Floral fraudulence: Do blue *Thelymitra* species (Orchidaceae) mimic *Orthrosanthus laxus* (Iridaceae)?

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Abstract

In Western Australia, *Thelymitra crinita* Lindl. and *T. macrophylla* Lindl. are pollinated by female, polylectic bees but offer no edible rewards. Flowers of *Orthrosanthus laxus* (Endl.) Benth. (Iridaceae) offer granular pollen and previous authorities suggest it is a Batesian model of *T. crinita*. We analyzed the floral fragrances and measured the floral dimensions of the orchid species, their putative hybrid, and *O. laxus*. Although the 'scentless' *T. crinita* emitted low levels of monoterpenoids and sesquiterpenoids, the pleasantly discernible fragrance of *T. macrophylla* was dominated by 2-phenylethanol. Their putative hybrid produced slightly lower levels of 2-phenylethanol compared with *T. macrophylla* and failed to produce any sesquiterpenoids associated with *T. crinita*. However, the hybrid produced higher volumes of the monoterpene linalool than either parent species. The fragrance of *O. laxus* contained 2-phenylethanol but lacked the sesquiterpenoids. We also measured perianth area and symmetry as well as the length and width of contrastingly pigmented floral centres for each taxon. Significant differences in floral area and symmetry were detected between the putative hybrid, the two parent species, and *O. laxus*. In contrast, the floral reward centre area (tuft of stamens) in *O. laxus* was significantly larger than the pseudo-reward centres (mitras) of both *Thelymitra* species and their hybrid. At the peak of their respective, but overlapping flowering periods, an inflorescence of *T. macrophylla* produced more than twice the number of open flowers as *T. crinita* and more than four times the number of open flowers on cymes of *O. laxus*. Based on scent production and visual displays, *T. macrophylla* appears more likely to be a Batesian floral mimic of *O. laxus*. We suggest that large-flowered *Thelymitra* species appear to produce a novel, visual and olfactory attractant pattern of fraudulence we call the 'New Again, More Again Effect'.

Introduction

Insect pollinated flowers usually attract their primary pollen vectors with a combination of visual and olfactory cues (Raguso 2008). However, the presence of vivid and distinctive visual and olfactory cues is not always associated with floral rewards. Evolutionary ecologists have documented pollination-by-deceit in four out of the five subfamilies in the family Orchidaceae (Tremblay et al. 2005). Floral mimesis in orchid flowers canalizes the behaviour of pollinators as they enter and exit the flower, facilitating cross-pollination (Dressler 1981). The majority of orchid species with mimetic flowers have been interpreted as food mimics (Tremblay et al. 2005). In a few cases, the scent, pigmentation patterns, and epidermal sculpturing of the flower suggests mimicry

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of edible fungi and/or the prey items of adult pollinators and/or their larvae (Kaiser 2006; Ren et al. 2011; Stökl 2011). However, in the most commonly described mode of food mimesis, the orchid flower lacks edible rewards but produces a floral display that attracts insects known to forage on the nectar and/or pollen of other co-blooming angiosperms (Cozzolino and Widmer 2005; Schiestl 2005).

Dafni and Bernhardt (1990) subdivided floral food mimesis into three overlapping modes of deceit. Generalist food mimics (e.g. species of *Dipodium* and *Orchis*) lack a model flower(s) and attract generalist foragers that visit a wide, unrelated number of co-blooming species. A few orchid species are classified as Batesian mimics (e.g. *Diurus* species; Dafni and Bernhardt 1990) because their floral presentation mimics specific co-blooming species (e.g. selected species of papilionoid legumes) and are pollinated, at least in part, by specialist foragers. A third, and relatively underexplored mode, is guild mimesis. In this case, the flowering period of the orchid overlaps with several co-blooming, but unrelated species that offer comparable rewards to the same subset of pollinators. While these rewarding flowers and non-rewarding orchids belong to several different families, their flowering periods and modes of presentation converge and overlap (Bernhardt 1996).

These three modes of food mimesis may all occur within the large-flowered *Thelymitra* species of Australasia (*sensu* Brown et al. 2008). Indeed, variation in floral presentation in outcrossing species of *Thelymitra* may drive speciation in this lineage (Edens-Meier and Bernhardt 2014). All cross-pollinated *Thelymitra* species studied to date are pollinated by polylectic/polyphagic insects (Edens-Meier and Bernhardt 2014). *Thelymitra epipactoides* (Cropper and Calder 1990) produces up to three distinct and discrete colour morphs and is an example of a food mimic lacking any model species. In contrast guild mimesis has been interpreted in the following species: *T. antennifera* (Lindl.) Hook.f. (Dafni and Calder 1987); *T. ixioides* Smith ex Sw. (Sydes and Calder 1993); and *T. megcalyptra* Fitzg. (*syn. T. nuda* R.Br.; Bernhardt and Burns-Balogh 1986). Thus far, Jones (2006) is the only authority to suggest that *T. crinita* is a Batesian mimic of *O. laxus* (Iridaceae).

Consequently, the large-flowered species in the genus *Thelymitra* may offer an opportunity to better understand the evolutionary ecology of food mimesis. In particular, it is obvious that primary, floral attractants (size, scent, and hood sculpturing) vary at the interspecific and intraspecific levels (Edens-Meier et al. 2013; Edens-Meier and Bernhardt 2014; Jones 2006; Sydes and Calder 1993). We know far more about reproductive success in different floral forms of the same orchid species (Smithson et al. 2007) than we do about the convergent characters expressed by food mimics and their models. Fragrance analyses of mimetic orchids are plentiful (Kaiser 1993; see Appendix in Raguso and Pichersky 1999; Schiestl et al. 1999) but attempts to relate the scent chemistry of the mimic flower to a Batesian model or guild are less frequent. Galizia et al. (2004) compared visual and olfactory displays of the model flower *Bellevalia flexuosa* and the Batesian mimic, *Orchis israelitica* H.Baumann & Dafni. They concluded that the mimic matched the model's visual display in the context of the bees' visual capabilities, but they found no evidence of scent mimicry. These authors argue that visual stimuli dominate in a fraudulent system at short distances.

The evolution of scent within an angiosperm lineage is usually labile and fragrance molecules detected by modern methods do not always reflect a common genetic ancestry (e.g. *Cypripedium,* Barkman et al. 1997). In particular, *Thelymitra* species offers an excellent opportunity to understand the inheritance of fragrance components for two reasons. First, although no known fragrance analyses have been completed to date on this genus, naturalists and botanists have commented on the qualitative range of odours produced by several species (Bernhardt and Burns-Balogh 1986; Dafni and Calder 1987; Jones 2006; Edens-Meier and Bernhardt 2014). Second, it is estimated that at least half of the c. 100 species of *Thelymitra* (*sensu* Brown; Nicholls 1964; Jeanes 2008, 2011), have blue flowers. Western Australia remains the centre of diversity for this genus (Brown et al. 2008) and is rich in unrelated angiosperm species with blue flowers (Neville and McQuoid 1998).

At a site in Lesmurdie, Western Australia, Edens-Meier et al. (2013) examined the floral biology of sympatric, vernal, co-blooming populations of blue-flowered *T. crinita* and *T. macrophylla*. They found that these two species were pollinated by female bees belonging to three native families. The pollen grains of up to three unrelated taxa were found on the hind legs of bees caught on *T. macrophylla*. The flowering periods of both *Thelymitra* species overlapped broadly with sympatric, co-blooming tufts (*sensu* Marchant 1987) of *O. laxus*. Furthermore, hybrids between the two species of *Thelymitra* were located and collected at this site by the Western Australian orchidologist, Dr. Andrew Brown. We collect data on scent production, respective floral displays, and flower sizes to test three hypotheses. First, if *T. crinita* is a Batesian mimic of *O. laxus*, then its scent biochemistry, floral display, and floral dimensions should converge with those of *O. laxus*. Second, if =*T. macrophylla* is a Batesian mimic of *O. laxus*, then its scent biochemistry, floral display, and floral dimensions should converge with those of *O. laxus*. Third, the hybrid, *T. crinita* × *T. macrophylla* should show intermediate characteristics between the scent emissions, floral display, and floral dimensions expressed by its parent species.

Materials and Methods:

Field vs. glasshouse populations: Field sites, identification, deposition of voucher specimens, and the use and origins of glasshouse populations of all *Thelymitra* species and hybrids studied from September 1 – October 23, 2009 are described in Edens-Meier et al. (2013). The population of *O. laxus* used in the research was located in Lesmurdie, Western Australia (intersection of Welshpool East Road and Pomeroy Road).

Study species, floral presentation, and floral measurements:

Thelymitra macrophylla: *T. macrophylla* flowered (Fig. 1) from the last week of August to the second week of October, producing 10 or more flowers/stem (mean = 16.5; n = 15; sd = 3.5; range = 10–23). During peak flowering periods, all the flowers on the scape opened sub-synchronously between 9:30 and 10:30 am and closed between 2:45 and 4:40 pm but the opening of its perianth segments was tardier on cool and cloudy days (Edens-Meier et al. 2013). We agree with Jones (2011) that these flowers produce a strong and pleasant scent. The morphology of the column and mitra follows Edens-Meier et al. 2013; Edens-Meier and Bernhardt 2014).

Thelymitra crinita: This species flowered (Fig. 2) from mid-September until the end of October producing <10 flowers/stem (mean = 9.8; sd = 2.8; n = 18; range = 5–15) at the field site. During peak flowering periods all the flowers on the scape opened simultaneously between 9:15 and 10:35 a.m. and closed between 2:30 and 4:40 pm without producing a discernible scent (Edens-Meier et al. 2013). The morphology of the column and mitra follows Edens-Meier et al. (2013) and Edens-Meier and Bernhardt (2014).

Thelymitra crinita × T. macrophylla: All measurements and scent analyses in this study were taken from a living collection of hybrids housed in the glasshouse at the Kings Park and Botanic Garden (Perth, Western Australia; see Edens-Meier et al. 2013). Six flowering stems were found at the Lesmurdie site from which Brown deposited vouchers in the Western Australian herbarium. A flowering scape produced 12 or fewer flowers (mean = 8.0; n = 8; sd = 3.16; range = 3–12). Flower opening and closing times were identical with parent species (above), producing a fragrance similar to *T. macrophylla*. The hybrid opened tardily on cool, cloudy days as did *T. macrophylla* (Bernhardt, personal observation). Flower and pollen morphology showed intermediacy between both parents (see Edens-Meier et al. 2013; Edens-Meier and Bernhardt 2014).

Orthrosanthus laxus: This species (Figs 1–3) is distributed throughout southwestern Australia and is a rhizomatous perennial herb forming tufts 0.15–0.55 m high flowering from August to early November (Marchant 1987). Individual tufts produced one to many inflorescences with the scape terminating in a compressed cyme containing two to many flower buds. We counted over 50 tufts with the majority located by the banks of the Welshpool Road within 0.5-3 m of adjacent flowering populations of *T. crinita* and *T. macrophylla*. The perianth has blue tepals and each flower produces three large, yellow anthers that open via longitudinal dehiscence. The staminal filaments bend inward toward the centre of the flower forming a triangular cluster. Conversely, the three blue arms of the style bend outward towards the tepals. Edens-Meier et al. (2013) did not observe or collect female bees on this species but they observed and collected pollinivorous flies (*Melangyna* species: Syrphidae). The same fly taxa were observed and collected on *T. crinita* and *T. macrophylla* (Edens-Meier et al. 2013).

Floral presentation of O. laxus, T. crinita, and T. macrophylla: Because *O. laxus* may serve as a Batesian model for *T. crinita*, we compared flowering patterns in 48 tufts (see above) while the flowering of *T. crinita* peaked and overlapped with *T. macrophylla* at Lesmurdie. Bernhardt observed that the population of *O. laxus* bloomed daily regardless of weather conditions. On every third day (10th, 13th, and 16th October 2009), we counted the number of inflorescences (cymes) in bloom within each tuft (Fig. 1). A cyme was recorded in bloom if it produced a minimum of one open flower. We also recorded the total number of open flowers produced on each cyme.

As *T. crinita* and/or *T. macrophylla* may be Batesian mimics of *O. laxus*, we compared flowering patterns in 18 tagged inflorescences of *T. crinita* and 15 of *T. macrophylla* sympatric within the Lesmurdie site. To compare and contrast the number of open flowers presented by those tagged inflorescences of *T. crinita* and *T. macrophylla,* we counted open flowers only on warm, sunny days over their respective flowering periods (see Edens-Meier et al. 2013) starting with the warm and sunny day before the first flower(s) opened. We made 141 counts of *T. crinita* inflorescences and 120 counts of tagged inflorescences of *T. macrophylla* (n = 8 warm, sunny days).

Floral Measurements: All *Thelymitra* flowers used for measurements came from the midpoint on the scape because the inflorescences of both species showed open development with terminal flowers reduced in size often failing to open. Only one flower per inflorescence was measured on the second day that the perianth opened. Fourteen flowers of *T. macrophylla* on 14 inflorescences and 13 flowers of *T. crinita* on 13 inflorescences were measured. Fifteen flowers of *O. laxus* were measured on September 23, 2009.

Fig. 1. Putative model (left; *O. laxus*) and mimic (right; *T. macrophylla*) Photos: Retha Edens-Meier.
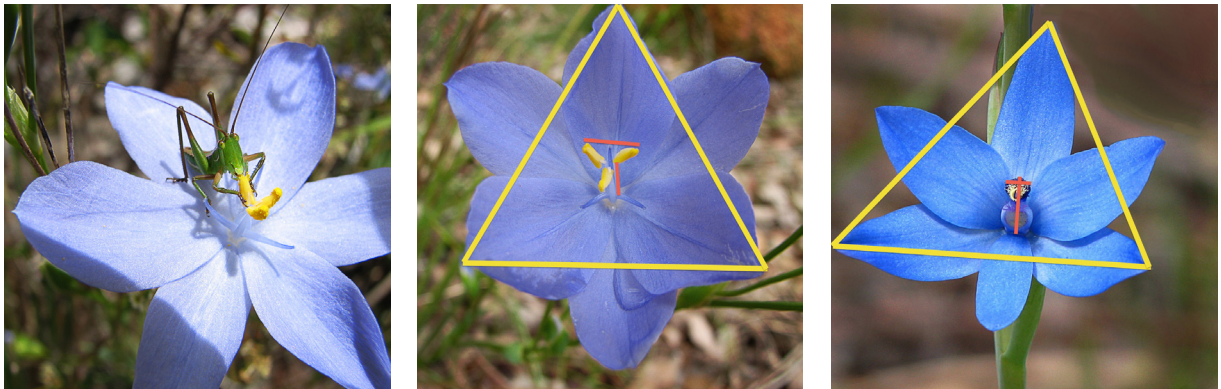

Fig. 2. High contrast yellow centers on blue flowers.

Left: Flower of *O. laxus* with unidentified orthopteran. Note how the insect forages specifically on one of the yellow anthers forming a central, triangular cluster.

Middle: Flower of *O. laxus* showing triangulation of the perianth and the location of the measurement units of the reward center (centralized anther cluster). Note how the stigma lobes are flattened against the tepals and extend beyond the centralized cluster of stamens.

Right: Flower of *T. crinita* showing triangulation of the perianth and the location of the measurement units of the mitra. Note the contrasting color and ornamentation of the median mitra lobe and the lateral "plumose-brushy" lobes. Photos: Retha Edens-Meier

In all cases, three physical measurements were taken using digital calipers for each flower regardless of species. First, perianth size (Figs 1, 5) was calculated using Heron's formula:

$$\text{Area} = \sqrt{p(p{-}a) * (p{-}b) * (p{-}c)}$$

where a, b, and c are lengths of each side;
p is half the perimeter of the triangle, p=(a+b+c)/2

Analysis of variance was used to test for differences in the area of the flowers among the different species. The variability of flower side lengths among species was compared, as measured by the standard deviation of side lengths. Flowers with equilateral geometry should have less variability in side lengths (smaller standard deviations) than species with asymmetrically shaped flowers (higher standard deviations). Analysis of variance was used to determine differences in the average standard deviations of flower side lengths among species.

Finally, we recorded the length and width of the floral centre because the centres in each species were contrasting in colour to that of the perianth segments and insects were observed to fly or crawl onto the floral centres of both *Thelymitra* species and *O. laxus*. In the focal *Thelymitra* species and their hybrid, the centre is represented by an ornamented hood with two trichome brushes (the mitra, Fig. 2). Therefore, we measured hood length and width. In *O. laxus*, the floral centre consists of three erect, elongated, stamens with blue staminal filaments and yellow anthers (Figs 4, 5). The length of the staminal filaments compared to the width of the anthers was measured. The area of the floral centre was calculated by multiplying the length and width of the stamen cluster for each flower. Analysis of variance was used to determine whether the area of the floral centre varied significantly among species. We also calculated the ratio of the floral centre to the floral area (perianth) and compared this ratio among *O. laxus, T. crinita, T. macrophylla,* and their putative hybrid (see Castillo et al. 2012). A one-way ANOVA tested for differences among species.

Fragrance collection: All fragrance collections on the orchids and their putative hybrids were completed on potted plants in the greenhouse (15°C – 22 °C day temperature regulation). We used only potted specimens of the orchids and their putative hybrids to collect fragrances because flowers of the *Thelymitra* species do not open on cool and cloudy days (Edens-Meier et al. 2103). The controlled greenhouse temperatures ensured that the flowers of the two parent species and their putative hybrid would remain open during the entirety of the fragrance collection (see Edens-Meier et al. 2013). In addition, ambient contaminants were easier to exclude in the greenhouse, as our study site bordered suburban homes and the sclerophyll woodlands included many co-blooming species. We used flowering plants of *O. laxus* at the original field site (above), as potted specimens of this species were not available in the greenhouse. Sampling was limited by plant availability and time constraints.

Flower buds on racemes in both *Thelymitra* species opened subsynchronously over a period of 3–5 days. Once flowers open on a raceme, they continue to open on a daily basis, as long as weather conditions are favourable. *Orthrosanthus laxus* was a steady state bloomer (*sensu* Gentry 1974) in which a flowering stem opened from 1 or 2 flowers every day and each flower wilted within 24 hours. An inflorescence of *T. macrophylla* produced 10–23 flowers while *T. crinita* ranged from 5–15 flowers. Both orchid species at Lesmurdie and in the glasshouse collections produced more open flowers per inflorescence (10–16) with individual orchid flowers living longer (14–17 days) than *O. laxus* (Edens-Meier et al. 2013).

Fragrances were collected as described by Galen et al. (2011), from living, intact inflorescences of each focal species. A headspace bag (nylon resin; Reynolds, Inc.) was positioned over 1–10 flowers and securely sealed at the bottom using a twist tie. An adsorbent trap, prepared using a Pasteur pipette with 10 mg Super Q (80/100 mesh; Alltech Associates, Inc.) packed between quartz wool was attached to a battery-operated PAS-500 vacuum pump (Spectrex, Inc.) with Tygon tubing, and was positioned above the flowers using a spring clip attached to a firmly secured bamboo stake (Fig. 3). The tip of the trap was then sealed within the top of the headspace bag with a twist tie. Floral scent was collected for either 1 or 3 hours, depending upon the strength of the fragrance as detected by the human nose, at a standardized flow rate of 200 ml air/min. Floral fragrances from ten *T. macrophylla* scapes and eight hybrid scapes were sampled for one hour since floral scents were easily discernable to the human nose. Flowers from ten *T. crinita* scapes, and ten *O. laxus* scapes (from 10 tufts) were sampled for three hours, since no discernable fragrance was apparent to the human nose. Vegetative and ambient air controls were included to account for non-floral compounds. Upon completion of the fragrance collection, scent traps were eluted into 1.5 ml borosilicate glass autosampler vials using 300 uL of GC-MS grade hexane. Each vial was capped, labeled, wrapped with parafilm, and stored at -20°C. All collected sample vials were placed in a plastic cooler surrounded with cold packs and shipped Australian Air Express to Cornell University, Ithaca, NY (USA) for GC-MS analyses.

Chemical analysis: Upon receipt, floral headspace samples eluted in hexane were concentrated to 50 μl under a flow of nitrogen gas (N_2). An internal standard of 5μl of a 0.03% solution of toluene in hexane was added to

each sample which allowed us to control for slight variation in final sample volume.

All floral scent samples were analyzed by gas chromatography-mass spectroscopy (GC-MS) using a Shimadzu GC17A gas chromatograph with a QP5000 mass spectrometer (EI, 70 electron volts, single quadrupole) as a detector (Shimadzu Scientific Instruments, Inc.). One µL sample aliquots were injected (splitless) at 240°C onto a polar (EC-wax, W.R. Grace & Associates, Inc.) fused capillary GC column, with an oven temperature program of a 3 minute hold at 40°C, followed by an increase of 10°C per minute, with a final 5 minute hold at the maximum oven temperature of 260°C. Compounds were identified by comparing the retention times and mass spectra of total ion current chromatogram peaks to those of authentic standard compounds, facilitated by strong (>90%) fits with entries from mass spectral libraries (NIST and Wiley). Crude emission rates (ng scent per flower per hour) were calculated by hand integrating peak areas and algebraically converting them to toluene equivalents (see Svensson et al. 2005) for quantitative comparisons.

Results

Floral presentation in *O. laxus, T. crinita* and *T. macrophylla*: Flowering of *O. laxus* tufts showed that 13–21 tufts had at least one inflorescence in bloom on each of the three observation days. We found that, while a tuft had as many as four inflorescences in bloom, the average number (n=55 flowering tuft counts) of inflorescences in bloom was less than two (mean = 1.7, sd = 0.96; range = 1–4). Ninety-six open flowers were recorded over the same period. While a terminal cyme could bear a maximum of four open flowers, on the same day, the average number of open flowers on each cyme was also less than two open flowers (mean = 1.6; sd = 0.63; range = 1–4) in bloom in each tuft. Each flower opened early in the morning and usually closed permanently12 hours later, with a few lasting as long as 24 hours on cool to cold and rainy days.

During its flowering season, a raceme of *T. crinita* consisted of less than four open flowers (mean = 3.18; sd = 2.75; range = 0–13) on warm, sunny days while *O. laxus* was in flower. A raceme of *T. macrophylla* produced less than nine flowers (mean = 8.43; sd = 5.58; range = 0–19) under the same weather conditions while *O. laxus* was in bloom.

Fig. 3. Fragrance collection of *O. laxus* at the Lesmurdie site in October, 2009. Note that the floral fragrance collection equipment is supported by bamboo stakes. Photo: Retha Edens-Meier

Floral measurements: Significant differences in perianth size were found among species (F=13.19, DF=3,46, P<0.00001), with post hoc analysis revealing that *O. laxus, T. crinita,* and *T. macrophylla* all were smaller than the hybrid (Fig. 4). Flowers of *Thelymitra macrophylla* were smaller than those of *O. laxus*. However, flowers of *T. crinita* were not found to differ significantly from those of *O. laxus*, or from those of *T. macrophylla*. Furthermore, some species were found to be less symmetrical than others (F=8.36, DF=3,46, P=0.00015) (Fig. 4). This post hoc analysis revealed that flowers of *O. laxus* and *T. macrophylla* both were more symmetrical than those of the *Thelymitra* hybrid. *Orthrosanthus laxus* was found to be more symmetrical than *T. crinita*. However, equal symmetry was identified between *T. crinita* and the hybrid; between *T. macrophylla* and *O. laxus;* and between *T. macrophylla* and *T. crinita*. Finally, significant differences were found in the size of the floral centre among species (F=24.65, DF=3,46, P<0.00001), with post hoc analysis revealing that the hybrid, *O. laxus,* and *T. macrophylla* all were smaller than *O. laxus* (Fig. 4). Furthermore, the floral centres of *T. crinita* and *T. macrophylla* were not found to be different from the hybrid and *T. macrophylla* was not found to be different from *T. crinita*. The one-way ANOVA for the ratio of the floral centre to the floral area (perianth) showed significant differences among species (F = 9.848, DF = 3, 46, and P <0.001). A Tukey's Honest Significant Difference post-hoc analysis revealed that *O. laxus* (mean = 0.034, sd = 0.014) had a significantly higher ratio than *T. crinita* (mean = 0.021, sd = 0.005), *T. macrophylla* (mean = 0.017, sd = 0.006), and their hybrid (mean = 0.016, sd = 0.001).

Fragrance analyses: The focal species differed markedly in total standardized scent emission per flower per hour. Flowers of *T. macrophylla* were 300-fold more strongly scented than those of *T. crinita*, and 16-fold more strongly scented than those of *O. laxus*, their putative Batesian model (Table 1). Only four scent compounds were detected in *T. macrophylla* of which three were structurally related benzenoids.

scent compound (class)	ret time min.	Thel. macrophyllum n = 10	Thel. crinita n = 10	Thel. mac x crin n = 8	Orth. laxus n = 10
"total emissions, ng/flw/hr"		642.86 + 144.20	1.82 + 0.47	185.52 + 23.20	41.33 + 11.21
"2PE emissions, ng/flw/hr"		624.72 + 140.49	absent	172.07 + 22.56	17.04 + 6.01
"rel % (mean, SEM)"					
(benzenoids)					
phenylacetaldehyde	14.66	0.30 + 0.10		0.41 + 0.07	
2phenethylacetate	16.65	1.38 + 0.13		1.25 + 0.29	
2phenylethanol	17.65	96.77 + 0.52		92.10 + 1.04	45.75 + 11.29
(monoterpenoids)					
"1,8 cineole"	8.32		11.23 + 3.75		
beta-phellandrene	8.83				trace
E-beta-ocimene	9.08		20.98 + 8.57		trace
linalool	13.28	1.61 + 0.50	1.86 + 0.99	6.24 + 0.98	
(sesquiterpenoids)					
alpha-copaene	12.71		25.23 + 8.43		
germacrene D	15.52		2.23 + 1.18		
"E,Z-alpha-farnesene"	15.61		0.94 + 0.62		
"E,E-alpha-farnesene"	15.90		6.65 + 3.99		
delta-cadinene	16.10		7.09 + 4.07		
(lox-derived)					
hexyl acetate	9.41				1.15 + 0.81
cis-3-hexenyl acetate	10.12				45.26 + 11.37
cis-3-hexen-1-ol	11.06				7.37 + 2.19

The most abundant of these compounds, 2-phenylethanol (96.77%) dominated the fragrance of *T. macrophylla*. The only volatile monoterpenoid detected from this species was linalool, at relatively low levels (1.61%). Although we were unable to smell the fragrance of *T. crinita*, chemical analysis revealed eight different volatile compounds of which three were monoterpenoids and five were sesquiterpenoids. The dominant monoterpenoid for *T. crinita* was E-β-ocimene (20.98%) while the dominant sesquiterpenoid was -copaene (25.23%). *Thelymitra crinita* also produced linalool in a relative amount (1.86%) comparable to that of *T. macrophylla* (1.61%). Additional sesquiterpenoids identified in floral fragrances collected from *T. crinita* included E,E-α-farnesene (6.65%) and δ-cadinene (7.09%).

Only four scent compounds were detected from flowers of the putative *Thelymitra* hybrid and these were the same four compounds detected in the floral headspace of *T. macrophylla*. In particular, the dominant floral compound produced by the hybrid 2-phenylethanol (92.10%), emitted at 1/3 of the rate measured for *T. macrophylla*. However, the same hybrid plants produced comparable actual amounts and greater relative amounts of the monoterpenoid linalool (6.24%) than did *T. macrophylla* (1.61%), and considerably more than did *T. crinita* (1.86%) of a very weak scent (Table 1). Unlike in *T. crinita*, we did not detect sesquiterpenoids in the floral scent of the hybrid.

Analyses of *O. laxus* revealed six volatile compounds - one benzenoid, two monoterpenoids, and three lipoxygenase (lox)-catalyzed fatty acid-derived molecules. The dominant compounds were the benzenoid 2-phenylethanol (45.75%) and the lox-derived cis-3-hexenyl acetate (45.26%), a common 'green leaf volatile'.

Discussion

***Thelymitra* species as *Batesian* mimics of *O. laxus*:** This study was motivated by the potential for different kinds of floral mimicry among blue and insect-pollinated *Thelymitra* species, due to their clear visual resemblance to rewarding, blue-flowered plants in Western Australia. Analyses of floral scent chemistry revealed that *T. macrophylla* closely produces a very similar, yet more intense, scent as does *O. laxus*. Below, we explore these findings in the larger context of evolution of floral cues. Morphometric analyses confirmed that the floral dimensions for *T. macrophylla* were smaller than those of *O. laxus*. In addition, the ratio of reward centre to floral area was found to be significantly smaller than *O. laxus*. *Thelymitra macrophylla* also showed more variability in the perianth (i.e. side lengths) than did *O. laxus*.

Based on results of fragrance analyses neither *Thelymitra* species produced a scent profile that exactly replicated the odour of the proposed model flower. Of the two *Thelymitra* species, *T. crinita* is least likely to represent a Batesian mimic of *O. laxus* based exclusively on its weak, terpenoid dominated scent, as the flowers of *O. laxus* lacked sesquiterpenoids. In contrast, the dominant scent constituent in *T. macrophylla* and *O. laxus* was the widespread benzenoid, 2-phenylethanol, a volatile common to many insect pollination systems (Bernhardt et al. 2003; Ashman et al. 2005; Galen et al. 2011). Interestingly, 2-phenylethanol is emitted at 30-fold higher amounts per flower of *T. macrophylla* than in *O. laxus*, which could potentially represent a super-normal mimetic stimulus (Schiestl 2005; see Edens-Meier et al. 2014), or one that exploits a pre-existing sensory bias of the pollinator species (Vereecken and Scheistl 2008; Schaefer and Ruxton 2009).

Schiestl (2005) and Schiestl et al. (1999) suggest that the presentation of super-normal floral stimuli is common in species expressing some mode of Batesian mimicry. That is, the visual and/or olfactory impact of the mimic surpasses that of a discrete model so that a naive pollinator responds to the greater stimulus provided by the fraudulent orchid. In a putative Batesian relationship between *T. macrophylla* and *O. laxus*, the overproduction of 2-phenylethanol is even more pronounced because *T. macrophylla* produces more flowers per inflorescence than are found in a single tuft of the putative model, *O. laxus* (Figs 2, 3). We acknowledge that the fake reward centre of *T. macrophylla* is smaller than the rewarding centre of *O. laxus*, but we suspect that the overall visual and olfactory display of one or more inflorescences of *T. macrophylla* (racemes were often clustered at Lesmurdie in 2009) is greater than the scent and visual cue produced by tufts of *O. laxus* on warm sunny days. Unlike the putative Batesian mimic system studied by Galizia et al. (2004), *T. macrophylla* potentially represents a superior olfactory mimic of *O. laxus*, with a similar colour scheme and a much larger floral display. Castillo et al. (2012) found that pollinators preferred small perianths and large rewarding structures in their studies on *Begonia gracilis* Kunth. The ratio of the fraudulent reward centre to its floral area (perianth) of *T. macrophylla* was always smaller than in the rewarding model of *O. laxus*. It is suspected that *T. macrophylla* 'cheats' (*sensu* Castillo et al. 2012) by offering a much larger blue flowering display than its model.

However, the production of exaggerated (hypermorphic) visual and olfactory cues does not guarantee superior reproductive success in a fraudulent orchid (Edens-Meier et al. 2013, 2014; Edens-Meier and Bernhardt 2014;
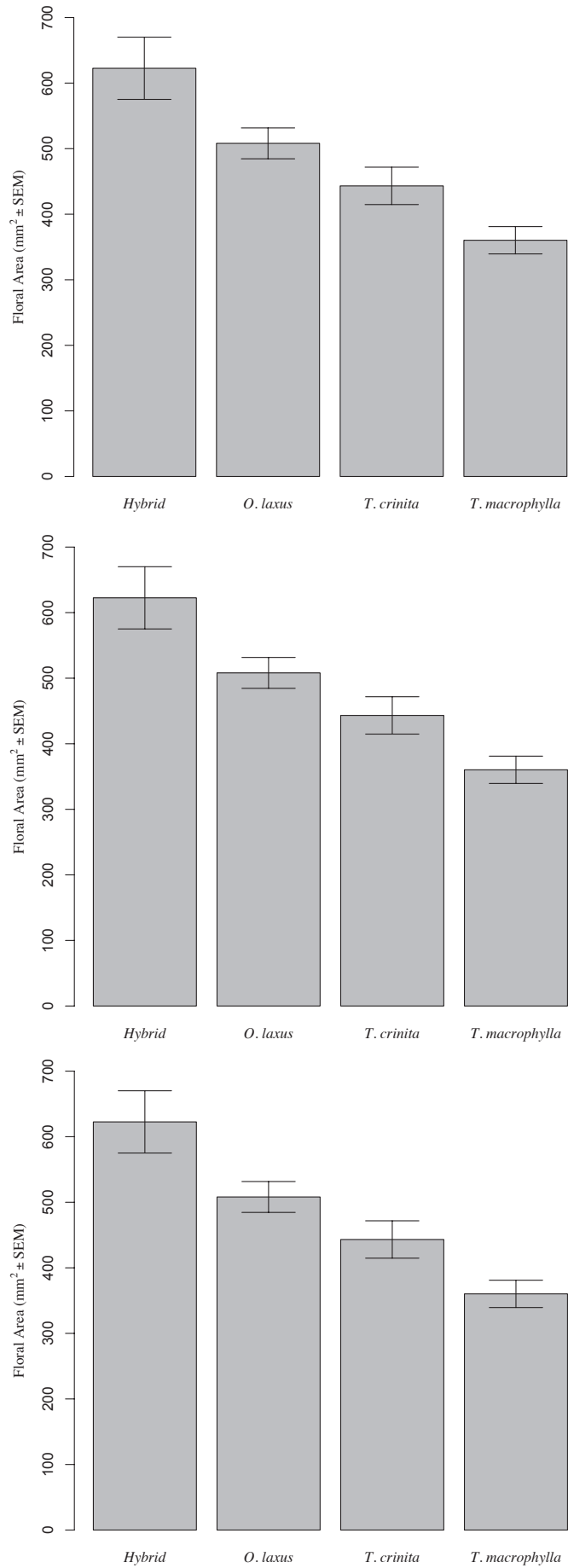
Fig. 4. Histograms of *T. crinita* × *T. macrophylla* comparing measurements of perianth size (top), floral symmetry (middle) and the floral centres (bottom).

Tremblay et al. 2005). The fraudulent floral displays of *T. macrophylla* resulted in poor pollination success in 2009. Although the rate of pollinarium removal by bees was greater than 16%, few bees deposited pollinia fragments on its stigmas, indicating that few bees visited the flowers of this species a second time. In fact, natural rates of pollinia deposition on receptive stigma surfaces were less than 3% on flowers of *T. macrophylla*. If this rate of pollination is consistent over several seasons, it would indicate that, as in many *Cypripedium* species, Batesian mimicry coupled with super normal stimuli works rather infrequently (Edens-Meier et al. 2013, 2014).

Kunze and Gumbert (2001) suggested that food deceptive orchids should be scentless, after experimentally demonstrating that bumblebees are less able to distinguish between artificial blue flowers with subtly different hues when scent is absent (also see Leonard et al., 2012). Therefore, in the absence of behavioural assays with known pollinators, we cannot yet determine whether the weak terpenoid floral scent of *T. crinita* serves any pollinator-related function. Alternatively, *T. crinita* may be a Batesian mimic of a second, unexamined, model species with blue flowers (e.g. shrubby Goodeniaceae were common *in situ*). In fact, flowers of *T. crinita* experienced higher pollination success than those of *T. macrophylla* in 2009. The rate of pollinarium removal in this orchid was almost identical to that of *T. macrophylla* but the rate of pollinia deposited on the stigmas of *T. crinita* flowers was over six times higher than in co-blooming *T. macrophylla* (Edens-Meier et al. 2013).

Apparent lack of intermediacy of floral cues between a *Thelymitra* hybrid and its parent species: The hybrid produced more linalool than either parent species and produced a significantly larger floral area. This contributes to earlier theories that new *Thelymitra* species evolve by interspecific hybridization (Burns-Balogh and Bernhardt 1986). More recently, Molloy and Dawson (1998) concluded that some *Thelymitra* species endemic to New Zealand evolved via hybridization followed by amphidiploidy. There is growing evidence that stabilized changes in polyploidy may be responsible for selectively advantageous differences in floral traits leading to diverging pollinator preferences (e.g. *Heuchera grossularifolia* Rydb*;* Segraves and Thompson 1999).

Comparative aspects of scent composition: Based on past analyses of floral fragrances in Orchidaceae (Kaiser 1993) and more recent reviews across the angiosperms (Knudsen et al. 2006), none of the scent compounds identified in this study are unique. It is interesting to note that flowers of *T. crinita* emitted a terpene-rich scent (Fig. 4) even though that scent was not detectable. It is likely that other published descriptions of odourless *Thelymitra* species (see Edens-Meier and Bernhardt 2014) may similarly underestimate the presence of low volatility compounds. For example, the sesquiterpene (E)-β-caryophyllene was emitted in low amounts by the flowers of four out of 14 'scentless' hummingbird pollinated plant species analyzed by Knudsen et al. (2004) in Ecuador. This compound is also emitted in low amounts by the tiny, autogamous flowers of *Arabidopsis thaliana* (L.) Heynh., where it has been shown to protect floral tissues against bacterial attack (Huang et al. 2012). Majetic et al. (2010) also found that the 'scentless' but colourful flowers of *Ipomoea purpurea* in fact emitted a complex blend of sesquiterpene volatiles, dominated by (E)-β-caryophyllene and germacrene D. Most of these species are not perceived as strongly scented to the human nose, probably because humans have a high average threshold of perception of sesquiterpenes (Ohloff 1994).

The significance of 2-phenylethanol vs. linalool: The pleasant scent of *T. macrophylla* was dominated by one volatile compound (2-phenylethanol). This compound has been detected extensively throughout the angiosperms including the Orchidaceae (Kaiser 2010). In fact, it is also found in the flowers of some basal angiosperms (Kaiser 2010; Bernhardt et al. 2003). However, in these basal species, 2-phenylethanol is usually expressed in small quantities often representing less than 4% of the total scent blend. Likewise, while 2-phenylethanol is also a scent component in dozens of orchid species, it is usually a minor component (see Kaiser 2010).

Studies that combine chemical analyses with pollinator behaviour and/or effectiveness suggest that 2-phenylethanol plays diverse, dosage-dependent roles in plant-pollinator dynamics. Roy and Raguso (1997) tested the importance of visual and olfactory cues in floral mimicry by rust fungi and found that artificial flowers augmented with a 0.1% solution of 2-phenylethanol attracted *Dialictus* bees (Halictidae). Similarly, Ashman et al. (2005) demonstrated that solitary bees preferred staminate flowers of *Fragaria virginiana* Mill. over female flowers because small amounts of 2-phenylethanol were released by pollen-bearing anthers. Galen et al. (2011) found that higher levels of 2-phenylethanol emission in *Polemonium viscosum* Nutt. reduced damage by nectar-thieving ants but also reduced visitation frequency by bumblebee pollinators, increasing pollen limitation and lowering seed fitness. Their study highlights the importance of carefully examining the effects of volatile dosages on both pollinators and predators. Predictions of how various floral visitors will respond to the same volatile are often difficult to assess (Galen et al., 2011). Field studies comparing insect visitation rates to *T. macrophylla* with those of *T. crinita* spiked with different dosages of 2-phenylethanol would allow us to assess the importance of this volatile to the different mimetic strategies utilized by these orchid species.

Linalool (3,7-dimethyl-1,6-octadien-3-ol), a monoterpene alcohol, was the only molecule shared by both species of *Thelymitra*; however, both produced it as minor constituents (< 2.0%) of their scent blends. Linalool, another compound with a fragrance pleasant to the human nose, is one of the most common components of floral scents (Raguso and Pichersky 1999; Knudsen 2006; Kaiser 2010) suggesting its importance and versatility in flower/pollinator communication (Parachnowitsch et al. 2013; Salzmann and Schiestl 2007). It can be present as a very minor component (and still show behavioural activity) in hawkmoth pollinated *Datura wrightii* Regel. (Riffell et al. 2009), as a nectar odour in bee pollinated *Penstemon* flowers (under positive selection; see Parachnowitsch et al. 2012, 2013), and as a dominant odour in the early spring flora (Borg-Karlson et al. 1994). Surprisingly, Parachnowitsch et al. (2012, 2013) found that floral scent in weakly scented *Penstemon digitalis* Nutt. experienced stronger (directional) selection (promoting seed fitness) than did floral colour or size and suggested that reproductive success benefits from being strongly scented in this species.

Introducing 'The new again, more again hypothesis': The evolutionary significance of the visual and olfactory display of these large and multi-flowered *Thelymitra* species is unknown. Specifically, it is not known why large-flowered *Thelymitra* species are the only orchids in Australasia that regularly, synchronously, and cyclically open and close their flowers over their flowering seasons. Edens-Meier et al. (2013) considered two overlapping possibilities: (1) these flowers may close after daily peaks of bee foraging to protect the exposed, naked pollinaria from pollen-eating insects (see Armbruster and Mziray 1987); (2) by opening for only part of the day, the *Thelymitra* flowers might produce a temporary but exaggerated floral display.

Cross-pollinated *Thelymitra* species are the only food-mimic orchids offering the following suite of characters: (1) large flowers (although flowers may be smaller than some model species, (2) hoods displaying a false reward centre, (3) when scapes are mutli-flowered their flowers open and close synchronously to sub-synchronously. Are these linked characters adaptive? We offer a novel hypothesis, 'The New Again, More Again Effect.' Until an inflorescence of a large-flowered *Thelymitra* species passes peak flowering, its floral presentation changes on a daily basis. Specifically, by mid-afternoon the flowers vanish by closing. The following day, if weather permits, these flowers reappear (new again). However, with the opening of the previous days flowers (an individual flower of both *Thelymitra* species lives 15–17 days; Edens-Meier et al. 2013) the inflorescence opens additional flowers. As the visual display increases, so does scent volume (more again). Because of the opening and closing of flowers coupled with the interrupted yet increasing levels of floral attractants, this effect continues to entice and attract both naïve and experienced resident bees belonging to the local pollinator guild.

This hypothesis should be relatively easy to test. One would simply wait for the blue orchid flowers to close during the afternoon hours and then attempt to catch native bees known to pollinate their blue Batesian models and/or guild flowers. If bees are found to be active at this time of day on these other, non-orchid, blue flowers, such results would support the proposed hypothesis. This finding would indicate that these bees forage on blue flowers after the mimics 'disappear'. In addition, we suggest field experiments on pollination rates of these orchid species by manipulating the number of potted orchids and/or prospective model species at various sites.

Focusing on Future Research: In most cases, floral signals form a multimodal stimulus combining olfactory and visual cues (Kunze and Gumbert 2001; Leonard et al. 2011). Specific floral colours and scent combinations can greatly influence the behaviour of potential pollinators (Galen and Kevan 1980; Raguso and Willis 2005). Recent experimental tests with bumblebees suggest that while multimodal floral signals can take longer to process (thus decreasing foraging efficiency), they generally lead to more accurate decision making by bees (thus enhancing floral constancy) once they are learned (Gegear 2005; Kulahci et al. 2008). Our field-based observations and collections have not confirmed that polylectic bees visit either *Thelymitra* species because they resemble *O. laxus*. Edens-Meier et al. (2013) did not observe a single native bee foraging on *O. laxus* in 2009. Bees collected on *T. macrophylla* carried pollen grains from a wide variety of co-blooming plants, blue-flowered and otherwise. Although syrphid flies in the genus *Melangyna* foraged on the dehiscent anthers of *O. laxus* and on the stigmas of *T. macrophylla*, they only carried a few grains of *O. laxus*. Furthermore, we never collected a syrphid fly carrying an entire pollinarium of *T. macrophylla*. We suggest targeting pollination ecology studies on *O. laxus* to determine whether its true pollinator(s) correlates with any insect species known to pollinate blue-flowered *Thelymitra* species.

Acknowledgments

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