

256 Semicovert IR model). For each of five flowering plants per population, two cameras were set up (30

257   cameras total) and remained in place until flowering was complete. Cameras were redeployed on

258   another plant if flowers were lost to herbivory by kangaroos (which happened six times). Methods of

259   camera deployment and programming for capturing vertebrate visitors to flowers followed Krauss et

260   al. (2018) and van der Kroft et al. (2019). Cameras were checked once every two weeks until

261   flowering was complete. Images were processed manually and for photographs that captured floral

262   visits, the following was recorded: the visiting species, its sex (if determinable), colour band pattern

263   (if present – see below), number of probes per visit per plant (including multiple probes to the same

264   flower), duration of the visit, and time and date of visit. Non-parametric Kruskal-Wallis rank sum

265   tests and post-hoc Dunn's test (Dinno 2017) were used to test for differences between populations

266   for pollinator visits per 10 days, using individual plants as the replicates (R Core Team, 2017).

267

268   To assess individual visitation patterns by floral visitors, 9 western spinebills and 14 brown

269   honeyeaters were captured by mist-netting and individually colour banded to enable identification in

270   camera trap images. At the time of capture, generally between 07:00 and 11:00 AWST, all banded

271   birds were swabbed for pollen with a fuchsin gelatine cube at locations where pollen was most likely

272   to have accumulated, namely the forehead, lores, gape and throat (Wooller et al. 1983; Kearns and

273   Inouye 1993). Each gelatine cube was melted on a glass microscope slide with a coverslip and sealed

274   with nail polish. Using a bifocal microscope, pollen grains were counted and classified to species,

275   genus or family level by comparison to a reference library created from flowering plant species at

276   INR in September 2018. Pollen counts were pooled from 30 captured honeyeaters across the three

277   INR populations and the percentage of *A. humilis* pollen in the total pollen load was quantified.

278   Species captured included; brown honeyeater (*Lichmera indistincta*, n = 14), new holland honeyeater

279   (*Phylidonyris novaehollandiae*, n = 5), red wattlebird (*Anthochaera carunculata*, n = 1), and western

280   spinebill (*Acanthorhynchus superciliosus*, n =10). The percentage of *A. humilis* pollen and co-

281   flowering species pollen for the three INR populations was calculated for individuals of both western

282   spinebills and brown honeyeaters.

283

Results

285

Genetic diversity

Null alleles were detected at significant frequencies in maternal genotypes from progeny arrays in two of the four loci (Am8 and Am76). From these, 14 of 17 (82%) apparently homozygous maternal loci were clearly shown to be null heterozygotes. There were no null alleles detected from these progeny arrays for Am71 and Am82. Overall, for 4 loci across 23 maternal plants (92 locus by plant combinations), only 3 locus-plant combinations (3.3%) were confirmed as homozygotes, 96.4% were heterozygous. For the complete dataset of 131 adult plants, allelic diversity was very high at these 4 loci (N_a = 20 (Am8), 18 (Am82), 32 (Am71) and 38 (Am76)) (Table 1). This is an under-estimate of the true allelic diversity because of known null alleles, which from the known maternal genotypes were detected at frequency (null) = 0.17 (Am8) and 0.13 (Am76). Due to null alleles, observed heterozygosity (H_O) was lower than the expected heterozygosity (H_E) (Table 1).

297

Mating systems, paternal diversity and realised pollen dispersal

From MLTR, the overall estimate of multilocus outcrossing rate (t_m = 0.85), and within population estimates (t_m = 0.77, 0.88, 0.90) were all high but significantly different from one (Table 2). The high variability at these markers also enabled the unambiguous identification of selfed from outcrossed offspring. Selfed offspring (n = 35, 16.6%) were clearly differentiated from outcrossed offspring as the former possessed no non-maternal alleles, the later typically possessed 3 or 4 non-maternal alleles across 4 loci, but never one nor zero. From these data, unambiguous outcrossing rates varied among populations (0.75 (B), 0.87 (C), 0.89 (A)), and were very similar to MLTR estimates. Outcrossing rates among the offspring of flowers within individual plants varied markedly, from complete outcrossing to complete selfing. The MLTR estimates of bi-parental inbreeding overall ($t_m - t_s$ = 0.04) and within populations ($t_m - t_s$ = 0.03, 0.06, 0.09) were all low. Estimates of correlated paternity were

309 high overall ($r_p = 0.32$) and within populations ($r_p = 0.22, 0.36, 0.46$), and consequently estimates of

310 effective number of pollen donors overall ($1/r_p = 3.1$) and within populations ($1/r_p = 2.2, 2.8, 4.5$)

311 were consistently low (Table 2).

312

313 Paternity was assigned by maximum likelihood analysis for 117 of the 211 (55%) offspring

314 genotyped. For 96 of 211 offspring (45%), all known potential sires were excluded as a true sire,

315 indicating that the true sire lay beyond the plots where plants were sampled. Multiple paternity rates

316 varied markedly among families and among populations, from entire full-sib families (in two

317 families, one completely selfed, one completely outcrossed) to near complete half-sib families

318 (almost all offspring with a different sire). From paternal inference of all offspring in COLONY,

319 mean ($\pm$ SE) number of sires for a mean of 9.2 offspring per family was 3.5 (±0.3). From COLONY,

320 overall estimates of the ratio of offspring half sibs to full sibs per population were 60:40 (A), 71:29

321 (B) and 85:15 (C).

322

323 From paternity assignments, realized pollen dispersal reflected near neighbour mating, where for

324 approximately 80% of all offspring, known sires were within 20 m of the maternal mate, including

325 selfs (Fig. 2). For offspring with paternity assigned, the maximum detected pollen dispersal distances

326 (PDD) were 129 m (A), 20 m (B), and 30 m (C). Median detectable pollen dispersal distances were

327 low (3 m (A), 3 m (B), 9 m (C)), with an overall mean ($\pm$SE) of 8.0 m ($\pm$ 1.4). These PDD values

328 underestimate the true extent of pollen dispersal due to the high percentage of offspring (45%) for

329 which all known potential sires were excluded, and therefore the true sire occurred beyond the known

330 candidate pool of sires (Fig. 2). Although the distance between mates for these unassigned offspring

331 is not known, and could be less than the maximum distance between known mates, the high

332 proportion of unassigned offspring does reflect an extended tail to the pollen dispersal distribution.

333

334 **Bird abundance and visitation to *Anigozanthos humilis* flowers**

335 The motion-triggered cameras detected 62 visits by birds (including repeat visits by banded

336 individuals) to *A. humilis* plants over a total of 21,552 recording hours across the three populations.

337 From the number of inflorescences monitored, this equates to approximately one visit per

338 inflorescence every 10 days. Given that most flowers are open and receptive for up to 3 days, this

339 means that most flowers are not pollinated. The most common floral visitors were western spinebills

340 ($N = 47$), followed by New Holland honeyeaters (*Phylidonyris novaehollandiae*; Meliphagidae) ($N =$

341 6), silvereyes (*Zosterops lateralis*; Zosteropidae) ($N = 6$), and brown honeyeaters ($N = 3$). Silvereyes

342 appeared to act as nectar thieves, as photos showed them feeding at the base of the corolla tube of *A.*

343 *humilis* flowers and not making contact with the anthers or stigma. This behaviour suggests that

344 silvereyes are unlikely to effect pollination for *A. humilis*. Despite the known local abundance of

345 honey possums and their high visitation rates to co-flowering *Banksia menziesii* and *B. ilicifolia*

346 (Krauss et al. 2018), they were not recorded visiting flowers of *A. humilis,* nor in an earlier study (van

347 der Kroft et al. 2019).

348

349 Pooled 2-ha survey data showed that, for those species that pollinate *A. humilis*, the average number

350 of honeyeater individuals in A ($\pm$SE) ($N = 21 \pm 1.6$) was approximately double that of B ($N = 10 \pm$

351 1.0) ($P = 0.0070$), and five times that of C ($N = 4 \pm 0.7$) ($P < 0.001$). Despite variation in number of

352 honeyeater individuals, the average visits to *A. humilis* per 10 days ($\pm$SE) were low in all populations

353 (1 ± 0.5 (A); 3 ± 1.6 (B), 0.1 ± 0.8 (C)) (Fig. 3). Populations A and B were significantly different in

354 number of visits from C ($P = 0.049$ (A), $P < 0.001$ (B)), but not each other ($P = 0.072$). The average

355 number of honeyeater flower probes per visit per plant ($\pm$SE) (2.7 ± 0.2 (A); 5.1 ± 2.5 (B), 1.9 ± 1.0

356 (C)) did not differ significantly between populations ($P = 0.54$).

357

358 Few of the banded western spinebills ($N = 9$) and brown honeyeaters ($N = 14$) (Supplementary

359 materials S.4) were recorded by motion-triggered cameras visiting *A. humilis* plants (site A = 3

360 individuals, site B = 1; site C = 0). These visits generally occurred in the morning, although some

361 vitiations occurred between midday and dusk. Three of these banded birds returned to *A. humilis*

362 plants more than once. One banded western spinebill (Australian Bird and Bat Banding Scheme

363 number: 01A46230) was detected returning to the same plant ten times over seven days between 8th

364 and 24th October 2018. Paternity analysis of seven offspring from this plant showed that four were

365 selfed and three were sired by a plant 20 m away. One brown honeyeater (01A46222) was detected

366 visiting a plant over two consecutive days (5th and 6th October 2018), and one western spinebill

367 (01A46216) was detected making three visits to the same plant over three days (12th, 13th and 24th

368 October 2018).

369

370 Pollen counts from 30 captured honeyeaters revealed that *A. humilis* comprised only 0.3% of the total

371 pollen load. For 10 western spinebills, *A. humilis* pollen comprised 4.1% of the pollen load, which

372 equated to an average of 3.9 pollen grains, and a maximum of 13 *A. humilis* pollen grains per bird.

373 (Table 3). For 14 brown honeyeaters, only one *A. humilis* pollen grain was identified. Across all birds,

374 *A. cygnorum* accounted for at least 79% of all pollen, although this value is underestimated as the

375 percentage of *A. cygnorum* pollen was calculated from counts that were capped at 1000 (recorded 3

376 times), and *Banksia* pollen comprised 8%. The abundance of pollen of co-flowering species varied

377 among population and honeyeater species (Table 3 and Supplementary materials S.5). For western

378 spinebills, pollen of an unknown Myrtaceae species was carried in the greatest abundance at A and

379 C, while *A. cygnorum* pollen was the most common at B. For brown honeyeaters, *A. cygnorum* pollen

380 was most abundant for all three populations.

381

382 **Discussion**

383

384 The mating system of the bird-pollinated *Anigozanthos humilis* was characterised by frequent

385 outcrossing, low paternal diversity, and a pollen dispersal distribution characterised by near

386 neighbour mating and an extended tail. These parameters were all similar among populations, despite

387 variation in the relative abundance of co-flowering bird-pollinated plants and the abundance of

388 honeyeaters. While high outcrossing rates, despite self-compatibility, are consistent with predictions

389 for plants pollinated by nectar-feeding birds, the low paternal diversity and predominantly near

390 neighbour mating for *A. humilis* are departures from those predictions (Krauss et al. 2017). The

391 estimates of mate diversity in *A. humilis* were also lower than that previously reported for most other

392 Australian plant species that rely in-part or completely on bird pollination, and lower even than most

393 insect-pollinated plants (Table 4).

394

395 Our observations on pollinator visitation rates and pollen loads provides a mechanistic understanding

396 of the low mate diversity in *A. humilis*. In this study, visitation rates by honeyeaters to the flowers of

397 *A. humilis* were extremely low (on average one visit every 10 days or less). As a consequence, the

398 amount of *A. humilis* pollen detected on individual honeyeaters was also extremely low (0-13 grains).

399 Further, mixed pollen loads on birds were dominated by co-occurring vertebrate-pollinated shrubs

400 and trees, especially *Adenanthos cygnorum, Banksia menziesii,* and *B. ilicifolia*. The combination of

401 these three factors provides the ecological context for the surprisingly low paternal diversity for this

402 bird-pollinated herb. These observations are also consistent with previous conclusions of severe

403 pollen limitation in *A. humilis*, where mean fruit set following cross-pollination by hand was 37 times

404 greater than that following natural pollination (van der Kroft et al. 2019).

405

406 Almost 96% of the pollen loads on western spinebills, the most frequent bird pollinator to *A. humilis*,

407 was heterospecific. This very low purity of pollen loads suggests that heterospecific pollen transfer

408 (Morales and Traveset 2008) may have a significant impact on the success of bird pollination for *A.*

409 *humilis* and consequences for parental diversity. For example, the abundant deposition of

410 incompatible pollen of other species can dilute competition among conspecific pollen donors, and

411 reduce access to ovules because of clogging of stigmas and styles with heterospecific pollen,

412 impacting male and female fitness through lower mate diversity, seed siring and production (Waser

413   1978; Snow et al. 1996; Morales and Traveset 2008; Mitchell et al. 2009a,b). A consequence of the

414   adaptation to pollination by generalist nectar feeders may be a tolerance to heterospecific pollen,

415   especially of phylogenetically distinct species (Fang et al. 2019; Streher et al. 2020). A tolerance may

416   mean foreign pollen does not have a negative competitive or physical effect on the pistil, and may

417   itself be an adaptation that contributes to the maintenance of high species diversity in plant

418   communities (Fang et al. 2019). Further research into the consequences of heterospecific pollination

419   is required to test these hypotheses, but would lead to a better understanding of the relationship

420   between the behaviour of generalist bird-pollinators and the competition by plants for their pollinator

421   services (Mitchell et al. 2009b). Our results in *A. humilis* do, however, highlight the influence that

422   the more rewarding members of a plant community can have on the behaviour of generalist nectar-

423   feeding bird pollinators, potentially resulting in inefficient pollination and lower mate diversity for

424   less common, less floriferous and less rewarding co-occurring plants.

425

426

427   Despite the poor delivery of pollen to the flowers of *A. humilis*, outcrossing rates were high and

428   comparable to other specialist bird-pollinated plants (Table 4). High outcrossing rates, despite self-

429   compatibility, reflect strong preferential outcrossing, demonstrated by hand-pollination studies that

430   have shown the number of seed per fruit following self-pollination can be 1% of that following cross-

431   pollination (van der Kroft 2019). Bird pollinator behaviour and the flowering phenology of *A. humilis*

432   further promotes outcrossing. Unfortunately, most of the birds banded were rarely captured by our

433   cameras, meaning that a behavioural estimate of whether repeat visitation to the same flower is likely

434   to regularly contribute to self-pollination is not possible. However, in *A. humilis,* only two flowers

435   are open and receptive at any given time for an inflorescence, of which there are typically only one

436   or two per plant (Hopper 1993). Having few receptive flowers on a plant at one time minimises

437   geitonogamous pollination and promotes outcrossing (Harder and Barrett 1995; Snow et al. 1996;

438   Mitchell et al. 2004). However, this strategy comes at a cost of low visitation rates by pollinators,

439 especially when other co-occurring bird-pollinated species outcompete for pollinators by providing a

440 more abundant and rewarding nectar resource (Ramsey 1988; Collins and Briffa 1982; Mitchell et al.

441 2009b).

442

443 Relatively infrequent visits by bird pollinators to the flowers of *A. humilis* compared to those of co-

444 occurring mass-flowering species appears to be a feature of populations of this species in banksia

445 woodlands (Whelan and Burbidge 1980; Collins and Briffa 1982; Ramsey 1988), and possibly other

446 *Anigozanthos* populations in natural habitats (Brown 1988; Ayre et al. 2020; though see Phillips et

447 al. 2014). Low visitation rates may be driven by a combination of low visibility of plant species with

448 short scapes (Dudash et al. 2011), comparatively low per plant nectar rewards (Thomson 1988) and

449 potentially low foraging efficiency (Linhart 1973; Pyke 1981). More generally, many other bird-

450 pollinated plant species are also visited infrequently, including some species of understory herbs (Kay

451 and Schemske 2003; Turner and Midgely 2016), small shrubs (Johnson et al. 2010) and epiphytes

452 (Ackerman et al. 1994; Micheneau et al. 2006). Like *A. humilis*, these species tend to be characterised

453 by comparatively low numbers of open flowers or total nectar rewards, suggesting that our findings

454 with *A. humilis* could be applicable to a wider range of understorey species that are also pollinated

455 by generalist species. As such, we shed new light on the conditions that can lead to departures from

456 high paternal diversity predicted for plants pollinated by nectar-feeding birds. Competition for bird

457 pollinators appears to be a limiting factor for herbaceous species, likely exacerbated in the presence

458 of heavily co-flowering trees and shrubs.

459

460 Given this ecological context, it is unsurprising that mating systems can vary significantly within and

461 among plant populations (Whitehead et al. 2018). Pollinator abundance, movement patterns and

462 behaviour, and competition for their services, may vary dramatically depending on plant population

463 sizes and flowering plant species composition (Linhart 1973; Collins and Briffa 1982; Phillips et al.

464 2014). The current study extends this understanding of variability in the mating system and

465   pollinators to a mechanistic understanding of the variability that exists in the mating portfolio (Barrett

466   and Harder 2017) among individuals and populations of plants pollinated by nectar-feeding birds.

467   For example, while low visitation rates were the norm for *A. humilis*, camera traps highlighted one

468   plant that was recorded being visited by the same banded bird on 10 occasions over 7 days, while

469   other plants were not visited at all. Between maternal plants and their offspring, mating was found to

470   vary from complete selfing to complete outcrossing for *A. humilis*. For species that have low visitation

471   rates from bird pollinators, self-compatibility may provide a bet-hedging strategy, enabling seed set

472   despite severe pollen limitation. Indeed, self-incompatibility may have evolved in response to more

473   reliable pollination by birds in co-occurring mass-flowering dominant tree species such as *Banksia*

474   *menziesii* (Ramsey and Vaughton 1991). However, for *Anigozanthos humilis*, extremely high

475   heterozygosity at the genetic markers deployed suggest that preferential outcrossing and fitness

476   benefits through heterosis could promote the evolutionary benefits of bird pollination, wide

477   outcrossing and paternal diversity, despite the inefficiencies associated with its pollination.

478

479   Our work with *A. humilis* suggests that the ecological and genetic consequences of pollination by

480   birds can differ greatly depending on the plant species involved. While studies on bird-pollinated

481   shrubs and trees have typically shown frequent visitation (e.g. Collins and Briffa 1982; Ramsey 1988;

482   Krauss et al. 2018) and a trend for high paternal diversity (Table 4; Krauss et al. 2017), here we have

483   seen a very different result. If this pattern occurs more broadly in bird-pollinated plants with few or

484   inconspicuous flowers, it raises the question of why bird pollination evolves in these lineages?

485   Reasons hypothesised for the evolution of bird pollination include more efficient pollen transfer

486   (Castellanos et al. 2003), low availability of insect pollinators in certain habitats, and higher mate

487   diversity (Krauss et al. 2017). However, these explanations may not apply to many of the less

488   floriferous bird-pollinated plants such as *A. humilis* that co-occur with species that have much higher

489   visitation rates. Given that there is a large diversity of herbs that are known to be bird pollinated or

490   conform to the bird pollination floral syndrome (e.g. Keighery 1980; Rosas-Guerrero et al. 2014), a

491 key question that remains is what are the ecological consequences of this strategy, and why did it

492 evolve in this group of plants? Given that genetic studies on this group of plants are surprisingly few

493 (e.g. Table 4), there is a need to test the generality of our genetic results, and understand the fitness

494 consequences of bird pollination under conditions of infrequent pollinator visitation.

495

505

506

Author contribution statement

508 All authors conceived of the concepts and ideas. JHK conducted the field and lab work, with

509 assistance from SLK, JA and RAD. JHK and SLK analysed the data. JHK, RDP and SLK led the

510 writing with significant contributions from all others.

511

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793 **Figures**

793 **Figures**

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795 **Figure 1.** *Anigozanthos humilis* growing in Ioppolo Nature Reserve (INR), Western Australia (left).

796 The inflorescences of *A. humilis* generally have a single scape with numerous subtubular flowers

797 forming an inflorescence. At INR, flowers of *A. humilis* are visited almost exclusively by honeyeaters,

798 with the western spinebill (right and below) the most frequent visitor, note the dusting of yellow

799 pollen on the birds head (right). Photos by Joshua Kestel.

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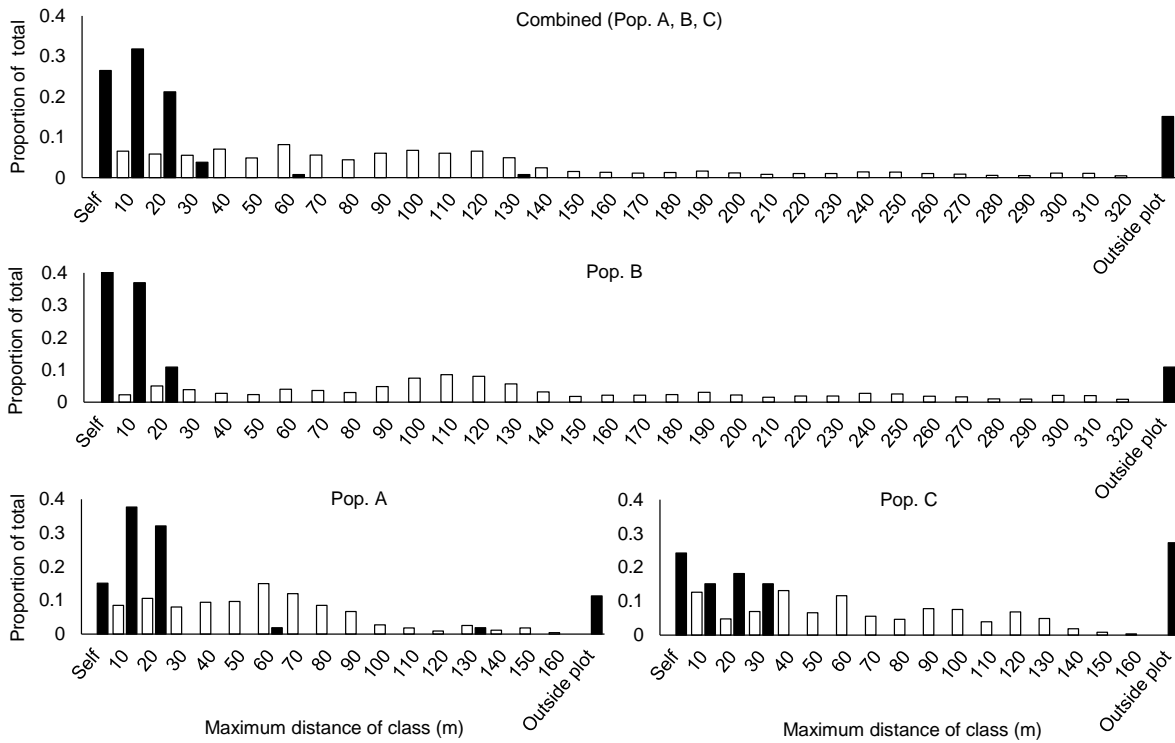
Figure 2. Realized pollen dispersal distributions from paternity assignment of offspring for *Anigozanthos humilis* in each of three populations (A,B,C), and combined. Y-axis shows the proportion of total, x-axis shows upper bound of distance in meters between mates (solid bars) and between all pairs of plants (open bars) for each distance class. Also shown are proportion of offspring that were the product of self-fertilization (self) and the proportion of offspring for which all known potential sires were excluded as the true sire, so pollen is inferred to have originated from outside the study plot area, but true distance is unknown.
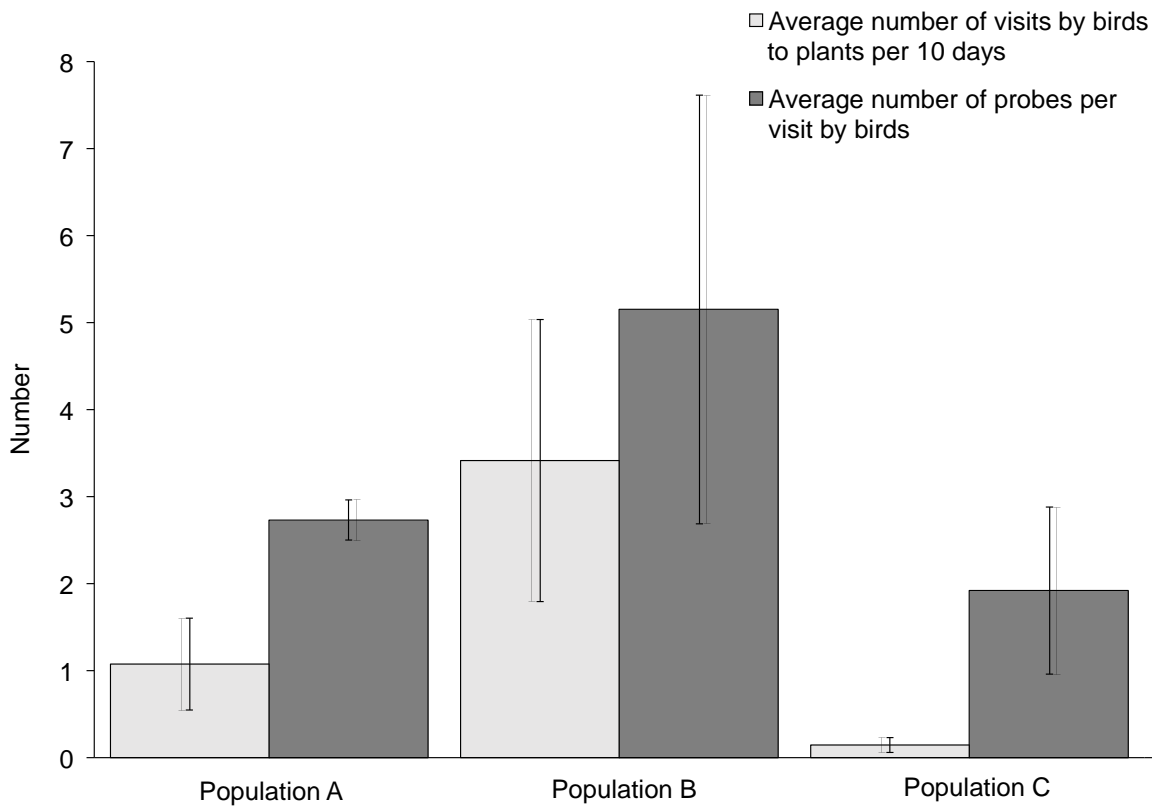
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820 **Figure 3.** Measures of bird visitation from 30 motion-triggered cameras monitoring 15 *Anigozanthos*

821 *humilis* plants across three sites (Populations A, B, C) in Ioppolo Nature Reserve, Western Australia.

822 Average number of visits by birds to plants per 10 days, and average number of probes per visit by

823 birds (counting multiple probes to the same flower) are shown with standard errors.

824

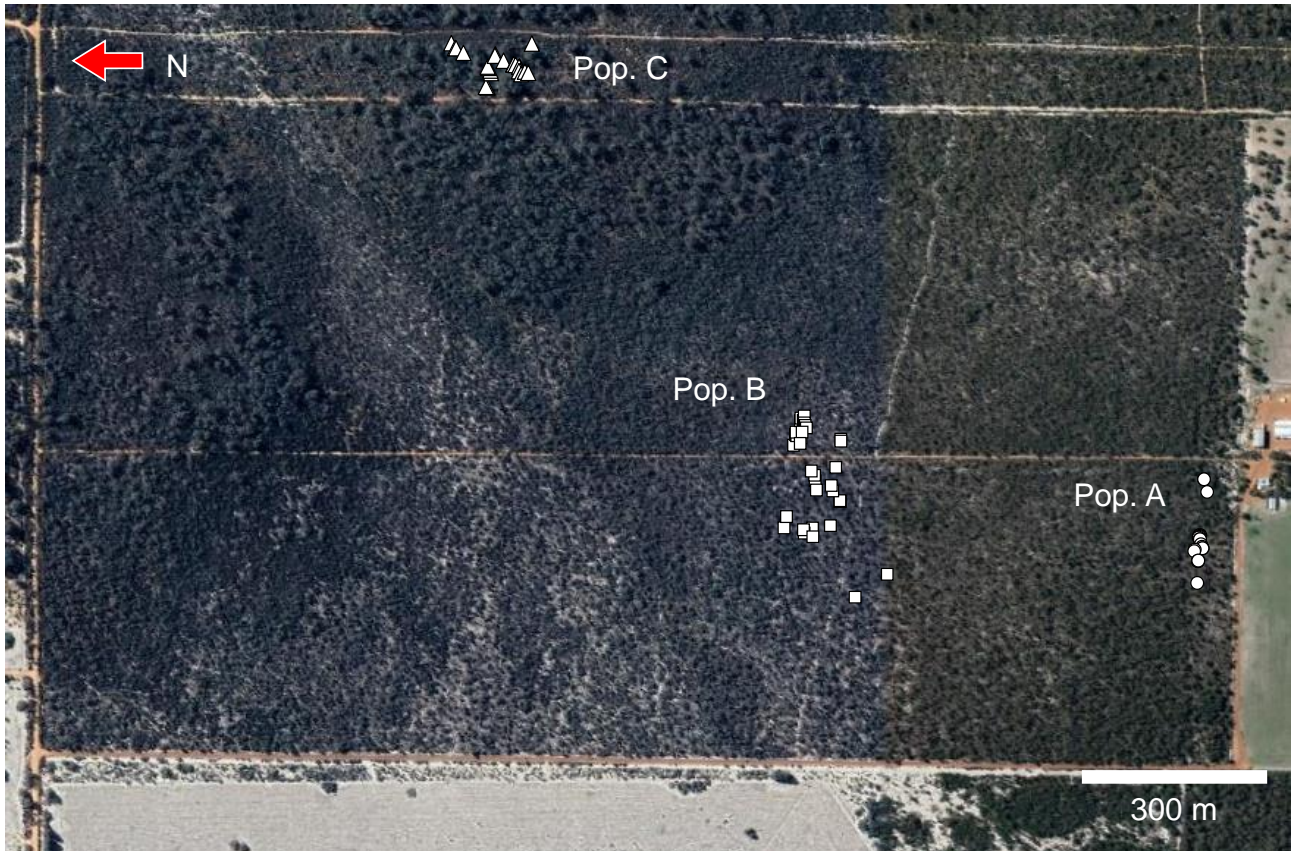825

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Electronic Supplementary materials

Figure S.1 Map of the three *Anigozanthos humilis* populations at Ioppolo Nature Reserve (INR),

Western Australia. Symbols indicate the locations of individual plants. Image from Google Earth.

Table S.2. Distance and density parameters for 131 *Anigozanthos humilis* plants surveyed after

herbivory, across three sites (Populations A, B, C) in Ioppolo Nature Reserve, Western Australia.

Parameters	A	B	C
Area surveyed (ha)	1.84	4.54	1.64
A. humilis plants surveyed	33	57	45
A. humilis density (plants per ha)	18	13	30
Average distance between *A. humilis* plants (m)	51.81 ± 1.44	129.08 ± 2.07	62.44 ± 1.21
Range of distances between *A. humilis* plants (m)	0.55 – 141.71	0.75 – 281.32	0.03 – 163.29

S.3 Canopy species flowering phenology

Within each of the three *A. humilis* populations, co-flowering canopy species known to be visited by bird species for nectar were surveyed on the 19th September 2018, 23rd October 2018, and 25th October 2018. Co-flowering canopy species were surveyed within each population by walking in a grid-like pattern across two 1-ha plots. The number of flowers/ inflorescences on each co-occurring co-flowering bird pollinated canopy species was counted by an observer at ground level. In pop. A, *B. ilicifolia* was the dominant co-occurring co-flowering canopy species, contributing 61% of the total floral resources available during the *A. humilis* flowering season. Within pop. B, *B. menziesii* was the dominant canopy species, providing 82% of the total floral resources available within the population. *A. cygnorum* was the only species flowering in pop. C, and in relatively small numbers.

S.4 Bird banding by population

Honeyeaters documented visiting *Anigozanthos humilis* in van der Kroft et al. (2019) were captured and banded over five sessions from 11th September 2018 - 28th September 2018. During every session, mist nets were opened at each site prior to sunrise and checked every 20 mins. Pre-recorded western spinebill and brown honeyeater calls were played from Bluetooth speakers located next to each of the nets. Captured western spinebills and brown honeyeaters were measured and fitted with a standard metal band and two to four colour bands subject to an approved marking scheme from the Australian Bird and Bat Banding Scheme. Across all three *A. humilis* populations, 25 birds were banded. Number of birds banded by site were: 5 western spinebills, 6 brown honeyeaters (population A); 5 western spinebills, 6 brown honeyeaters (B); 1 western spinebill and 2 brown honeyeaters (C).

Table S5. Pollen counts from 30 netted honeyeater species in Ioppolo Nature Reserve, Western Australia. Names of bird species are abbreviated to published four letter abbreviations as follows; BrHo = brown honeyeater, NHHo = new holland honeyeater, ReWB = red wattlebird, and WeSp = western spinebill. Pollen was not counted above 1000 grains per sample. For calculations, individuals

869   with more than 1000 grains were rounded down to 1000. ID code refers to band numbers for the

870   Australian Bird and Bat Banding Scheme.

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Pop.	ID code	Bird sp.	Number of grains				
			Adenanthos cygnorum	*Banksia* sp.	*Anigozanthos humilis*	Unknown pollen sp. 2	Unknown Myrtaceae
A	01A46221	BrHo	1000	15	0	0	68
A	03727926	NHHo	47	379	6	0	12
A	03727925	NHHo	0	1	0	0	4
A	03727924	NHHo	33	21	0	3	14
A	03727923	NHHo	9	54	0	0	1000
A	03727922	NHHo	34	0	0	0	1
A	07312571	ReWB	5	27	0	0	98
A	01A46215	BrHo	303	0	0	0	0
A	01A46217	BrHo	935	79	0	0	18
A	01A46225	BrHo	10	122	0	0	0
A	01A46234	BrHo	450	29	0	0	0
A	01A46223	WeSp	22	59	13	0	122
A	01A46222	BrHo	910	98	0	0	0
A	01A21624	WeSp	18	13	0	0	22
A	01A46216	WeSp	12	0	1	0	22
B	01A46219	WeSp	0	5	0	0	0
B	01A46218	WeSp	19	5	8	1	1
B	01A46234	BrHo	800	0	0	0	0
B	01A46232	WeSp	11	14	4	0	0
B	01A46233	BrHo	1000	0	0	0	0
B	01A46231	WeSp	5	1	1	0	1
B	01A46230	WeSp	107	0	0	0	0
B	01A46229	BrHo	750	25	1	0	0
B	01A46219	WeSp	39	58	7	0	3
B	01A46228	BrHo	760	30	0	0	0
B	01A46227	BrHo	270	1	0	0	1
B	01A46226	BrHo	1000	28	0	0	0
C	01A46220	WeSp	50	7	5	19	310
C	01A46236	BrHo	1000	0	0	1	0
C	01A46235	BrHo	1000	0	0	0	1

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