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1 The bees don't know and the flowers don't care: the effect of heterospecific pollen on
2 reproduction in co-occurring lilies (*Thysanotus* spp.) with similar flowers.

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9 Running title: Heterospecific pollen and reproduction in lily species

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47 ABSTRACT

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49 Species integrity relies on the maintenance of reproductive isolation, particularly between
50 closely related species. It has been hypothesised that the presence of heterospecific pollen
51 on flower stigmas adversely affects plant reproduction with increasing effect in closely
52 related species. Using two pairs of co-occurring buzz pollinated *Thysanotus* lilies in the
53 Mediterranean climate region of Perth, Western Australia we quantified the effect of
54 heterospecific pollen on fruit and seed set. We first determined mating systems of the two
55 focal species using self and outcross pollen followed by separate treatments with
56 heterospecific pollen within each species pair. Of the two species receiving pollen,
57 *Thysanotus triandrus* had a mixed mating system but with significantly lower fruit and
58 seed set from self pollen relative to outcross pollen. *Thysanotus tenellus* was autogamous
59 with no difference in fruit or seed set between autogamous, self or outcross pollinations.
60 Heterospecific pollen had no effect on fruit or seed set of either focal species. This
61 outcome points to a post-pollination means of reproductive isolation consistent with a
62 flower morphology that causes a low specificity of pollen placement and thus a poor
63 capacity for pre-pollination discrimination. Negative effects of heterospecific pollen,
64 therefore, do not appear to play a role in reproduction within this fascinating group of
65 buzz pollinated flowers.

66

67 ADDITIONAL KEYWORDS: Autogamy - buzz pollination - fringe lily - heterospecific
68 pollen transfer - mixed mating - selfing.

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78 INTRODUCTION

79

80 Sexual reproduction in angiosperms is achieved through the medium of flowers with
81 abundant evidence that flowers have been integral to the diversification of the group
82 (Harder & Barrett, 2006). Critical to diversification is the subsequent maintenance of
83 species integrity and effective reproductive isolation. The use of animals that have their
84 own agendas for gamete transfer in most flowering plants means plants have needed to
85 develop ways of either restricting which pollinators visit their flowers or limiting the
86 interference of heterospecific pollen on their own reproduction. Pre-zygotic barriers
87 between species may be driven by phenological differences, divergent floral morphology
88 or differences in pollinator attraction signals including colour, scent (Okamoto *et al.*,
89 2015) or rewards (Johnson, 2010). Post-zygotic barriers include incompatibility systems
90 (McClure & Franklin-Tong, 2006), low viability of hybrids or hybrid sterility (e.g.
91 Fishman & Wyatt, 1999).

92

93 Recently there has been considerable attention paid to how reproductive isolation
94 may occur between closely related species with divergent floral morphologies (e.g.
95 Armbruster, 2014; Devaux *et al.*, 2014). Specialised floral forms permit flower visitors to
96 effectively access resources of one species and not others and accurately deliver pollen
97 from one flower to another only within the same species (Johnson, 2006; 2010; Harder &
98 Johnson, 2009). Structural modification leads to reproductive isolation of some plants in
99 a population and eventually leads to speciation. In many of the iconic examples of
100 radiation (Goldblatt *et al.*, 2000, 2001; Goldblatt & Manning, 2006) modifications are
101 associated with altering the location or accessibility of a food reward (usually nectar), so
102 that flowers may become unattractive to more generalist pollinators unable to access the
103 reward. Importantly, unsuitable flower visitors are likely to be ineffective pollinators and
104 selection is predicted to refine and optimise the fit between flower morphology and
105 appropriate pollinators. In radially symmetrical (actinomorphic) flowers, in contrast,
106 there is less likelihood that flowers will be specialised for a narrow range of pollinators
107 and isolating mechanisms are more likely to be physiological (post pollination) than in
108 bilaterally symmetrical flowers. Examining the pollination biology of species with

109 actinomorphic flowers and relatively specialised pollination can help understand how
110 reproductive isolation can be maintained in species with apparently similar, relatively
111 unspecialised floral morphology.

112

113 One group of plants with mostly actinomorphic flowers, however, do rely on a rather
114 narrow range of pollinators. Flowers that are buzz pollinated automatically select a
115 restricted pollinating fauna of particular bee species that sonicate the anthers to release
116 pollen (Buchmann, 1983). Therefore, given generalist flower morphology yet specialised
117 pollination, how do these species maintain reproductive isolation? Although some buzz
118 pollinated species may have accurate pollen deposition on a flower visitor and
119 subsequently accurate pollen reception (Harder & Wilson, 1997) others may not (Huang
120 & Shi, 2013). Taxa with the *Solanum* type floral structure (many *Solanum* spp.,
121 *Dodecatheon*, Tremandraceae, Boraginaceae) have a focused type of pollen delivery to a
122 site on bees near where the stigma is also likely to contact the visitor, thereby increasing
123 successful pollen transfer. However species in other groups with poricidal anthers (which
124 release pollen through pores) such as in the Melastomataceae have anther configurations
125 that do not provide pollen delivery onto a consistent part of a flower visitor. In Australia
126 there is a group of species with open lily-type flowers, some of which also have an anther
127 configuration similar to that in *Melastoma*. In *Thysanotus*, a genus of approximately 50
128 species (Brittan, 1987) there is little possibility of specialised bee-floral matching as
129 buzzing tends to produce a rather messy pollen release. This makes specific, accurate
130 pollen delivery to a stigma and hence reproductive isolation by this method, difficult.

131

132 If closely related species with very similar flowers that lack morphological
133 divergence co-occur and pollinators are indiscriminate in their visitation patterns, flowers
134 likely experience heterospecific pollen transfer (HPT, Morales & Traveset, 2008) or
135 pollen loss (Buide *et al.*, 2015). If pollen is incompatible this may just cause stigma
136 clogging (De Jager, Dreyer & Ellis, 2011), where heterospecific pollen prevents
137 conspecific pollen contacting the stigmas. If interbreeding barriers are weak or absent,
138 female function may be adversely affected due to ovule usurpation or production of
139 infertile hybrids at the expense of fertile offspring (Fishman & Wyatt 1999).

140

141 A number of studies have examined the effect of heterospecific pollen on fertility of
142 the recipient species (Thomson, Andrews & Plowright, 1981; Kwak & Jennerston, 1991;
143 Morales & Traveset, 2008) but few have specifically examined the effect of
144 heterospecific pollen from a closely related congener on reproductive success. One
145 exception, Arceo-Gómez & Ashman (2011), found that heterospecific pollen decreased
146 seed set and elevated seed abortion. De Jager *et al.* (2011) also found that application of
147 heterospecific pollen prior to conspecific pollen reduced seed set in coflowering *Oxalis*
148 species.

149

150 Our aim was to determine both the pollination biology and what effect heterospecific
151 pollen had on the reproductive output of two species, each one of which was matched
152 with another with almost identical superficially actinomorphic flowers. To achieve this
153 we measured flowering period to investigate phenological isolation and observed bee
154 visits to two species. We used hand pollination to determine mating system and the effect
155 of HPT on fruit and seed set of two focal species. Collectively, these investigations
156 permitted us to evaluate evidence for negative impacts of heterospecific pollen transfer
157 and pre- vs. post-zygotic mechanisms of species maintenance. Our null hypothesis was
158 that heterospecific pollen would have no effect on fruit or seed set. However, because the
159 focal species coflower, are closely related (Sirisena, 2010) and have such similar flowers,
160 bees may not be able to distinguish between species. Thus, an alternative that may be
161 expected is that heterospecific pollen exchange may be common, and this may decrease
162 the reproductive output of the species.

163

164

MATERIALS AND METHODS

165

166

STUDY SPECIES AND AREA

167

168 Pollination trials on *Thysanotus tenellus* Endl. and *T. triandrus* R.Br. (Asparagaceae)
169 were recorded at the Nicholson Road Reserve (32° 02' 56.89", 115° 55' 58.75") in the
170 Perth metropolitan region of southwestern Western Australia. The study site is an urban

171 reserve in a low lying area of the Bassendean landform system of the Swan Coastal Plain,
172 supporting relatively natural heathland vegetation with scattered emergent trees. The soil
173 profile is described as duplex with a sandy A horizon and a dense sandy clay B horizon
174 that becomes waterlogged in the lower areas in winter and early spring. The climate is
175 Mediterranean-type with cool winters and hot summers (average maximum annual
176 temperature 25.5°C, minimum annual 13.4°C) and rainfall is approximately 822mm/year
177 (Bureau of Meteorology, 2014, for the City of Gosnells, 1991-2013). All species occur in
178 the same area but do vary somewhat in density in different parts of the area (Supporting
179 Information Fig. S1).

180

181 The flowering period of each species overlapped with a congeneric species that had an
182 almost identical flower form. *Thysanotus* species have very similar coloured and shaped,
183 broadly campanulate flowers. The flower colour is usually described in published
184 descriptions as purple but there may be slight variation between species (Fig. 1). The
185 flower colours of the four *Thysanotus* species in this study were quantified using an ASD
186 FieldSpec 4 Spectroradiometer (standard resolution) to confirm similarity of colour
187 across the visible and near-visible UV spectrum (350-700 nm; Supporting Information
188 Fig. S2).

189 Flowers last only a few hours, opening in the morning and closing about 5 – 6
190 hours later. All species have a gynoeceium with a single papillate stigma, three locular
191 ovary and two ovules per loculus.

192

193 Most of the species of *Thysanotus* are enatiostylous, buzz pollinated and there are
194 three anther configurations, two of which are represented in the species studied here (Fig.
195 1). There is no nectar and pollen is the only reward. *Thysanotus tenellus* is very similar to
196 a currently unpublished *Thysanotus* (*Thysanotus* sp. Coastal plain (N.H. Brittan 66/63)
197 referred to hereafter as *T. sp.*) and has six suberect, equal sized anthers (Table 1) that
198 generally cluster to one side of the gynoeceium. Although *T. sp.* has not been
199 phylogenetically assessed, from the study by Sirisena (2010) it is likely to be in the same
200 clade as *T. tenellus*. In *T. tenellus* the style is only slightly longer than the anthers, while
201 in *T. sp.* the style is longer and hooked so the stigma is well above the anther pores but

202 faces towards them when the flower is open. Pollen is deposited on the ventral surface of
203 bees and in *T. tenellus* would be picked up from the same surface but in *T. sp.* pollen
204 reception is from the dorsal surface of a visitor. While this would seem an inefficient
205 method of pollen collection, the buzzing ejects pollen in all directions and some will land
206 on the side and back of any visiting bee. In *T. triandrus*, sister to the very similar *T.*
207 *multiflorus* R.Br., (Sirisena, 2010) there are three curved anthers (Table 1) to one side of
208 the gynoeceum and the curved style brings the stigma to face the anther pores. Bees grasp
209 the anthers and pollen is deposited on the ventral surface but as in *T. sp.*, pollen reception
210 is from the dorsal surface of the visitor.

211

212 The plants were examined in three separate areas within the reserve that were
213 delimited on the basis of fire history. Area A was burnt in 2000, is adjacent to (separated
214 by a 3m wide track) area B burnt in 2002 and area D is long unburnt (> 20 years) and
215 over 500m away from the other two areas with species varying in abundance but largely
216 co-occurring (Supporting Information Fig. S1).

217

218 FLOWERING PHENOLOGY AND POLLINATOR VISITATION

219

220 In 2013 all flowers of the four species were counted on most days during the flowering
221 season in three areas (A, B and D) where they occur in the reserve. Phenological
222 reproductive isolation was calculated between *T. tenellus* and *T. sp.* and between *T.*
223 *multiflorus* and *T. triandrus* following Martin & Willis (2007) for the areas in which they
224 co-occur. Phenological isolation quantifies the synchrony of flowering based on the
225 proportion of each species flowering each day and is one where there is complete
226 isolation and zero when there is complete overlap in time and number of flowers.

227

228 At the same time as flowers were counted, pollinator activity was also measured in two
229 ways. First, during flower counting flowers were scrutinized for pollinators as observers
230 (PGL, EEB) travelled through the study site. These observation periods occurred from
231 20-30 days per year with each visit ~30 minutes in duration and included all focal species
232 with qualitative estimates of pollinator activity recorded. Second, in November 2013,

233 focal flowers were regularly sampled to quantify pollinator visitation rates. *T. triandrus*
234 and *T. multiflorus* plants growing in close proximity were observed for 10 minute periods
235 during the morning (total 5.8 hours, 2700 flower hours) and bee visits were recorded. *T.*
236 *tenellus* was not sampled to quantify visitation rate because over the entire month of
237 flower counting not a single pollinator was observed (further, examination of open
238 flowers near their closing time in 2011, 2012, and 2013 showed that the vast majority of
239 flowers had no pollen on the petals indicating little to no visitation by bees; pers obs. P.
240 Ladd).

241

242

POLLINATION EXPERIMENTS

243

244 Mating system

245

246 Mating system manipulations were performed on plants in the field that were bagged
247 using black plastic mesh (2mm opening) that excluded any potential pollinators. Pollen
248 was obtained by slitting an anther to expose the pollen and applying the pollen to the
249 stigma of a flower while using a X12 jeweler's lens to ensure the pollen was correctly
250 placed. Treatments were autogamy (flowers unmanipulated), self (pollen from the same
251 flower), outcross (pollen from a plant over 4m from the recipient, flower emasculated).
252 From a floral anatomical point of view there are two pairs of species (Table 1) and these
253 have overlapping flowering times. Thus hybrid pollen crosses were also done to see if the
254 species would produce hybrid seed (manipulated flowers emasculated). In the two focal
255 species supplementary pollination of flowers on unbagged inflorescences was compared
256 with unmanipulated flowers tagged at the same time as the supplementary pollinations to
257 test for pollen limitation under natural conditions. Flower pedicels were tagged with
258 different coloured beads for different treatments. A self-incompatibility index (ISI) was
259 calculated from the mating system study (Zapata & Arroyo, 1978) as $1 - \text{hand self}$
260 $\text{pollination (or autogamous treatment)}/\text{hand cross pollination}$.

261

262 Heterospecific pollen

263

264 To test whether heterospecific pollen inhibited the production of fruit, *T. tenellus* and *T.*
265 *triandrus* were used as recipients for pollen from *T. sp.* and *T. multiflorus* respectively.
266 Six *T. triandrus* plants with six or more inflorescences were selected (in area B) and
267 covered with mesh bags to prevent pollinator interaction. Six inflorescences were labeled
268 per plant, using different coloured labels for self pollination (3), and outcross pollination
269 (3). At 7.30am each day every flower on the labeled inflorescences was hand pollinated
270 with *T. multiflorus* pollen. At least an hour later, the flowers were pollinated with either
271 self or cross *T. triandrus* pollen, according to their tag. It was found that *T. tenellus* is
272 autogamous at the Nicholson Reserve but from the mating system experiment hybrid
273 pollen seemed to decrease fruit set so in a separate trial flowers were pollinated with
274 hybrid (*T. sp.*) pollen. At flower opening in the morning *T. sp.* pollen was applied to the
275 stigma of *T. tenellus* flowers (12 plants used) and flowers were tagged – a similar number
276 of non-manipulated flowers on each plant were also tagged as controls. As the interest
277 was in whether heterospecific pollen interfered with fruit set if the flowers were visited or
278 not, inflorescences were not bagged. As self pollen seems to be placed on stigmas before
279 flowers open, it was not possible to have a treatment of *T. sp.* pollen applied to
280 emasculated *T. tenellus*. In all cases any fruits that formed were harvested to count seeds
281 and the numbers of tagged flowers that did not set fruit were recorded. Unfortunately, it is
282 not possible to examine pollen tube growth in the styles because in *Thysanotus* aniline
283 blue-stained pollen tubes do not clearly fluoresce under ultraviolet light.

284

285 Heterospecific pollination of *T. triandrus* on *T. multiflorus* and *T. tenellus* on *T.*
286 *sp.* were assessed but only one plant of *T. multiflorus* and *T. sp.* was used so the results
287 are preliminary and indicative only.

288

289

STATISTICAL ANALYSIS

290

291 Fruit set and seed production data were analysed using generalised linear mixed models
292 (GLMMs) in R 3.0.2 (R Core Team, 2013), using the lme4 package (Bates, Maechler &
293 Bolker, 2012). Mean proportions and 95% confidence intervals were calculated and

294 comparisons between pollination treatments were made graphically. Statistical
295 significance was assessed against an alpha level of 0.05.

296

297 Analysis followed the suggestions of Bolker *et al.* (2009) for analysing non-
298 normally distributed data that also include random effects. GLMMs were fit by maximum
299 likelihood using a binomial distribution with a logit link for fruit set data
300 (presence/absence). For seed set data (counts between 1 and 6), GLMMs were
301 implemented using a Poisson distribution with a log link. Parameter estimates were
302 determined by adaptive Gauss-Hermite Quadrature (GHQ), as recommended by Bolker *et*
303 *al.* (2009) for models with binomial and Poisson distributions that include less than three
304 random effects. GLMMs were also tested for overdispersion using `overdisp_fun()`, and
305 no overdispersion was detected in any of the models.

306

307 Mating system

308

309 GLMMs were implemented for the proportion of flowers setting fruit, and the mean
310 number of seeds per fruit. For these GLMMs, pollen treatment was the predictor, and
311 plant number was the random effect. Open pollination was the baseline (control) to which
312 each pollination treatment was compared.

313

314 Heterospecific pollen transfer

315

316 To determine whether interspecific pollen transfer reduced reproductive success, the fruit
317 and seed set data from the HPT experiment were compared to the results obtained from
318 the mating systems experiment. The proportion of fruit with each possible seed number
319 (1-6) was also graphed for self and outcross pollination for *T. triandrus*, again comparing
320 interspecific pollen transfer to the mating systems experiment. Chi tests were used to
321 compare the frequencies for outcross and self pollinations between the mating system and
322 interspecific pollen transfer experiments. This occurred for the proportion setting fruit
323 and mean number of seeds per fruit.

324

325

RESULTS

326

327 Fringe lilies flower sequentially from September (austral spring) through to December
328 (Fig. 2). *T. tenellus* and *T. sp.* are the first to flower followed by *T. multiflorus* starting in
329 early October and *T. triandrus* in early November (Fig. 2). Flowering of *T. tenellus* and
330 *T. sp.* is episodic with peaks and troughs relatively consistent between species, while
331 peak flowering of *T. tenellus* and *T. sp.* occurred at a similar time in both areas where
332 they co-occur (Table 1). While synchrony of flowering was broadly similar across the
333 three populations studied, variation in plant densities and therefore flower abundance
334 contributed to variation in phenological reproductive isolation (RI). More flowers of *T.*
335 *tenellus* occurred in area D and less of *T. sp.* giving *T. tenellus* a higher RI value than the
336 other species while the opposite was true in area B. In the second species pair synchrony,
337 peak flowering of *T. triandrus* was 19 days later than that of *T. multiflorus* in area A and
338 RI was higher for *T. triandrus* than *T. multiflorus*. Across their range, these two species
339 rarely co-occur (Brittan, 1987), but were intermixed in area A but not area B (only
340 separated by 30-70m, well within pollinator flight distance).

341

342 Flowers of *T. triandrus* and *T. multiflorus* have very similar measured colour
343 spectra, as do *T. tenellus* and *T. sp.* (Supporting Information Fig. S2) with peaks
344 coincident with bee blue spectral reception.

345

346 *T. triandrus* has a mixed mating system (ISI 0.56) however self pollen results in
347 significantly reduced (< 50%) fruit and seed set (Fig. 3, Supporting Information Table
348 S1) compared with outcross pollen. Self pollination resulted in only one or two seeds per
349 fruit while outcross pollination generally produces three to six seeds. Sixty-one percent of
350 fruits from outcross pollen had six seeds while only 5% had one seed. There was no
351 evidence of autogamy.

352

353 In open pollinated flowers, there was a low number of seeds per fruit. This
354 matched the number of seeds per fruit in the self pollination treatment, which indicates

355 that most fruit were the product of geitonogamy (Fig. 3). So, while there is some
356 reproductive assurance, this is relatively weak.

357

358 Fruit and seed set did not differ in *T. tenellus* between autogamous, self and
359 outcross pollination. (ISI = 0.1, Fig. 3, Supporting Information Table S2). The species
360 has prior selfing (*sensu* Lloyd, 1979) as it was found that 88% of stigmas (n = 20) from
361 just opened (unvisited) flowers carried 4.7 ± 1.0 (SE) pollen grains. Hybrid pollination
362 between *T. tenellus* and *T. sp.* with *T. tenellus* as the pollen recipient produced lower
363 (although not significantly lower) fruit and seed set with more variability than
364 intraspecific pollination (Fig. 3B; Fig. 3D).

365

366 Pollen content for *T. tenellus* is about half that of *T. sp.* (Table 1) while the pollen
367 content and colour spectra for *T. multiflorus* and *T. triandrus* are almost identical (Table
368 1, Supporting Information Fig. S2). Blue-banded bees (*Amegilla chlorocyanea* [Cockerell
369 1914]) visit both *T. triandrus* and *T. multiflorus* indiscriminately. In area A, where *T.*
370 *multiflorus* and *T. triandrus* grow together, *A. chlorocyanea* were seen flying from
371 flowers on one species to flowers of the other (often backwards and forwards) 11 times
372 during 500 minutes of pollinator observations (2700 flower hours). Visits to *T.*
373 *multiflorus* plants only were observed on two occasions, and to *T. triandrus* only on one
374 occasion. No bees were observed on the other two species and observations of flowers to
375 assess possible visitation in 2013 showed few flowers had been visited (often none) on
376 the days they were observed.

377

378 Pollination of unbagged flowers of *T. tenellus* with pollen of *T. sp.* produced the
379 same fruit and seed set as control (open pollinated) flowers (Fig. 3). The aim of this HPT
380 trial was to determine if heterospecific pollen had any effect on fruit or seed set and the
381 results show there was no effect. However it is not possible to decide if the seed produced
382 is the result of early acting autogamy or hybridization, although the lack of seed set in the
383 reverse cross of *T. tenellus* pollen on *T. sp.* stigmas suggests it is unlikely hybrid seed
384 would be produced (Table 2).

385

386 In *T. triandrus* heterospecific pollen produced no effect on fruit ($\chi^2_{[1]} = 0.29$,
387 $p > 0.05$) or seed set ($\chi^2_{[1]} = 0.86$, $p > 0.05$; Fig. 3A; Fig. 3C). Subsequent application of self
388 or outcross pollen (after heterospecific pollen) produced almost exactly the same fruit and
389 seed set as in the mating experiment (Fig. 3A; Fig. 3C). Self pollination produced fewer
390 fruit than outcross and fruits contained one or two seeds/fruit from self pollen and greater
391 than three for outcross pollen. In the present study there was only one fruit with one seed
392 produced from 39 hybrid pollinations with *T. triandrus* as the female (Table 2).

393

394

DISCUSSION

395

396 The study of the effect of heterospecific pollen on reproductive output in *T. tenellus* and
397 *T. triandrus* examined the extent of reproductive isolation between two pairs of species
398 that are closely related and have similar flowers. It might be expected that the more
399 distantly related the heterospecific pollen donor is to the recipient the less likelihood there
400 would be of an adverse effect (Ashman & Arceo-Gomez, 2013) unless there is some sort
401 of allelopathic interaction (e.g. as found by Sukada & Jayachandra, 1980 and Thomson *et*
402 *al.*, 1981). Heterospecific pollen made no difference to fruit or seed set for *T. triandrus*
403 when conspecific pollen was later applied. In contrast to the results for *Mimulus guttatus*
404 DC. (Arceo-Gomez & Ashman, 2014) self pollen did not have a more severe reduction in
405 seed set than outcross pollen added after the heterospecific pollen. The proportion of
406 flowers setting fruit after receiving heterospecific pollen was almost exactly the same as
407 found in the mating system experiment for both self and cross pollen. Similarly, *T. sp.*
408 pollen had no effect on fruit or seed set in *T. tenellus*. Other studies have also found no
409 detectable effect after heterospecific pollination (e.g. Kwak & Jennersten, 1991; Caruso
410 & Alfaro, 2000; Harder, 2000) but in most cases the species were not closely related. In
411 contrast experiments on the effect of heterospecific pollen in *Mimulus guttatus* using a
412 closely related congener (*M. nudatus* Curran ex Greene) showed the *M. nudatus* pollen
413 mimicked the pollination reaction of conspecific pollen but seed set was reduced due to
414 ovule usurpation (Arceo-Gomez & Ashman, 2011). In *T. triandrus* there is no evidence
415 of such a deleterious effect. Unpublished data, reported in Brittan (1981), suggested that
416 *T. triandrus* and *T. multiflorus* are capable of interbreeding. However, tests for

417 hybridisation with plants from our study site showed that *T. triandrus* success with *T.*
418 *multiflorus* pollen is very low (Table 2).

419

420 Pollination in *T. tenellus* at Nicholson Road Reserve is autogamous and the
421 species has prior selfing (*sensu* Lloyd, 1979). This has not been reported in other buzz
422 pollinated species that would normally need a bee to release the pollen from the anthers.
423 Autogamy has been considered to provide reproductive assurance under conditions of
424 pollen limitation (Morgan & Wilson, 2005; Kennedy & Elle, 2008; De Waal, Anderson
425 & Ellis, 2015; Buide *et al.*, 2015) and has been invoked as a strategy that reduced the
426 effect of heterospecific pollen deposition from *Arenaria glabra* Michx. on the closely
427 related *A. uniflora* Poir. (Fishman & Wyatt, 1999). However for *T. tenellus* (unlike *A.*
428 *glabra*) heterospecific pollen had no significant detrimental effect on fruit or seed set.
429 This might be considered to have been due to the fact that the stigmas have conspecific
430 pollen on them when flowers opened. While it is not entirely clear when the pollen
431 reaches the stigma in buds of *T. tenellus*, this is unlikely to be a long time before the
432 flower is opened. When the stigmas of just-opened flowers were examined, only 0.7% of
433 pollen grains on the stigmas had emergent pollen tubes and these were very short. Thus
434 pollen tube growth would have been at a similar time for both the con- and heterospecific
435 pollen. The prior selfing does however provide good reproductive assurance as shown by
436 consistent high fruit and seed set at the same sites (Ladd unpublished data), so the species
437 has escaped pollen limitation. Because *T. tenellus* has prior selfing it is not possible to be
438 certain if hybrid pollination can produce fruit or not. However the reciprocal cross of *T.*
439 *tenellus* pollen on *T. sp.* female produced only 2 fruit with 1 and 2 seeds indicating that
440 there is a low probability of hybrid progeny being produced (Table 2).

441

442 Unlike *T. tenellus*, fruit and seed set in *T. triandrus* is pollen limited, as is
443 commonly found in animal pollinated plants (Aizen & Harder, 2007) and species with
444 evolutionarily specialised flowers tend to be more pollen limited than species with
445 unspecialised flowers (Lázaro, Lundgren & Totland, 2014). Buzz pollination is a
446 relatively specialised system in which there is selection for flower visitors that can
447 sonicate the anthers. It has been suggested that it is a system that provides precise pollen

448 deposition on a bee's body (Harder & Wilson, 1997) so this should be important in
449 limiting HPT. This may be the case in flowers with the *Solanum* L. and *Dodecatheon* L.
450 type of floral morphology where pollen deposition and collection is on a relatively
451 restricted part of the ventral surface of a bee but other floral morphologies associated
452 with buzz pollination tend to have less precise pollen placement on a visitor. In three
453 species of *Pedicularis* L. there was variation in the modal location of pollen deposition on
454 bumblebee visitors but there was also deposition on other parts of the visitor's body.
455 Similarly, stigma contact from the same species on the pollinator was not 100% precise
456 so although the pollen deposition and receipt from the insect's body could contribute to
457 reproductive isolation it would not eliminate interspecific pollen transfer (Huang & Shi,
458 2013). Thus the adaptive accuracy (*sensu* Armbruster *et al.*, 2009) is relatively low. This
459 is also the case with the *Thysanotus* species. In the four species studied here pollen is
460 mainly deposited on the ventral surface of the bee and the position of anthers and stigma
461 in *T. tenellus* would easily lead to facilitated selfing if the population at our study site was
462 not already autogamous. In the other species however, the stigma location leads to pollen
463 collection from the dorsal surface of the bee – poor adaptive accuracy. In *Thysanotus*,
464 buzz pollination is “messy” and is one of the features that allows assessment of whether
465 flowers have been visited or not. In visited flowers pollen is usually obvious on the
466 tepals, style and outer anther surfaces. In a buzz pollinated species dichogamy is not
467 adaptive as stigmas need to be receptive when pollinators visit for pollen, especially if the
468 flower has a very limited lifespan. As noted by Armbruster *et al.*, (2009) the low adaptive
469 accuracy may be related to increased herkogamy (the three non autogamous species have
470 herkogamy distances of over 2mm) that is more useful to buzz pollinated species to limit
471 selfing than is dichogamy.

472

473 The almost identical flower colour and morphology point towards a lack of any
474 isolating factor that would allow a visitor to discriminate between flowers of *T. tenellus*
475 and *T. sp.* or between *T. triandrus* and *T. multiflorus*. Reproductive isolation cannot be
476 effected by morphological differences between the flowers of the two species pairs and
477 pollinators are unlikely to distinguish between them.

478

479 It has been suggested that interspecific pollinator visits incur a cost to any visited
480 species through lowered male fitness due to pollen “misplacement” (Muchhala &
481 Thomson 2012). This is unlikely in *Thysanotus* species because during buzzing pollen is
482 often scattered over the pollinator and adjacent parts of the flower. This makes it difficult
483 for a pollinator to gather all the pollen it has released so some will always still be
484 available for transfer to a stigma. There is the possibility that differential growth rates of
485 self and outcross pollen tubes may explain the lack of effect of HPT in the species
486 examined here. Despite heterospecific pollen being applied at least an hour before
487 conspecific pollen in *T. triandrus*, if heterospecific pollen tubes grew more slowly than
488 conspecific tubes it may be that with only six ovules available, faster growing conspecific
489 tubes could reach all the ovules before heterospecific tubes. Unfortunately it is not
490 possible to examine pollen tube growth in the style due to a lack of clear pollen tube
491 fluorescence.

492
493 While complex flowers with specialised requirements for successful pollination
494 may have strong pre-mating isolation this limits their opportunities for reproduction if
495 their specialised pollinator is absent (e.g. Bond, 1994). Post mating isolation is a more
496 appropriate strategy if poor pollinator visitation is a regular problem, as overall
497 community flowering will ensure some pollinators are present that might visit the
498 flowers. Buzz pollinated species have an advantage as pollen is necessary for bee
499 reproductive success (Carr *et al.*, 2015) and flowers that increase an insect’s learning time
500 tend to engender a greater constancy in pollinators (Armbruster, 2014). A consistent
501 search image is likely to benefit buzz pollinated species overall as bees that are
502 accustomed to the floral form (Goulson, 1999) and colour will reliably visit even if the
503 flowers are of different species. At Nicholson Road Reserve there are seven *Thysanotus*
504 species that flower sequentially from late winter through to summer (Ladd pers obs). The
505 closely similar flower form and colour would ensure consistent pollinator servicing as
506 pollinators would swap from the species declining in flowering as another species began.

507
508 The focal species have different forms of reproductive assurance, as found in
509 *Silene ramosissima* Desf. and *S. nicaeensis* All. (Buide *et al.*, 2015). Heterospecific

510 pollen had no detrimental effect on fruit or seeds set of the focal species and the
511 formation of hybrid seed from *T. triandrus*, *T. multiflorus* and *T. sp.* was very low or did
512 not occur at all, so it is likely there is post mating reproductive isolation between the
513 species. This is similar to results reported for buzz pollinated *Pedicularis* (Mao, 2010
514 cited in Huang & Shi, 2013) and may be a feature of buzz pollinated species in general.
515 The situation for *T. tenellus* remains unclear as it is autogamous with prior selfing and it
516 is not possible to tell if hybrid seed can be produced from *T. sp.* pollen. Nevertheless, this
517 seems unlikely as the reverse cross of *T. tenellus* pollen on *T. sp.* stigmas produced
518 almost no seed set.

519

520

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524

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688

689 Table 1. Characteristics of the species, * indicates the focal species with a floral form almost identical to the species listed below it in
 690 the table. HC – hemicryptophyte, abs – absent, na – not assessed
 691

| Species | Plant form | Flower diam (cm) | Inflorescence type | Anthers | Pollen/flower \pm CI | Style form | Infertile fruit retained for some time | Phenological isolation in 2013 (Date of maximum flowering) | | |
|---------------------------------------|------------|------------------|--------------------|-------------------------|------------------------|------------|--|--|----------------|---------------|
| | | | | | | | | Area A | Area B | Area D |
| * <i>T. tenellus</i> Endl. | Geophyte | 1.9 \pm 0.1 | Open panicle | 6 equal, straight, free | 36,158 \pm 3,077 | Straight | No | abs | 0.322 (16 Oct) | 0.057 (9 Oct) |
| <i>T. sp.</i> | Geophyte | 2.5 \pm 0.1 | Open panicle | 6 equal, straight, free | 93,118 \pm 11,201 | Hooked | No | abs | 0.075 (16 Oct) | 0.327 (5 Oct) |
| * <i>T. triandrus</i> (Labill.) R.Br. | HC | | Large umbel | 3 equal, curved, free | 361,806 \pm 30,537 | Curved | Yes | 0.662 (18 Nov) | 1 (18 Nov) | abs |
| <i>T. multiflorus</i> R.Br. | HC | 3.0 \pm 0.1 | Large umbel | 3 equal, curved, free | 374,445 \pm 54,107 | Curved | Yes | 0.549 (31 Oct) | 1 (27 Oct) | na |

692

693

694 Table 2. Fruit and seed production from hybrid trials between the two pairs of species. Comparisons are with outcross conspecific
 695 pollination of the female.
 696

| Female (pollen recipient) | <i>T. tenellus</i> | <i>T. sp.</i> | <i>T. triandrus</i> | <i>T. multiflorus</i> |
|---------------------------|--------------------|---------------|---------------------|-----------------------|
| Number of crosses | 184 | 24 | 39 | 25 |

| Pollen donor | <i>T. sp.</i> | <i>T. tenellus</i> | <i>T. multiflorus</i> | <i>T. triandrus</i> |
|--------------------|---------------|--------------------|-----------------------|---------------------|
| Fruit and seed set | No change | Very small | Very small | 0 |

697

698 Figure captions

699

700 Figure 1. A) *T. tenellus*, B) *T. sp.*, C) *T. triandrus*, D) *T. multiflorus*, flower diameters are listed in Table 1.

701

702 Figure 2. Flowering phenology of the four *Thysanotus* species in 2013. Flower counts for *T. multiflorus* and *T. triandrus* were from
703 area A, *T. sp.* from area B and *T. tenellus* from area D.

704

705 Figure 3. Results of the breeding system on mean fruit set (A, B) and mean seed set (C, D) in *T. triandrus* (left) and *T. tenellus* (right).
706 Results of the heterospecific pollination trial are shown in light grey. HPT cross: *T. multiflorus* pollen was added to *T. triandrus*,
707 followed by later outcross pollen. HPT self: *T. multiflorus* pollen was added to *T. triandrus*, followed by later self pollen. HPT only: *T.*
708 *tenellus* received *T. sp.* pollen only. Sample sizes are given at the base of each column, and bars are 95% confidence intervals.

709

710 Supporting Information Figure S1. Distribution of the four species in the three study areas at Nicholson Road Reserve that were
711 originally delimited by time since fire. A – Fire in 2001; B fire in 2002; D date of last fire unknown but before 2000. Blocks indicate
712 notional density of each species.

713

714 Supporting Information Figure S2. Colour spectra of the flowers of four *Thysanotus* species and photoreceptor spectra for the bee
715 *Osmia rufa* (Menzel *et al.*, 1988).