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Floral biology of large-flowered *Thelymitra* species (Orchidaceae) and their hybrids in Western Australia

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Abstract

Historically, only a few large flowered species in the genus *Thelymitra* were identified as obligate out-breeders. We compared floral presentation, pollen-pistil interactions, pollination ecology, and interspecific hybridization in two populations of *T. macrophylla* where its flowering periods overlapped with *T. antennifera* (Tenterden) and *T. crinita* (Lesmurdie) respectively. Pollen-pistil interactions were studied using glasshouse collections of *T. crinita* and *T. macrophylla* at KPBG. The number of flowers per inflorescence in *T. macrophylla* varied significantly between sites. Climatic conditions influenced flower opening and closing regimes differently in *T. crinita* vs. *T. macrophylla*. While all three *Thelymitra* species opened on warm, sunny mornings and closed by late afternoon, *T. crinita* at Lesmurdie was significantly more likely to open its perianth segments on cool days compared to the co-blooming, sympatric flowers of *T. macrophylla*. The floral lifespans of individual flowers of *T. macrophylla* and *T. crinita* were reduced significantly following application of *Thelymitra* pollen onto the stigmatic surface but were not reduced by pollinarium removal. Flowers of both species were self-compatible but neither species self-pollinated mechanically (autogamy). Fluorescence microscopy also showed that both species were inter-compatible. Natural rates of pollinarium removal by insects were low in all three species at both sites. Natural rates of pollen deposition on receptive stigmas were significantly higher in *T. crinita* vs. *T. macrophylla* but pollen deposition was less than 12% in both species. Observations and collections of pollinators were infrequent and pollinaria vectors were restricted to a few polylectic, female bees in the families Apidae, Colletidae, and Halictidae. We found a large number of hybrids between *T. antennifera* and *T. macrophylla* at Tenterden but few obvious hybrids between *T. crinita* and *T. macrophylla* at Lesmurdie. As expected, all hybrids showed characteristics intermediate between their two putative parent species, including pollen configuration in the *T. crinita* × *T. macrophylla* specimens. Due to malformations of the column, the majority of *T. antennifera* × *T. macrophylla* flowers appeared unable to attach their pollinia to their respective rostella.

Introduction

Ongoing revisions of *Thelymitra* J.R.Forst. & G.Forst. have expanded this genus to over 100 species from the southern Philippines to seasonally moist-temperate Australasia (Brown *et al.*, 2008; Jeanes 2004, 2006, 2009, 2013). While a range of vegetative and floral characters is used to segregate species of *Thelymitra*, orchid taxonomists rarely use labellum traits in this genus. Most of the species in the genus have a radially symmetrical perianth with a labellum identical to the two lateral petals. When taxonomists compare and segregate taxa in the genus *Thelymitra*, they are more likely to focus on the relative size, degree of fusion, inflation, and ornamentation of structures confined to the hooded column (see Bishop 1996; Erickson 1965; Jeanes 2006; Rupp 1942). Variation in the physical size of the flowers and mitra ornamentation in *Thelymitra* species was

used in the interpretation of major trends in floral evolution by Burns-Balogh and Bernhardt (1988).

When the Australian botanist, Robert David Fitzgerald (1830–1892) examined the flowers of several *Thelymitra* species, he subdivided them into two reproductive systems based on his observations and an experimental procedure he derived from Darwin (1862). Fitzgerald noted that small-flowered species had friable pollinia (contents of each anther cell crumbled instead of remaining in a cohesive and entire pollinium). Their fragments fell onto their receptive stigmas spontaneously as their solitary anthers dehisced. In contrast, the large-flowered species produced entire and paired, pollinia that attached to a viscidium (naked and detachable rostellum) upon anther dehiscence. In large-flowered species the pollinia didn't fragment or fall onto the stigma. Fitzgerald showed that the paired pollinia in a large-flowered, *Thelymitra* species could be withdrawn completely, when the tip of a pin adhered to the viscidium. The pollinia did not contact the stigma lobes when the pollinarium (two pollinia pairs + one viscidium) was removed in this manner.

Therefore, Fitzgerald's published texts and folios (Fitzgerald 1875–1894) concluded that self-pollinating *Thelymitra* species could be recognized on the bases of their smaller flowers and tardily opening perianth segments. However, Fitzgerald warned that the transition from cross- to self-pollination was gradual and so discrete it could occur within the same species. Recent taxonomic revisions of the genus suggest that almost half of the >100 (A. Brown, pers. comm.) described species are facultative to obligate self-pollinators (Jeanes 2004, 2006, 2009; Jones, 1988). Regardless of flower size, Fitzgerald never described insect-mediated pollination in a *Thelymitra* species Cheeseman (1880), working in New Zealand, found thrips feeding on pollen of *T. longifolia,* a small-flowered species, but discounted these tiny insects as pollinators.

In fact, insect-mediated and putative cross-pollination has been described, thus far, in only six large-flowered species. Jones (1981) was the first to observe bees carrying the pollinaria of Australian *T. media* and *T. aristata,* both large-flowered species, on the last two abdominal segments of their abdomens. The bees were observed to clasp the ornamented and pigmented clinandrium or mitra (*sensu* Burns-Balogh and Bernhardt 1988) hooding the exposed pollinarium and stigma. Bernhardt and Burns-Balogh (1986) also observed and caught a few, female bees on large-flowered, *T. megcalyptra* (syn. *T. nuda*) and these bees also landed on and clasped the ornamented and pigmented mitra. Remnants of the pollinia were found, once again, on the dorsum of the bees' abdomen. Similar observations, collections, and abdominal depositions of pollinaria were found on bees collected on large-flowered *T. antennifera* (Dafni and Calder 1987), *T. epipactoides* (Cropper and Calder 1990), and *T. ixioides* (Sydes and Calder 1993). The dominant pollinators of all three species were female, native, and polylectic bees in families Apidae and Halictidae. The exception to the rule was *Syrphus damaster* (Syrphidae) and the solitary wasp (*Eurys* species) carrying pollinaria of *T. antennifera* on their heads. All *Thelymitra* species studied failed to secrete nectar and did not offer edible pollen to insect foragers. Dafni and Bernhardt (1990) concluded that insect-pollination within the lineage was most likely based on guild mimesis in which the *Thelymitra* flower mimicked co-blooming species offering pollen and/or pollen and nectar to female bees foraging for their offspring. These included the yellow flowers of *Goodenia* and *Hibbertia* species for *T. antennifera* and a wide range of blue-purple flowers of various petaloid monocotyledons for *T. megcalyptra* and similar species. The bee's abdomen appears to clasp the underside of the mitra. When the bee prepared to fly away, the abdomen unclasped and swung downward, contacting the viscidium. As the same bee prepared to clasp the mitra of a second column, the pollinia-laden abdomen should swipe against the stigmatic lobes.

It is curious that all six studies described above were completed in eastern Australia when the south western corner of the state of Western Australia is the centre of diversity for the genus *Thelymitra* and appears to support the largest populations of large-flowered species (Brown *et al*. 2008; Jeanes 2006, 2009; Jones 1988). As a number of species have overlapping flowering periods and distributions, interspecific hybrids were recorded frequently (Bates and Weber 1990; Brown *et al*. 2008).

We selected *T. antennifera, T. crinita,* and *T. macrophylla* for additional studies on the floral biology of large-flowered species for three reasons. First, living collections of *T. crinita, T. macrophylla* and their putative hybrid were included in the glasshouse collection at the Kings Park and Botanic Garden (KPBG) in West Perth, Western Australia. This afforded opportunities to test breeding systems of two large-flowered species at the intra- and interspecific levels. Second, extensive populations of *T. macrophylla* were found at two, disjunct sites. At one site, it overlapped with the flowering of an extensive population of *T. antennifera* and their putative hybrid while, at the second site it overlapped with *T. crinita* and their putative hybrid. With two large populations of *T. macrophylla*, it became possible to compare the inflorescence length vs. the number of flowers per inflorescence at two sites and then compare the influence of climate on the opening and closing of flowers at each site. Third, the morphology of the mitra of *T. crinita* is similar to eastern *T. ixioides* (Sydes and Calder 1993) while the mitra of *T. macrophylla* is similar to eastern *T. megcalyptra* (Bernhardt and Burns-Balogh 1986). This offered the opportunity to compare prospective pollinators and their visitation rates of two, co-

blooming species *in situ*. It also gave us the opportunity to compare characters of parent species and their putative hybrids.

Comparing the floral biology of three, large-flowered species in large populations allowed us to test hypotheses regarding the evolution in a lineage. This required a combination of techniques and protocols using biometry, breeding experiments, and field and glasshouse observations, as floral presentation in flowers 'pollinated-by-deceit' is usually a multi-layered syndrome (Dafni and Bernhardt 1990).

Materials and methods

Study species. We studied populations of *T. antennifera*, *T. crinita, T. macrophylla,* and their putative hybrids (Figs 1–8). Descriptions of floral morphology of all three species and the *T. antennifera* × *T. macrophylla* hybrid follow Brown *et al*. 2008.

Study sites, field populations *vs* glasshouse collections. Field and glasshouse studies on *T. macrophylla* (scented sun orchid), *T. crinita* (blue lady orchid), and *T. antennifera* (lemon-scented orchid) represented one season of study (1 September–23 October, 2009). The field site for *T. antennifera* (approximately 500 flowering stems), *T. macrophylla* (approximately 100 flowering stems) and the hybrid between *T. antennifera* and *T. macrophylla*, (here after *T. antennifera* × *T. macrophylla*; approximately 50 flowering stems) was located near Tenterden, Western Australia. Pressed vouchers were deposited in the National Herbarium of New South Wales (NSW) and the herbarium of the Missouri Botanical Garden (MO).

The second site for field research included *T. crinita* (approximately 300 flowering stems), *T. macrophylla* (approximately 85 flowering stems) and hybrids between *T. crinita* and *T. macrophylla* (here after *T. crinita* × *T. macrophylla*; 6 flowering stems) was located in Lesmurdie, Western Australia (intersection of Welshpool and Pomeroy). At the Lesmurdie site, we tagged 15 stems in bud of *T. macrophylla* and 17 stems in bud of *T. crinita* and observed them from 6–23 October 2009. Data collected on these 32 flowering stems is described below in the subsections on 'Flowering patterns' and 'Natural rates of pollinaria removal *vs*. pollinaria deposition'. Deposition of herbarium vouchers for *T. crinita* and *T. macrophylla* were as above. Vouchers of the putative hybrid between *T. crinita* and *T. macrophylla* were collected and identified on 22 October 2009, by Dr. Andrew Brown.

Glasshouse collections. All hand-pollination work was completed using potted plants in a glasshouse (15°C–22 °C day temperature regulation) located at KPBG. All glasshouse specimens of *T. crinita* and *T. macrophylla* were collected from Canning Mills Road, east of Perth, WA, in September 1998. All glasshouse hybrid specimens (*T. crinita* × *T. macrophylla*) were collected from the Fiona Stanley Hospital site south of Perth, WA, in September 2009.

Statistics. All statistical analyses were performed using the R statistical language and programming environment (R Development Core Team 2011). We used the following abbreviations in our results: 'F' = test statistic for an analysis of variance test; 'W' = Wilcoxon rank sum test; 'χ^2'= Chi-square test; 'DF' = degrees of freedom; 'sd' = standard deviation of a sample; 'n' = sample size; and 'P' = probability of obtaining a given test statistic's value or greater at random.

Flower counts and flowering patterns

Flower bud number *vs*. length of the inflorescence. We counted the number of flower buds on *T. antennifera* and *T. macrophylla* at the Tenterden site. At the Lesmurdie site, we counted the flower buds on 17-tagged inflorescences of *T. crinita* and 15-tagged inflorescences of *T. macrophylla* from 6–23 October 2009 (see above). We tested for a difference in the average number of flowers per stem produced by *T. macrophylla* between the Tenterden and Lesmurdie sites using One-Way ANOVA. We recorded the number of flowers that opened daily on inflorescences of *T. crinita* and *T. macrophylla* at the Lesmurdie site from 6–23 October 2009. We also recorded daily weather conditions.

Flower bud opening *vs*. ambient temperature (*in situ*). A few previous studies suggest that mature, perianth segments do not open unless there is an absence of cloud cover and ambient temperatures exceed the low 20's (see review in Bernhardt and Burns-Balogh 1986). During September and October 2009, mean daily temperatures varied from 14.3–37.0 °C. Therefore, each day we observed flowers opening at the Lesmurdie site, the day was recorded as cool (<21°C) or warm (to at least 21°C).

Cloud cover. We recorded whether the day was cloudy or sunny. A cloudy day refers to whether the sun

remained occluded by stratus clouds at least from 9 a.m. – Noon. All rainy days were recorded as cloudy days. A sunny day refers to a day in which the sun was not occluded by clouds from 9 a.m. – Noon. A Three-Way ANOVA was used to test for differences in the ratio of open to the total number of flowers per stem to determine the role of weather effects on *T. crinita* and *T. macrophylla*. These effects were identified as sunlight, (sunny day *vs*. cloudy day), and temperature (warm *vs.* cold). This gave us the three-way interaction of species, sunlight, and temperature. These ratios were transformed using the arcsine transformation for ratio data to better fit a normal distribution (Sokal and Rohlf 2003). Post-hoc analysis of the three-way interaction was done using pairwise t-tests.

Floral lifespans (glasshouse collection). To determine the floral life spans of *T. crinita* and *T. macrophylla* under controlled conditions, we used glasshouse plants exclusively. The first day a bud in the glasshouse opened, it was labeled with a jeweler's tag and subjected to one of two treatments (control *vs.* pollinarium removed). Specimens labeled controls were not manipulated. We recorded the number of days they opened and closed prior to withering. A withered flower was defined as a flower with a browned or crustose stigma and its labellum covered the stigma before the flower closed for the last time. Such flowers never reopened the following day. The second group of flowers had their pollinaria removed with clean toothpicks the first day they opened. They were recorded as withered using the same criteria as above. A mixed effect ANOVA model was used to test for differences in lifespan between the main treatment effects (control and pollinarium removed) and species (*T. crinita* and *T. macrophylla*), as well as the interaction of treatment and species. Since individual plants received both the control and experimental treatments, individual plant identifiers were included in the model as a blocking (random) effect.

Peduncle (flowering stem) length *vs.* number of flowers per inflorescence (glasshouse collection). The length of the stem and the number of flowers produced were measured for 13 specimens of *T. crinita*, 15 *T. macrophylla* specimens, and eight specimens of their putative hybrid. We used a One-Way ANOVA to test for differences among species and the hybrid in the number of flowers per stem. A second One-Way ANOVA was used to test for differences in the length of flowering stems. Tukey's HSD post-hoc tests were used to determine the sources of differences in the One-Way ANOVAs.

We counted the number of flowers on stems of the recurrent hybrid, *T. antennifera* × *T. macrophylla*, at the Tenterden site. We recorded which flowers on the stem had a normal column in which their pollinia connected to the rostellum and which flowers had malformed columns so that the pollinia did not connect to the rostellum. We calculated the average proportions of normal and malformed columns.

Hand-pollination experiments

Hand-pollination of flowers. To determine the compatibility system in glasshouse plants of *T. crinita* (n = 13 flowering stems) and *T. macrophylla* (n = 15 flowering stems) we divided all flowers on all stems into four experimental groups – control (unmanipulated), self-pollinated, cross-pollinated, and reciprocal hybridization. In all cases the entire pollinarium was removed from the anthers using a wooden toothpick (used once and discarded) and transferred to the receptive stigma. Because flowers opened only on warm, sunny days and because flowers closed by early afternoon, hand-pollinations were completed as soon as the flowers opened each morning. Self-pollinated flowers received pollen from the same flower (autogamy) whereas cross-pollinated flowers received pollen from the anther of a second flower of the same species in a separate container. In the reciprocal hybridizations, the entire pollinarium was removed from the anther of one species and its pollen was rubbed onto the stigma of the second species until a pollen smear was visible. The controls received no treatment. Each flower (one bud on each peduncle) was identified using a small jeweler's tag along with the type of treatment the flower received.

Analyses of hand-pollinations (pollen tube/pistil interactions). Experimental samples were harvested six days following the initial treatment, or in the case of the control flowers, six days after opening. These flowers were then collected, fixed, and preserved (*sensu* Lipow *et al.* 2002). Protocols used to split pistils lengthwise using a razor blade followed Edens-Meier *et al.* (2011) in order to locate pollen tubes on stigmas and/or within pistils. Softening, staining, and observing under epifluorescence followed Lipow *et al.* (2002).

Statistical analyses. A Multivariate Analysis of Variance (MANOVA) was used to test for differences in the three response variables (grains on stigma, pollen tubes in stigma, pollen tubes in ovary) among the independent variables for pollination treatment (control, cross, self, reciprocal hybridization) and species (*T. crinita* and *T. macrophylla*). Since data were recorded as counts, a square root transformation was used to develop a normal distribution (Sokal and Rohlf 2003).

Natural rates of pollinaria removal *vs* pollinaria deposition

Tenterden site. On 27 September and 2 October 2009, we examined peduncles of *T. antennifera*, *T. macrophylla*, and the hybrid of *T. antennifera* and *T. macrophylla* at the Tenterden site at random. We counted the number of flowers per inflorescence and the number of flowers on each stem in which the entire pollinarium was missing.

Statistical analyses (Tenterden). Kruskal-Wallis test were used to assess differences in the ratios of pollinaria removed to total flowers among *T. antennifera*, *T. macrophylla* and their hybrid.

Lesmurdie site. Seventeen flowering stems of *T. crinita* were tagged during the observation period from 7 October–23 October 2009 and 15 flowering stems of *T. macrophylla* from 6–23 October 2009. Every day we checked columns on each flower on each inflorescence for missing pollinaria and whether the stigma retained pollinaria fragments and/or a visible pollen film on the stigma. In both species, stigmas retaining pollinia fragments and/or a visible pollen film, closed and failed to reopen within 24 hours (see below). We excluded all flowers showing evidence of insect attack as these flowers closed early following physical damage. Pollen deposition on stigmas was observed using 3x optivisors.

Wilcoxon Rank Sum tests were used to compare the ratios of pollinated/total number of flowers and the ratios of pollinaria-removed/total number of flowers between *T. crinita* and *T. macrophylla* at the end of the 17-day observation period.

Insect observations, collections, and pollen load analyses

Catching insects (both sites). Insects were observed from September–October 2009, for approximately 200 hours. We arrived at the field site by 8 a.m. and left at 5 p.m. Insects landing on flowers were collected with butterfly nets and euthanized in a killing jar with ethyl acetate. Specimens were pinned, labeled, and identified. Bees less than 9 mm in length flew very rapidly and landed on *Thelymitra* flowers for a second or less. Consequently, it was not possible to follow these small bees from flower to flower. We had to catch them as soon as we saw them. We failed to catch more than half the bees we observed (see results). Pinned specimens were donated to the Entomology Department of the Western Australian Museum (Welshpool, WA) and identified by Dr. Terry Houston.

Identification of pollinaria and pollen load analyses (both sites). Pollinaria worn by insects were removed with a probe and stained with Calberla's fluid (Bernhardt and Dafni 2000). To determine whether the insect collected pollen on other species flowering within the same site, each insect was placed on a glass slide and washed in two–four drops of ethyl acetate. The scopae of bees were scraped with a probe to dislodge additional pollen loads. Staining and mounting of pollen residue left on the glass slide followed Bernhardt and Dafni (2000). The pollen slide was co-referenced with the pinned insect specimen. The pollen of a particular plant species was counted as present on a slide if we counted in excess of 25 grains based on protocols established for insect-pollinated Australian species (see Bernhardt 1989).

Hybrids

***T. antennifera* × *T. macrophylla* (Tenterden).** We counted and collected specimens resembling standard descriptions of *T. antennifera* × blue, large-flowered *Thelymitra* species as described by Brown *et al.* (2008). We recorded the number of flowers per inflorescence, floral characters of fresh specimens, and observed whether pollinia connected to the rostellum in open flowers. Pollen grains were removed from pollinia and stained with Calberla's fluid (see Bernhardt and Dafni 2000). Pressed vouchers were deposited as above.

***T. crinita* × *T. macrophylla* (Lesmurdie).** We counted and observed what we thought were intermediate specimens between *T. crinita* × *T. macrophylla*. These specimens were later collected by Andrew Brown for herbarium deposition. Consequently all morphometric analyses of *T. crinita* × *T. macrophylla* were based exclusively on known intermediate specimens (K. Dixon, pers. comm.) grown in a glasshouse (KPBG). We examined macroscopic and microscopic characters as above.

Results

Floral characters and flowering patterns

***Thelymitra antennifera* (Tenterden):** *Thelymitra antennifera* produced a mean of 1.7 flowers per stem (Table 1). Flowers of *T. antennifera* opened sluggishly on 26, 27 September and 2 October 2009, starting at 10:00 a.m. By 11:45 a.m. the flowers of only half of the population were fully opened. Flowers did not begin closing

until after 4:00 p.m. Perianth segments were yellow (Fig. 1). The hood lacked a discernable midlobe and the prominent yellow papillose anther was flanked by two brownish burgundy lateral lobes (staminodia, Figs 1, 6). On 27 September, a mean of 93.3% of flowers were open per stem (sd=16.8). On 2 October, all flowers were open. A Wilcoxon rank sum test confirmed that a significantly higher percentage of flowers was open on 2 October than on 27 September (W=1396.5, P=0.0071).

Table 1. Floral Characters of three *Thelymitra* spp. and their hybrids (includes greenhouse = 'A' and field sites); NA = not assessed; Tenterden site = 'B'; Lesmurdie site = 'C'.

Reproductive Characters	*T. antennifera*[B]	F1 *T. antennifera* × *T. macrophylla*[B]	*T. macrophylla*	F1 *T. macrophylla* × *T. crinita*	*T. crinita*[C]
Mean length scape (in mm)	NA	NA	585.5	491	491
Mean number of flowers/scape	1.7[B]	1.2[B]	4[B]; 16.5[C], 12.3[A]	8[A]	9.8[C], 7.7[A]
Perianth color	yellow	red/orange/pink often with yellow margins	ranges from white, blue, to pink	blue to light purple	light blue to cobalt blue
Hood midlobe	absent; anthers dark yellow	reduced; anthers light yellow	smooth; yellow + blue band	'bumpy', light pappilose; magenta or indigo + yellow	prominent papillae; 'fuzzy'; purple blue + yellow
Trichome brush presence	absent	absent	present	present	present
Approximate number of hairs/trichome brush	absent	absent	>160	<150	<40
Color of trichome brush if present	absent	absent	white	pink to purple	pink to cobalt blue
Pollen	monads	monads	monads	monads, dyads, and tetrads	tetrads
Floral odour discernable to Human nose	strong	weak	strong	strong	absent

***Thelymitra crinita* (Lesmurdie site and glasshouse collection):** An inflorescence of *T. crinita* at the Lesmurdie site produced a mean of 9.8 flowers per stem (sd = 2.8, n = 18, range = 5–15; see Table 1). Flower buds in the glasshouse collection and at Lesmurdie opened acropetally. Perianth coloration graded from a dull, pale blue with pink highlights to a vivid, cobalt blue in *T. crinita* (See Fig. 2, Table 1).

On warm sunny days, glasshouse flowers of *T. crinita* began opening from 9:15–10:35 a.m. with perianth segments closing from 2:30–4:40 p.m. Individual flowers lived 9–26 days (mean=13.71, sd =4.46, n = 21) regardless of whether their pollinaria were removed or not (see results of ANOVA in the Comparative Flowering section below). However, if the flowers were selfed, crossed, or hybridized, the perianth segments always closed 24–48 hours later and never reopened.

***Thelymitra macrophylla* (Lesmurdie, Tenterden, and glasshouse collection):** An inflorescence of *T. macrophylla* produced between 10–23 flowers at the Lesmurdie site and between 1–11 flowers at the Tenterden site (see Table 1; Fig. 9). *Thelymitra macrophylla* produced significantly more flowers per stem (mean=16.5, sd=3.5) at the Lesmurdie site than at Tenterden (mean=4.0, sd=1.9) as determined by a One-Way ANOVA ($F=274.34$, $DF=1,49$, $P<2.2*10^{-16}$). Flower buds in the glasshouse collection, at Lesmurdie, and at Tenterden always opened acropetally in this species. Perianth coloration graded from light to deep blue in both populations and in the glasshouse plants (Fig. 3). On warm sunny days, glasshouse flowers of *T. macrophylla* began opening from 9:30–10:30 a.m. and began closing from 2:45–4:40 p.m. These flowers lived an average of 17.10 days (sd = 4.46, n = 20) whether their pollinaria were removed or not. If the flowers were selfed, crossed or hybridized the perianth segments always closed 24–48 hours later and never reopened.

Comparative flowering of *T. crinita vs. T. macrophylla* (glasshouse). Flowers of *T. crinita* lived an average of 15.3 days if they kept their pollinaria and an average of 11.7 days if the pollinarium was removed the first day the flowers opened. Flowers of *T. macrophylla* lived an average of 18.7 days if they kept their pollinaria and 15.11 days if the pollinaria were removed the first day the flowers opened. However, no significant differences were detected by the mixed effect ANOVA among treatments ($F=4.60$, $DF=1.13$, $P=0.051$), species ($F=4.51$, $DF=1.13$, $P=0.054$), nor the interaction of treatment and species ($F=0.94$, $DF=1.13$, $P=0.349$).

Flowering patterns of *T. crinita* and *T. macrophylla* at Lesmurdie. The Three-Way ANOVA revealed that *T. crinita* flowers opened regardless of whether it was sunny or cloudy. *Thelymitra crinita* also opened on days that were cold provided it was sunny.

In contrast, *T. macrophylla* flowers did not open on cold days, even if the sun were shining. The detailed results of the ANOVA were as follows: For the main effect of Species, the average ratio of open/total number of flowers was significantly lower (F=12.789, DF=1,547, P=0.0004) for *T. crinita* (mean=0.20, sd=0.26) than for *T. macrophylla* (mean=0.26, sd=0.24) at the Lesmurdie site. The average ratio of open/total flowers was significantly higher (F=235.70, DF=1,547, $P<2.2*10^{-16}$) on sunny days (mean=0.38, sd=0.31) than on cloudy days (mean=0.05, sd=0.17). The average ratio of open/total flowers was significantly higher (F=103.71, DF=1,547, $P<2.2*10^{-16}$) for warm days (mean=0.41, sd=0.30) than cold days (mean=0.02, sd=0.10). The three-way interaction effect of Species:Sunlight:Temperature was also significant (F=10.30, sd=4,547, $P=4.8*10^{-8}$). Post hoc analysis of the three-way interaction showed that *T. crinita* had similarly higher ratios of open flowers on warm and/or sunny days than it did on cold and cloudy days. *Thelymitra macrophylla* however, had higher ratios of open flowers on warm days than cold, regardless of sun exposure.

Fig. 1. *Thelymitra antennifera* and putative hybrids of *T. antennifera* × *T. macrophylla* (Tenterden, Western Australia).
Fig. 2. Flower of *Thelymitra crinita* (Lesmurdie, Western Australia).
Fig. 3. Flower of *Thelymitra macrophylla* (Lesmurdie, Western Australia).
Fig. 4. Flower of putative hybrid *Thelymitra antennifera* × *T. macrophylla* (Tenterden, Western Australia). Note that the rostellum is retained but no pollinia connect to the viscidium.

Flowers produced by the hybrid, *T. antennifera* × *T. macrophylla* at Tenterden. The mean number of flowers per stem produced by the *T. antennifera* × *T. macrophylla* hybrids was 1.2 (sd=0.84). However the mean number of flowers with malformed columns (rostellum never contacted pollinia) per stem was 0.59 (sd=0.81) for a final ratio of 2.03 normal flowers for every malformed flower (Figs 4, 5).

Flowers produced by *T. crinita, T. macrophylla,* and *T. crinita* × *T. macrophylla* (glasshouse collections). *Thelymitra crinita* produced a mean of 7.7 flowers per stem (sd=2.69). *Thelymitra macrophylla* produced a mean of 12.3 flowers per stem (sd=3.27), and the hybrid produced a mean of 8.0 flowers per stem (sd=3.16). We found significant differences in the number of flowers per stem among the two species and their putative hybrid (F=9.68. DF=2,33, P=0.0005). Post-hoc analyses revealed that *T. macrophylla* produced significantly more flowers than *T. crinita* and the hybrid, but that the number of flowers produced by *T. crinita* and the hybrid did not differ significantly from one another. *Thelymitra crinita* produced a mean stem length of 411.7 mm (sd=63.6). *Thelymitra macrophylla* produced a mean stem length of 585.5 mm (sd=63.8), and the hybrid produced a mean stem length of 491.0 mm (sd=97.9). We found significant differences in stem lengths among the species and their hybrid (F=20.23, DF=2.3, P=1.8 *10^{-6}). Post hoc analyses revealed that *T. macrophylla* had longer stems than both *T. crinita* and their hybrid, while *T. crinita* and their hybrid produced stems that did not differ significantly in height (Table 1).

Fig. 5. Flower of putative hybrid *Thelymitra antennifera* × *T. macrophylla* (Tenterden, Western Australia). Note difference in colour with Fig. 4 and also note that both pollinia pairs dropped attaching to rostellum.

Fig. 6. Flower of putative hybrid *Thelymitra crinita* × *T. macrophylla* (Lesmurdie, Western Australia). Note the white trichome brushes and the much reduced papilla on the median lobe of the hood.

Fig. 7. Flower of putative hybrid *Thelymitra crinita* × *T. macrophylla* (Lesmurdie, Western Australia). Note the pinkish/purple trichome brushes.

Fig. 8. Flower of *Thelymitra antennifera* (Tenterden, Western Australia) with red blotches. This specimen was found with *T. antennifera* (all perianth segments yellow) and putative hybrids of *T. antennifera* × *T. macrophylla*.

Fig. 9. Inflorescence of *Thelymtira macrophylla* in situ (Tenterden, Western Australia). Note the few flowers and flower buds on the peduncle.

Hand-pollination experiments *T. crinita* and *T. macrophylla* (glasshouse). Flowers labeled as controls in the glasshouse failed to self-pollinate in both species. Flowers deprived of pollinaria the first day they opened did not set fruit by agamospermy in either species. This does not mean, however, that each species was self-incompatible. Hand-manipulated pollination events resulted in a full response of pollen tubes germinating and growing through ovary tissue in both species. Both *T. crinita* and *T. macrophylla* were self-compatible (Figs 10, 11) based on the evidence that they each accepted their own pollen and produced pollen tubes that extended through the ovary within six days following pollen deposition on their stigmas. Based on results of hand-manipulated hybridizations, *T. crinita* and *T. macrophylla* showed degrees of interspecific compatibility. When pollen from *T. crinita*, was transferred to the stigma of *T. macrophylla*, we observed a number of swollen grains on the surface of the stigma although most pollen tubes penetrated into the ovary. We observed a similar response in pollen tubes of *T. macrophylla* when placed on stigmas of *T. crinita* (Figs 12, 13).

The MANOVA revealed that, regardless of species, fewer pollen tubes were found in the styles and entering the ovaries under the control treatment compared to the three manipulation treatments for both species. Furthermore, that same pattern held true for the number of grains on the stigmas of *T. crinita* flowers. In *T. macrophylla*, the control treatment yielded fewer grains on the stigmas than in the cross-pollination treatment. Detailed results are as follows:

The MANOVA detected significant differences in the response variables among the pollination treatments, and the interaction of species and treatment, but no significant difference between the two species (Table 2). Examination of individual ANOVA tables for each response variable revealed significant differences in the number of grains on stigmas among the treatments and the interaction of treatment species, significant differences in the number of tubes on stigmas among treatments, and significant differences in the number of tubes in ovaries among treatments (Table 3). Post-hoc analyses of the significant treatment main effect for all dependent variables (grains on stigma, tubes in stigma, tubes in ovary) via pairwise t-tests showed that the control treatment yielded significantly fewer ($P<0.05$) grains and tubes than the three manipulated treatments (cross, self, and reciprocal hybridization). Post-hoc analysis of the significant interaction effect for the number

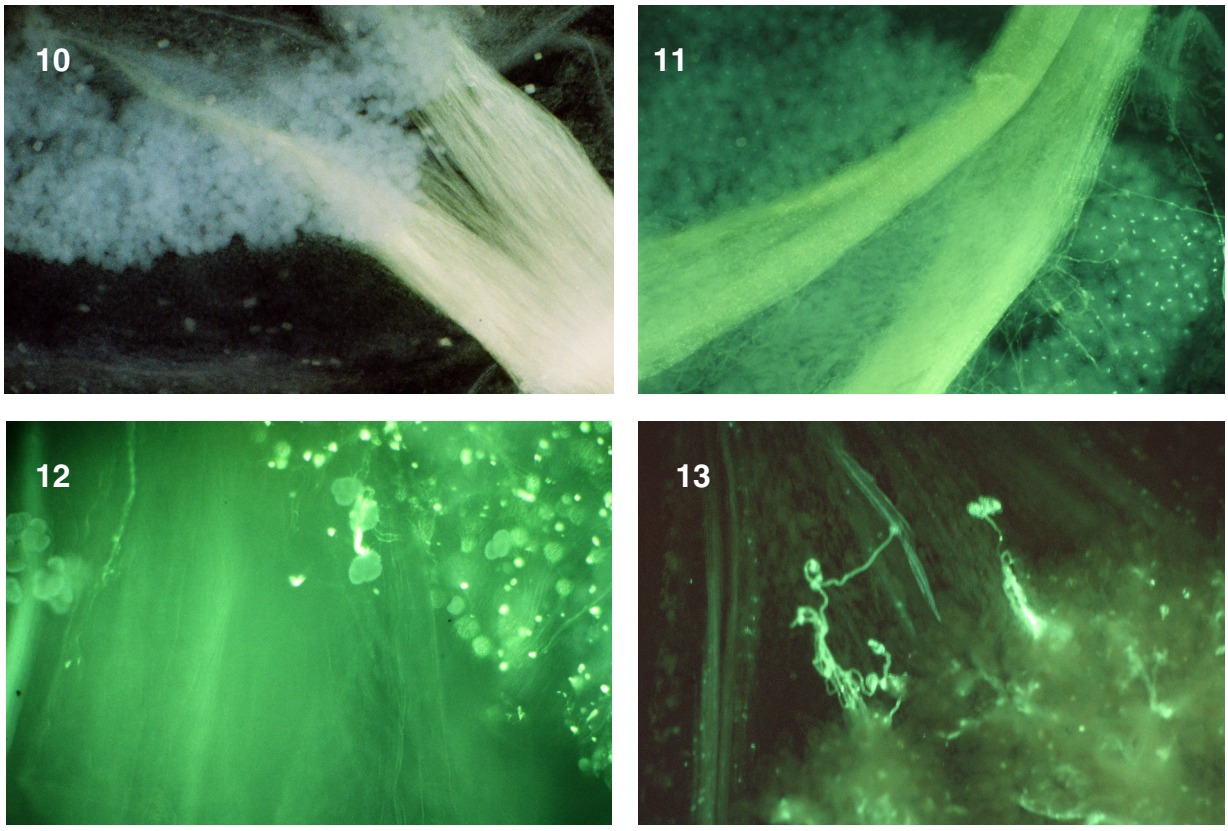

Fig. 10. Pollen tubes reach ovules in the ovary of *Thelymitra crinita* following glasshouse, hand-manipulated self-pollination.

Fig. 11. Pollen tubes reach ovules in the ovary of *Thelymitra crinita* following glasshouse, hand-manipulated cross-pollination.

Fig. 12. Swollen pollen grain with bloated pollen tube of *Thelymitra crinita* on stigma of *T. macrophylla* (glasshouse collection).

Fig. 13. Aberrant pollen tubes of *Thelymitra macrophylla* in ovary of *T. crinita* (glasshouse collection).

of grains on stigmas via pairwise t-test revealed significantly fewer (P<0.05) grains on stigmas for the control treatment than the three manipulated treatments for *T. crinita*. In contrast, *T. macrophylla*, had significantly fewer (P<0.05) grains on stigmas under the control treatment compared to the cross treatment. All other pairwise comparisons were not significant (P>0.05) (Tables 2, 3).

Natural rates of pollinaria removal *vs* pollinaria deposition

***Thelymitra antennifera* and *T. macrophylla* (Tenterden).** Time and budget restraints made it impossible for us to record rates of pollinaria removal in the *T. antennifera* population throughout its flowering season. On September 27 we counted 50 flowering stems of *T. antennifera* and found that only five flowers on five stems lacked their pollinaria. We were unable to detect the presence of pollinia or pollinia fragments on receptive lobes. On 2 October, we checked an additional 31 stems and found that only three flowers on three different stems lacked their pollinaria (no evidence of pollinia deposition on stigmas) even though all flowers on all stems were open. On 2 October, we found that on 31 flowering stems of *T. macrophylla* that a total of seven flowers had their pollinaria removed on four stems. On the same date we found that on 47 stems of the hybrid, *T. antennifera* × *T. macrophylla,* four flowers had their pollinaria removed on four separate stems. The mean ratio of flowers at the Tenterden site with pollinaria removed/total number of flowers was 0.081 (sd=0.261) for *T. antennifera*, 0.054 (sd=0.156) for *T. macrophylla*, and 0.043 (sd=0.141) for *T. antennifera* × *T. macrophylla*. The Kruskal Wallis test revealed no significant difference between these species and their hybrid (χ^2=0.340, DF=2, P=0.844) at Tenterden.

***Thelymitra crinita* and *T. macrophylla* (Lesmurdie).** Over a 17-day period of observations at Lesmurdie, a total of nine inflorescences of tagged *T. crinita* experienced pollinaria removal by insects. A total of twenty-one flowers of *T. crinita* had their pollinaria removed but only four flowers showed fragments of pollinia on their stigmas. A total of 11 inflorescences of tagged *T. macrophylla* experienced pollinaria removal. Fifty flowers of these tagged stems had their pollinaria removed and five flowers had pollinia fragments left on their stigmas. The mean ratio of pollinated/total number of flowers was 0.117 (sd=0.136) for *T. crinita*, and 0.023 (sd=0.048) for *T. macrophylla*. The Wilcoxon Rank Sum test revealed that *T. crinita* appeared to have a higher ratio of pollinated/total number of flowers than *T. macrophylla* (W=177.0, P=0.048). The mean ratio of flowers with pollinaria removed/total flowers was 0.160 (sd=0.169) for *T. crinita*, and 0.178 (sd=0.178) for *T. macrophylla*. No significant difference in these ratios was detected by the Wilcoxon test (W=117.5, P=0.7155).

Table 2. MANOVA (multiple analyses of variance) Table for pollen grains on stigma, pollen tubes on stigma, and pollen tubes in ovary by treatment and species. DF = degrees of freedom; Den-DF = denominator degrees of freedom; F = F-statistic; Num-DF = numerator degrees of freedom; P = P=value; Wilks = Wilk's λ.

Source	DF	Wilks	F	Num-DF	Den-DF	P
Treatment	3	0.06322	69.6	9	297.07	$<2.2\times10^{-16}$
Species	1	0.97284	1.1	3	122.00	0.337572
Treatment*Species	3	0.77335	3.7	9	297.07	0.000221
Residuals	124					

Table 3. ANOVA (analysis of variance) Tables for pollen grains on stigma, pollen tubes on stigma, and pollen tubes in ovary by treatment and species. DF = degrees of freedom; F = F-statistic; P = P-value.

Response Variable	Source	DF	F	P
Grains on stigma	Treatment	3	25.149	6.3×10^{-13}
	Species	1	1.0282	0.3125
	Treatment*Species	3	9.1596	1.6×10^{-5}
	Residuals	124		
Tubes in stigma	Treatment	3	561.2700	$<2\times10^{-16}$
	Species	1	1.6821	0.1970
	Treatment*Species	3	1.5846	0.1965
	Residuals	124		
Tubes in ovary	Treatment	3	163.7622	$<2\times10^{-16}$
	Species	1	0.0096	0.9223
	Treatment*Species	3	1.1176	0.3447
	Residuals	124		

Insect observations, collections, and pollen load analyses

***Thelymitra antennifera* (Tenterden).** Insects were observed infrequently on all three species comparable to their low rates of pollinaria removal (see above). At 11:30 a.m. on 26 September 2009, at the Tenterden site, we observed but were unable to collect a small black bee entering a half-open flower of *T. antennifera*. It then gathered pollen from an open flower of *Chamaescilla corymbosa* (blue squill) before it left the site. On 6 October 2009, we collected one female bee (*Leioproctus* species, subgenus *Leioproctus*) on a *T. antennifera* flower but it did not carry pollinaria or pollinia fragments. Other visitors to *T. antennifera* included flies in the family Bombyliidae. These insects never carried pollinaria.

***Thelymitra macrophylla* (Tenterden).** We collected *Lasioglossum* (subgenus *Chilalictus*) on *T. macrophylla* at Tenterden, and it carried remains of pollinaria from the host flower. Fragments of pollinia were found on the stigma of the same flower visited by this bee.

***Thelymitra macrophylla* (Lesmurdie).** We collected five bees between 16–22 October 2009 on *T. macrophylla*, of which three were females of *Lasioglossum* (subgenus *Chilalictus)*, one female of *Leioproctus*, (subgenus *Leioproctus*) and one female of *Homalictus* species (Halictidae). Pollinaria were found on two specimens of *Lassioglossum* species and one specimen of *Leioproctus* species. In all three cases, the viscidium was attached dorsally towards the tip of the abdomen (Fig. 14). The pollen loads found in the scopae of all bees were mixed with pollen of co-blooming members of the Myrtaceae, papilionoid legumes and unidentified monocotyledons. One syrphid fly carried the remains of the viscidium on its proboscis. We did not find whole pollinaria on any of these flies but two specimens carried individual grains of *T. macrophylla* suggesting they may have consumed pollen mixed with stigmatic fluids. Syrphid flies also carried mixed loads of pollen from co-blooming species including Asteraceae, *Hakea* species, Goodeniaceae, Myrtaceae, and several unidentified *Stylidium* species. We note that the two syrphid flies carrying grains of *T. macrophylla* also carried the distinctive pollen of *Orthrosanthus laxus* (large blue flag: Iridacae). The flowers of *O. laxus* did not secrete nectar and syrphid flies were observed probing the longitudinally dehiscent anthers. Syrphid flies were observed on the flowers of *O. laxus* for the duration of their flowering period. We never observed bees on *O. laxus*.

***Thelymitra crinita* (Lesmurdie).** We caught only three bees on *T. crinita* on 21 and 22 September 2009. One female *Leioproctus* species, (subgenus *Leioproctus*) was found to carry the pollinarium of *T. crinita* with the viscidium attached to her stinger. The other two bees were females of *Amegilla chlorocyanea* (Fig. 15). They did not carry any pollinia. However, on 23 October, 2009, at 10:10 a.m., another bee of the same species (recognized by its large bluish, green-banded abdomen) was observed visiting flowers on six plants of *T. crinita*

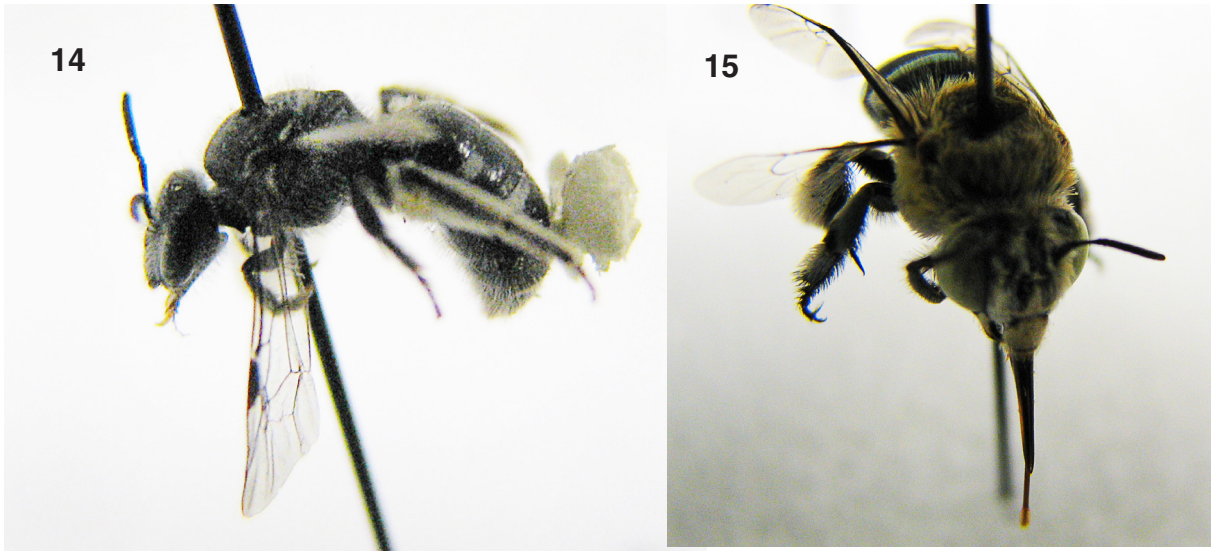

Fig. 14. Pinned specimen of female *Leioproctus* sp. carrying pollinaria of *T. macrophylla* (Lesmurdie, Western Australia).

Fig. 15. Pinned specimen of female *Amegilla chlorocyanea* collected on flowers of *T. crinita* (Lesmurdia, Western Australia).

remaining less than a second on each flower. Its legs touched the mitras. Stigmas were examined after the bee left the area and pollinia residue was found on seven flowers on six flowering stems. All seven flowers closed the following day. We observed and collected both syrphid and bombyliid flies visiting *T. crinita*. None carried the pollen of the host flower.

Hybrids

***Thelymitra antennifera* × *T.* macrophylla (Tenterden).** The flowers of the hybrid between *T. antennifera* and *T. macrophylla* (Tenterden) resembled the description and photograph of flowers of *T.* ×. *macmillanii* (*sensu* Jones 2006). These hybrids showed intense variation of pigmentation patterns grading from pale pink with whitish perianth margins through deep red with yellowish perianth margins (Table 1; Figs 4, 5). Floral fragrance of these hybrids resembled a weak lemon scent compared to the much stronger lemon scent of

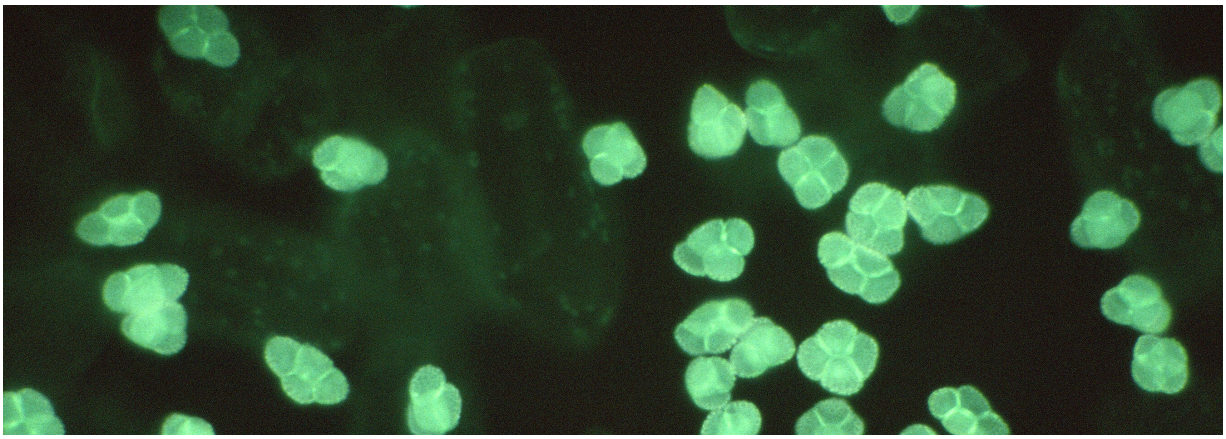

Fig. 16. Tetrads of *Thelymitra crinita* under epifluorescence.

Fig. 17. Adjacent inflorescences of *Thelymitra macrophylla* and *T. crinita* (Lesmurdie, Western Australia).

Fig. 18. Mature flower bud of *Thelymitra crinita* bearing the pollinarium of *T. macrophylla* following physical contact with the open flower.

T. antennifera sensu stricto. However, we report for the first time the malformation of the column of these putative F_1 hybrids (see above). In most cases the pollinia did not drop onto the rostellum at maturity. When the flower was tapped lightly with a probe, the pollinia fell out of the anther onto the ground. The viscidium was always retained by the rostellum after the pollinia fell from the flower. In other cases we observed the pollinia never dropped out of the anther and never connected to the rostellum. Field investigations on 3 October 2009, showed that out of a total of 49 flowering hybrid stems, 20 inflorescences bore 1–3 open flowers in which the pollinia never connected to the viscidium.

We also report flowers of *T. antennifera* with red splotches on their perianth segments (Fig. 6). These flowers were never used in any of the data subsets described above. The plants producing these flowers were found adjacent to pure yellow *T. antennifera* and/or the putative F_1 hybrids. We never saw plants with splotched flowers growing adjacent to plants of *T. macrophylla*. These splotched flowers retained the normal brownish burgundy lateral lobes found in *T. antennifera sensu stricto.* (Table1). In addition, the pollinia in these splotched flowers were attached normally to the viscidium.

***Thelymitra crinita* × *T. macrophylla* (Lesmurdie).** It was more difficult to discriminate between hybrids produced by *T. crinita* and *T. macrophylla* at the Lesmurdie site as both were blue flowered species (Figs 7, 8) and putative F_1 plants showed a more subtle gradation of characters (Table 1). Indeed, our four putative hybrids required collection and confirmation by Dr. Andrew Brown, one of Western Australia's most prominent orchidologists. The gradation of characters was most striking at the microscopic level. Specifically, pollen grains of *T. macrophylla* were dispersed as monads while pollen grains of *T. crinita,* were dispersed as tetrads (Fig. 16), as in *T. ixiodes* (Sydes and Calder 1993). In the putative hybrids at Lesmurdie and in the glasshouse collections, we found a mixture of tetrads, triads, dyads, and monads in the putative hybrids (Table 1). Molloy and Dawson (1998) had much the same results when they compared F_1 hybrids and species of hybrid origin in some *Thelymitra* species in New Zealand. When *T. ixiodes, T. formosa, T. cyanea*, or *T. aemula* all containing tetrad pollen, crossed with a second species with monad pollen, the offspring produced pollinia containing a mixture of monad and tetrad grains.

We did not observe insects alternating their foraging on the flowers of both species at Lesmurdie because our protocol was to capture any bee as it landed on the flower, with the exception of the much larger (>10 mm in length) *Amegilla chlorocyanea* (Apidae, see above). However, sympatric, co-blooming populations of both species often grew so close to each other that flowers touched. On one occasion, when the wind was blowing, a bud of *T. crinita* was blown into the interior of a *T. macrophylla* flower and its perianth segment removed the pollinarium from the anther (personal observation by R. Edens-Meier, Figs 17, 18).

Discussion

Variation in floral characters and flowering patterns. Differences in the numbers of flowers per peduncle varied between the three, large-flowered species and showed some degree of intermediacy when two species hybridized (Table 1). This was anticipated based on previous descriptions of all three species (Brown *et al.* 2008; Jones 1988). However, we did not anticipate the significant difference between the number of flowers per peduncle in disjunctive populations of *T. macrophylla* with the southern (Tenterden) population offering fewer flowers than the northern (Lesmurdie) population. If fewer flowers per peduncle in the southern population were genetic in origin, it would explain the fewer flowers per stem in the recurrent hybrid, *T. antennifera* × *T. macrophylla* (Tenterden). *Thelymitra antennifera* produced less than two flowers per peduncle (as usual; see Jones 1988) while *T. macrophylla* (Tenterden) had a mean of only four flowers per peduncle. Does a consistent and positive correlation exist between peduncle length, the number of flowers per peduncle, and northern distributions of *T. macrophylla* in Western Australia? We cannot answer this question, unfortunately, because the most recent treatment of the taxonomy of *T. macrophylla* (Jeanes 2013) did not consider variation in the number of flowers per peduncle along a geographical distribution.

Tagged flower buds of *T. macrophylla* and *T. crinita* showed variation in the floral life spans of these large-flowered *Thelymitra* species. The literature on pollen-pistil interactions in some orchid flowers showed that removal of the pollinarium and/or the deposition of pollen on the stigma interrupted and shortened their lifespans (Huda and Wilcock 2012). In both *T. crinita* and *T. macrophylla* hand-pollination (both cross and self) triggered premature closing of the perianth segments. However, when freshly opened flowers of both species were deprived of their pollinaria the decline in their floral lifespans was not statistically significant in either species. Much the same response was recorded previously in controlled experiments on flowers of *Mystacidium venosum* (Luyt and Johnson 2001) and *Chloraea alpina* (Clayton and Aizen 1996). Glasshouse collections of *Thelymitra crinita* and *T. macrophylla* failed to self-pollinate in the absence of pollinarium vectors. Field records at Lesmurdie showed that insect-mediated pollinations occurred in less than 12% of

the flowers on tagged stems in both species. We argue that if a large-flowered *Thelymitra* species were to close its perianth segments permanently following pollinarium removal, without pollen deposition on its stigma, that this process would be maladaptive. In fact, it would be especially maladaptive in outcrossing orchids with mimetic, 'food flowers'. A review of pollination in orchids with mimetic, 'food flowers' showed that this system was visited infrequently by pollinators (see below and see Tremblay *et al*. 2005). Allowing *Thelymitra* flowers to reopen after the pollinarium is removed, (provided preferred climatic conditions prevail) may be interpreted as selectively advantageous. It may improve female fitness in these two species. Both species were pollinated infrequently because they were so pollinator limited (*sensu* Vance *et al*. 2004). In particular, insect-mediated, pollinarium removal in *T. macrophylla* was far higher than actual rates of pollen deposition on the stigma. This indicated that most bees visited *T. macrophylla* only once (see below).

This was also the first field study that showed that opening and closing perianth segments varied between two *Thelymitra* species as *T. crinita* continued to open on cool days, provided it was sunny, while *T. macrophylla* remained closed even if it were a cloudless day. Why do large-flowered *Thelymitra* species open and shut at all? We did notice that pollen-eating syrphid flies remained active on cloudy, cool days, and were often observed foraging on the large, blue flowers of *Orthrosanthus laxus* (Lesmurdie; Bernhardt and Edens-Meier, unpublished) through late afternoons. By the time a large-flowered *Thelymitra* species opens its perianth segments for the first time, its anther has already dehisced, and all of its pollinia are connected to the rostellum. Consequently, both pollinia pairs are now naked and exposed while the perianth segments are open. Hover flies (Syrphidae), thrips, or other pollinivorous insects have access to these exposed pollinia and may damage them while feeding. As hoverflies were observed feeding on stigmatic fluids in this study, and in earlier publications (Cady and Rotherham 1970; Dafni and Calder 1987) these insects may also contribute interspecific hybridization when they carry pollinia fragments on their mouthparts between co-blooming species. A second explanation may be based in part on visual *vs*. pollinator memory. By opening for only part of the day, these mimetic flowers produce a temporary but vivid, visual cue on an erratic basis based on climate. The prospective pollinators may be less able to discriminate and reject the mimics on their standard foraging routes as the mimics close and thus 'vanish' from sight only to reappear at another time.

Therefore, variation in the opening and closing of large-flowered *Thelymitra* species may increase some reproductive success at two levels. First, it may increase the ability of flowers of some species to exploit the density-limited guild of pollinating bees that visit a mimetic flower more than once. We note, for example, that *T. crinita* opened on cold but sunny days, unlike *T. macrophylla*, and the rate of pollinated stigmas on tagged peduncles of *T. crinita* was 9% higher than in *T. macrophylla.* Second, when two or more sympatric *Thelymitra* species have overlapping flowering periods, but respond to different climatic cycles, this may afford some interspecific isolation as different pollinators may be active under different temperature and cloud cover regimes.

Breeding systems. Rogers (1913) dissected fresh flowers of *T. antennifera* and insisted this species was unable to self-pollinate. This was confirmed by the field studies of Dafni and Calder (1987). *Thelymitra crinita* and *T. macrophylla* now join a small, but increasing number of populations of large-flowered *Thelymitra* species that fail to self-pollinate when pollinators fail to arrive (Cropper and Calder 1990; Sydes and Calder 1993). Based on our glasshouse experiments, both *T. crinita* and *T. macrophylla* should be classified as self-compatible. This is not atypical for the Orchidaceae as floral presentation encouraging out-crossing tends to accompany self-compatibility in the majority of orchid genera studied to date based on recent reviews. For example, Edens-Meier *et al.* (2010) showed that all outcrossing *Cypripedium* species studied to date lack pre-zygotic self-incompatibility (Bernhardt and Edens Meier 2010; Tremblay *et al.* 2005).

Pollinarium removal *vs*. insect visitations. Darwin (1862, 1877) was among the first to note that orchid flowers received, at best, infrequent visits from their pollinators. He was also unaware (Darwin 1862) that the *Orchis* and *Ophrys* species he studied were food and sexual mimics, respectively. As Darwin did not have the time or energy to spend the entire day and the entire flowering season observing insect visitors, he recorded the natural removal of pollinaria in the flowers of populations near his house. This allowed him to determine natural rates of insect visits and pollinaria dispersal over several seasons. Our own records of pollinarium removal in 11 inflorescences of *T. macrophylla* at Lesmurdie in 2010 indicated strongly that the rate of pollinarium removal in flowers of tagged plants was ten times higher than the rate of pollinia deposition on the stigmas of the same flowers.

Of nine, tagged flowering stems of *T. crinita*, only 21 flowers had their pollinaria removed while 11% of all the flowers on these stems were insect-pollinated. In fact, *T. crinita* produced fewer flowers per stem than *T. macrophylla* (Table 1) at Lesmurdie so *T. crinita* had a higher rate of reproductive success than its congener growing at the same site. There are two possible and interrelated explanations for the very 'modest' success of the flowering stems of *T. crinita.* First, *T. crinita* flowered later than its congener and its flowering peak occurred

under warmer, sunnier, mid-spring conditions when bee numbers presumably increased. Second, *T. crinita* was the only species to attract females of *Amegilla chlorocyanea* and, as described above, at least one female visited several flowers on several stems without receiving an edible reward. Bees in this genus are unusually large by Australian standards (>10 mm in length) so they may carry more pollinia on their abdomens leaving a broader film of pollinia fragments upon contacting stigmatic lobes.

Otherwise, bee visitation to both species was similar to collections and descriptions in previous publications on the pollination biology of large-flowered, blue *Thelymitra* species. Insects carrying pollinaria on their abdomens were all female bees that clasped the mitra but their visits were, at best, dismally infrequent (Bernhardt and Burns-Balogh 1986; Sydes and Calder 1993). Cropper and Calder (1990) suggested that the infrequent visits of female *Nomia* species to *T. epipactoides* provided an explanation of why this orchid was so rare. However, our populations of *T. crinita* and *T. macrophylla* were common *in situ* (Brown *et al*. 2008). In fact, pollinator visits to food mimicking, orchid species are rarer than pollinator visits to sexually mimetic orchids (see review in Tremblay *et al.* 2005). Reproductive success in food mimicking, orchid species should be highest when their flowers occur at far lower frequencies than those of the rewarding, co-blooming species mimicked by the orchids (Dafni and Bernhardt 1990). Our sites offered hundreds of large-flowered, *Thelymitra* species in bloom. We suspect that their rates of pollinaria removal and deposition were probably low in part because they equaled or even outnumbered their pollen and/or nectar producing model flowers. Large-flowered *Thelymitra* species seem less likely to fool prospective pollinators when they grow *en mass* in large populations compared to a few flowering orchid stems interspersed among their model, co-blooming species. Perhaps this is why at least half the species in the genus *Thelymitra* show such a consistent shift towards facultative autogamy to subcleistogamy (Bates 1999; Jeanes 2004). Jeanes (2013) placed *T. macrophylla* in the *T. nuda* complex. It contains 15 species (*sensu* Jeanes 2013), of which two appear to self-pollinate. An additional explanation may be based on foraging speed of bees *vs*. pollen load. Morse (1981) found that foraging speeds of both *Bombus terricola* Kirby and *B. vagans* Smith visiting milkweed flowers (*Asclepias syriaca*) decreased with increasing numbers of attached pollinia. As flowers of *T. crinita* and *T. macrophylla* attached pollinaria to the abdomens of their visiting bees this, presumably, represented a form of drag that could alter a bee's speed and foraging ability, especially if pollinaria proved difficult to remove. For this reason, most small to medium sized bees (halictids and *Leioproctocus*: see Cropper and Calder 1990) may have chosen to avoid these flowers after a single visit while a large bee (*e.g. Amegilla chlorocyanea*) might visit flowers on several stems before abandoning a mode of floral presentation that failed to offer edible rewards.

While *Leioproctus fulvescens* (Colletidae) pollinates some flowers of *T. longifolia* in New Zealand (Lehnebach *et al*. 2005) this is the first time members of this bee genus have been found visiting flowers of *Thelymitra* species in Australia. This study also adds members of the genera *Homalictus* (Halictidae) and *Amegilla* (Apidae) to the list of potential pollinators of large, blue-flowered species. *Thelymitra macrophylla* and *T. crinita* also appear to belong to a small but increasing number of large-flowered species exploiting females of *Lasioglossum* (subgenera *Chilalictus*, *Parasphecodes*; Halictidae; see Bernhardt and Burns-Balogh 1986; Dafni and Calder 1987; Sydes and Calder 1993). What do the genera *Amegilla*, *Exoneura*, *Homalictus, Leioproctus* and *Nomia*, representing three different families, have in common aside from some species making infrequent visits to *Thelymitra* species? All appear to be genera dominated by species in which the females show polylectic foraging habits. In these bee genera, many species appear to collect pollen from a wide variety of unrelated angiosperm species and a single bee may carry pollen from three to five different plant families in the same pollen load (Bernhardt 1989, 1995, 1996). In fact, some of these bee species forage on flowers offering pollen as the only reward including *Acacia* (Bernhardt 1989), *Dianella* (Bernhardt 1995) and *Hibbertia* (Bernhardt 1984, 1986, 1996; Dafni and Calder 1987). As some *Dianella* and *Hibbertia* species have porate-porose anthers, bees forage using sonication (Bernhardt 1984, 1986, 1995, 1996), hence these insects attempt to clasp (and vibrate?) the mitras or ornamented anthers (*e.g. T. antennifera*; Dafni and Calder 1987) of some large-flowered *Thelymitra* species.

Evolutionary significance of recurrent hybrids? Interspecific hybridization between some *Thelymitra* species appears unavoidable in Western Australia (Brown *et al*. 2008) as populations are often large, pollination events are few and different species probably share the same pollinators. As the pollinator quickly recognizes and rejects the floral presentation of the mimetic flower of one species it may actually be more likely to visit the flower of a second species in which color, odor and mitra sculpturing differ. Therefore, what is the significance of the hybrids observed and collected in this present study aside from their morphological intermediacy? Burns-Balogh and Bernhardt (1988) hypothesized that introgression was a powerful evolutionary mechanism in mitra diversification and speciation within the *Thelymitra* lineage. There is no genetic evidence to back up this hypothesis although McAlpine (1978) found morphological evidence for backcrossing between the recurrent hybrid, *T.* × *truncata*, and one of its parent species, *T. ixioides*. Instead, the origin of some *Thelymitra* species, endemic to New Zealand, reflected a history of interspecific hybridization and amphidiploidy according

to Molloy and Dawson (1998). In contrast, Bower (2001) noted that introgression in *Thelymitra* species was often prevented following recurrent interspecific crosses as the first generation of hybrids were sterile.

Flowers of *T. antennifera* (Tenterden) with red blotches, in the company of many, typical *T. antennifera* plants and many flowering stems of *T. antennifera* × *T. macrophylla* make us wonder if backcrossing occurred in the past even though the majority of F1 hybrids produced malfunctioning columns? While this hybrid was and is an inefficient disperser of pollinia, we still do not know whether its stigma can accept and process the pollinia of *T. antennifera* if transported by a potential pollinator? Considering the size of the F1 population hybridization between *T. antennifera* and blue large-flowered *Thelymitra* species may be increasing in Western Australia when we compare Brown *et al.* (2008) to Nicholls (1951, Plate 44). The text in Nicholls describes *T.* × *macmillanii* (Jones 2006) but it is clear that some of the flowers illustrated (Nicholls 1951, Plate 44 b, e, g, h) are obviously *T. antennifera* × a blue and large-flowered species (see Brown *et al.* 2008). Nicholls wrote that *T. macmillanii* was present but very rare in Western Australia. It is now obvious that those red-orange hybrids are not rare today.

In fact, taxonomists with molecular laboratories might consider analyses of *T. crinita* × *T. macrophylla* and its parent species. The F_1 hybrid provided such a subtle gradation between the two parents, its presence went largely unnoticed prior to this study, except for some rescued plants collected and now grown in a glasshouse at KPBG (see above). Pollinarium structure showed no obvious dysfunction. The combination of tetrad, dyad, and monad grains in the pollinia did not indicate obvious male sterility as grains stained with Calberla's fluid remained as large as those in either parent species and filled with granular cytoplasm (Edens-Meier and Bernhardt, unpublished). Genetic analyses may provide an older history of gene filtration/migration between one or both of the parental species.

We think that we have made it clear that this genus makes an excellent field model system for floral evolution for interested evolutionary botanists with sufficient funding. Our study of one field season demonstrates the need to extend this study over a number of years to gather additional information to expand upon preexisting data and hypotheses. What is needed in particular is for populations in this study to be monitored over a far longer period of time (at least five years), especially during this period of global climatic confusion.

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References

Bates RJ (1999) Self-pollinated sun orchids of the *Thelymitra pauciflora – T. longifolia* alliance in Australia. *The Orchadian* 13: 65–72.

Bates RJ, Weber JZ (1990) *Orchids of South Australia.* (AB Caudell, Government Printer, South Australia)

Bernhardt P (1984) The pollination biology of *Hibbertia stricta* (Dilleniaceae). *Plant Systematics & Evolution* 174: 266–277.

Bernhardt P (1986) Bee-pollination of *Hibbertia fasciculata* (Dilleniaceae). *Plant Systematics & Evolution* 152: 231–241.

Bernhardt P (1989) The floral ecology of Australian *Acacia.* Pp. 127–155, in Stirton CH, Zarucchi JL (eds). Advances in legume biology. (Monographs in Systematic Botany from the Missouri Botanical Garden, St. Louis, MO)

Bernhardt P (1995) The floral ecology of *Dianella caerulea* var. *assera* (Phormiaceae). *Cunninghamia* 4: 1–17.

Bernhardt P (1996) Anther adaptations for animal pollination. Pp. 192–220, in D'Arcy W, Keating R (eds). The biology of anthers. (Cambridge University Press, New York, NY)

Bernhardt P, Burns-Balogh P (1986) Floral mimesis of *Thelymitra nuda* (Orchidaceae). *Plant Systematics and Evolution* 151: 187–202.

Bernhardt P, Dafni A (2000) Breeding system and pollination biology of *Mandragora officinarum* L. (Solanaceae), in northern Israel. Pp. 215–224, in Totland, O. (ed.), The Scandinavian Association for Pollination Ecology honours Knut Faegri. *Det Norske Videnskaps – Academi*, Series 39 (Oslo, Norway)

Bernhardt P, Edens-Meier R (2010) What we think we know *vs* what we need to know about orchid pollination and conservation: *Cypripedium* L. as a model lineage. *Botanical Review* 76: 204–219.

Bishop T (1996) Field guide to the orchids of New South Wales and Victoria. (University of New South Wales Press, Sydney)

Bower C (2001) Pollination [T*helymitra*]. Pp. 208–213 in Pridgeon AM, Cribb PJ, Chase M, Rasmussen FN (eds) *Genera Orchidacearum* Volume 2 Orchidoideae (Part 1). (Oxford University Press, Oxford, England)

Brown A, Dundas P, Dixon K, Hopper S (2008) Orchids of Western Australia. (University of Western Australia Press, Nedlands, Western Australia)

Burns-Balogh P, Bernhardt P (1988) Floral evolution and phylogeny in the tribe Thelymitreae (Orchidaceae: Neottioideae). *Plant Systematics & Evolution* 159: 19–47.

Cady L, Rotherham ER (1970) Australian native orchids in colour. (Charles E. Tuttle Co.: Publishers. Rutland, Vermont, U.S.A.)

Cheeseman T (1880) On the fertilization of *Thelymitra. Transactions and proceedings of the New Zealand Institute* 13: 291–296.

Clayton S, Aizen M (1996) Effects of pollinia removal and insertion on flower longevity in *Chloraea alpine* (Orchidaceae). *Evolutionary Ecology* 10: 653–660.

Cropper SC, Calder DM (1990) The floral biology of *Thelymitra epipactoides* (Orchidaceae) and the implications of pollination by deceit on the survival of this rare orchid. *Plant Systematics and Evolution* 170: 11–27.

Dafni A, Bernhardt P (1990) Pollination of terrestrial orchids in Southern Australia and the Mediterranean region. Systematics, ecological, and evolutionary implications. Pp. 192–252, in Hecht MK, Wallace B, Macintyre RJ (eds). *Evolutionary Biology* 24. (Plenum Publishing Corporation, New York)

Dafni A, Calder DM (1987) Pollination by deceit and floral mimesis in *Thelymitra antennifera* (Orchidaceae). *Plant Systematics and Evolution* 158: 11–22.

Darwin C (1862) On the various contrivances by which British and foreign orchids are fertilized by insects, and on the good effects of intercrossing. (John Murray, London, England)

Darwin C (1877) The various contrivances by which orchids are fertilized by insects, 2nd edition. (D. Appleton & Company: New York, USA)

Edens-Meier R, Vance N, Luo Y, Li P, Westhus E, Bernhardt P (2010) Pollen-pistil interactions in North American and Chinese *Cypripedium* L. (Orchidaceae). *International Journal of Plant Science* 171: 370–381.

Edens-Meier R, Arduser M, Westhus R, Bernhardt P (2011) Pollination ecology of *Cypripedium reginae* Walter (Orchidaceae): Size Matters. *Telopea* 13: 327–340.

Erickson R (1965) Orchids of the West, 2nd edition. (Paterson Brokensha Pty Ltd: Perth, Australia).

Fitzgerald RD (1875–1894) *Australian Orchids*, Vols 1 & 2. (Government Printer: Sydney, NSW, Australia)

Huda MK, Wilcock CC (2012) Rapid floral senescence following male function and breeding systems of some tropical orchids. *Plant Biology* 14: 278–284.

Jeanes JA (2004) A revision of the *Thelymitra pauciflora* R. Br. (Orchidaceae) complex in Australia. *Muelleria* 19: 19–79.

Jeanes JA (2006) Resolution of the *Thelymitra fuscolutea* R. Br. (Orchidaceae) complex of southern Australia. *Muelleria* 24: 3–24.

Jeanes JA (2009) Resolution of the *Thelymitra variegata* (Orchidaceae) complex of southern Australia and New Zealand. *Muelleria* 27: 149–170.

Jeanes JA (2013) An overview of the *Thelymitra nuda* (Orchidaceae) complex in Australia including the description of six new species. *Muelleria* 31: 3–30.

Jones DL (1981) The pollination of selected Australian orchids. Pp. 40–43, in Lawler L, Kerr RD (eds). Proceedings of the Orchid Symposium held as a satellite function of the 13th International Botanical Congress, Sydney, Australia, 1981. The Orchid Society of New South Wales. (Harbour Press: Sydney, N.S.W., Australia)

Jones DL (1988) *Native orchids of Australia*. (Reed Books Pty Ltd: Australia)

Jones DL (2006) Native orchids of Australia: including the Island Territories. (Reed New Holland, Sydney, Australia)

Lehnebach C, Robertson AW, Hedderley D (2005) Pollination studies of four New Zealand terrestrial orchids and the implication for conservation. *New Zealand Journal of Botany* 43: 467–477.

Lipow SR, Bernhardt P, Vance N (2002) Comparative rates of pollination and fruit set in widely separated populations of a rare orchid (*Cypripedium fasciculatum*). *International Journal of Plant Sciences* 163: 775–782.

Luyt R, Johnson SD (2001) Hawkmoth pollination of the African epiphytic orchid *Mystacidium venosum*, with special reference to flower and pollen longevity. *Plant Systematics and Evolution* 228: 49–62.

McAlpine DK (1978) On the status of *Thelymitra truncata* Rogers. *The Orchadian* 5: 179–81.

Molloy BPJ, Dawson MI (1998) Speciation in *Thelymitra* (Orchidaceae) by natural hybridism and amphidiploidy, in R. Lynch (ed.), 'Ecosystems, entomology and plants: proceedings of a symposium held at Lincoln University to mark the retirement of Bryony Macmillan, John Dugdale, Peter Wardle and Brian Molloy', 1 September 1995. *Royal Society of New Zealand Miscellaneous Series* 48: 103–113.

Nicholls WH (1951) Orchids of Australia; Drawn in Natural Colour. (Georgian House, An Australian Society Publication: Melbourne, Australia) R Development Core Team (2011) R: A language and environment for statistical computing. ISBN 3-900051-07-0, URL http://www.R-project.org/. (R Foundation for Statistical Computing:Vienna, Austria)

Rogers RS (1913) Mechanisms of pollination in certain Australian orchids. Transactions and Proceedings of the Royal Society of South Australia 36: 48–64.

Rupp HMR (1942) *The Orchids of New South Wales*. (Australian Medical Publishing Company Limited: Glebe, NSW, Australia)

Sokal RR, Rohlf FJ (2003) *Biometry*, 3rd Edition. Pp. 409–422. (W.H. Freeman and Company: New York, U.S.A.)

Sydes MA, Calder DM (1993) Comparative reproductive biology of two sun-orchids; the vulnerable *Thelymitra circumsepta* and the widespread *T. ixioides* (Orchidaceae). *Australian Journal of Botany* 41: 577–589.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo R (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: A spasmodic journey to diversification. *Biological Journal of the Linnean Society* 84: 1–54.

Vance NC, Bernhardt P, Edens RM (2004) Pollination and seed production in *Xerophyllum tenax* (Melanthiaceae) in the Cascade Range of Central Oregon. *American Journal of Botany* 91: 2060–2068.

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