

Does morphology matter? An explicit assessment of floral morphology in sexual deception

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Summary

1. Sexually deceptive orchids are renowned for their olfactory mimicry of female insect sex pheromones to lure male pollinators, but the role of floral morphology remains unknown.
2. Here, we reveal compelling new experimental evidence that morphology also matters in sexual deception. Our study exploited two morphologically distinct *Chiloglottis* orchids that both employ the same semiochemical (chiloglottone 1) to attract their respective primary pollinator. In these experiments, we monitored attempted copulation of pollinators with orchid labella as this likely impacts plant fitness.
3. Reciprocal pollinator choice tests revealed significant reductions in the frequency and duration of attempted copulation when pollinators were presented with alternate orchid species that differ in floral morphology, but nevertheless exhibit identical semiochemicals. Experimentally shortening the labellum also reduced the duration of attempted copulation in one of the species.
4. Pollinators exhibited contrasting orientations during attempted copulation and pollination, which seem to be correlated with fundamental differences in the morphological adaptations for both mechanical fit and female mimicry in these orchids.
5. Our findings confirm the overlooked importance of floral morphology for sexually deceptive orchid pollination and indicate that pollinator behaviour could impose strong selection on specific floral traits.

Key-words: adaptation, *Chiloglottis*, female mimicry, mating behaviour, mechanical fit, pollination, pollinator-mediated selection, specialization

Introduction

Pollinator-mediated selection on floral morphology is predicted to be driven by two non-exclusive mechanisms: selection exerted via the sensory preferences of pollinators on attractive traits and selection driven by the mechanical fit between pollinators and flowers. Several studies have revealed patterns consistent with selection for mechanical fit to primary pollinators (Steiner & Whitehead 1990; Muchhala 2007; Anderson & Johnson 2008). For example, floral tube length of plants pollinated by long tongue flies typically matches the tongue lengths of their local pollinators (Pauw, Stofberg & Waterman 2009). Pollinator preference may also influence floral morphology with contrasting pollinator preferences potentially driving divergence in floral colour (Bradshaw & Schemske 2003;

De Jager & Ellis 2014a) and odour (Shuttleworth & Johnson 2010). Strong adaptive responses to pollinator preference are illustrated by the matching flower colours between the rewardless food-deceptive orchids and the rewarding plants they mimic (Peter & Johnson 2008; Newman, Anderson & Johnson 2012).

Pollinator sensory preferences are also predicted to be a key driver in sexually deceptive orchids. These plants attract male insects as pollinators by mimicking female sex pheromones (Schiestl *et al.* 1999, 2003; Ayasse, Stökl & Francke 2011) and achieve pollination when mate-seeking males attempt copulation with their flowers. Variation in floral odour has been linked to speciation in sexually deceptive orchids (Peakall *et al.* 2010; Xu *et al.* 2011; Peakall & Whitehead 2014), indicating a key role for chemistry in their evolution. Beyond sex pheromone mimicry, these orchids exhibit unusual, often morphologically complex insectiform labella that have long been interpreted as morphological mimicry of the females of the pollinator species (Kullenberg 1961; Paulus & Gack 1990; Peakall

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1990). Alternatively, complex floral morphology in sexually deceptive orchids may be under selection via mechanical fit with male pollinator morphology to enhance copulation behaviour with the labellum and/or contact with the floral reproductive structures.

To date, no studies of sexually deceptive orchids have comprehensively investigated the role of flower morphology in either female mimicry or mechanical fit. Some support for female mimicry is found in *Cryptostylis*, where the labella exhibit similar reflectance spectra as the abdomen of its pollinator's female (Gaskett & Herberstein 2010). Floral colour in *Ophrys* has also been found to enhance pollinator detection over short distances (Streinzer, Paulus & Spaethe 2009). *Ophrys* may also provide some support for mechanical fit as a significant correlation between labellum length and pollinator length has been reported (Paulus 2006). However, due to a general size overlap between male and female sexes in pollinators of *Ophrys* (Amiet *et al.* 2010), it may not be possible to disentangle mimicry of female form from mechanical fit to male pollinators within this system, as no clear predictions can be made for each hypothesis. Much needed experimental manipulations are also lacking, and as a consequence, the function of these intriguing and morphologically complex labellum structures remains poorly understood.

The parasitoid thynnine wasps that pollinate the vast majority of Australian sexually deceptive orchids exhibit unique mating behaviour and unusual sexual dimorphism. The smaller wingless females spend most of their adult lives underground, while the larger winged males search above-ground for emerging females (Ridsdill Smith 1970). This sexual dimorphism offers a unique opportunity to tease apart the role of female mimicry from mechanical fit. Here, we conduct an explicit assessment of the role of floral morphology in the thynnine-pollinated sexually deceptive orchid genus *Chiloglottis*. The highly specific orchid–pollinator interactions (average of 1.1 pollinators/orchid species) in the genus involve 1–3 floral volatile components from a pool of six related 2,5-dialkylcyclohexane-1,3-diones or 'chiloglottones' (Schiestl *et al.* 2003; Franke *et al.* 2009; Peakall *et al.* 2010).

Pollinators can be readily attracted to and may even attempt copulation with plastic beads (as simplistic female dummies), when spiked with the appropriate synthetic chiloglottone(s) (Schiestl *et al.* 2003; Peakall *et al.* 2010). *Chiloglottis* species, however, exhibit consistent differences in the size and shape of their labella, including the placement of insectiform calli structures (Bower 1996; Peakall *et al.* 2002; Peakall & Whitehead 2014). Pollinators have also been reported to exhibit higher tendencies to attempt copulation with flowers than with spiked beads (Whitehead & Peakall 2014). Thus, despite the critical importance of semiochemicals, morphology may also be important for pollination.

Our study takes advantage of two strategically targeted species, *Chiloglottis trapeziformis* Fitzg. and *C. valida* D.L. Jones. These orchids are drawn from distinct clades

(Peakall *et al.* 2010) and represent the two divergent floral morphologies that characterize the genus (Peakall *et al.* 1997). Yet, both orchids employ chiloglottone 1 for pollinator attraction (Schiestl & Peakall 2005). Each species has a distinct primary pollinator, male *Neozeleboria cryptoides* Smith and male *N. monticola* Turner wasps, respectively (Peakall *et al.* 2010). While essentially allopatric, both orchids may share each other's pollinator when distributions overlap and rare F1 hybrids are known (Peakall *et al.* 1997, 2002).

Each wasp species represents a distinct clade within *Neozeleboria*, and they exhibit some behavioural differences that likely reflect their phylogenetic divergence (Schiestl & Peakall 2005; Griffiths *et al.* 2011). For example, orientations during attempted copulation at the two species are consistently different (Figs 1 and S1, Video S1, Supporting Information). Thus, working with this pair of orchids, and their respective pollinators, provides a powerful opportunity to evaluate the role and importance of morphology while minimizing the confounding effects of chemistry.

Our study asks two main questions. First, we ask, does morphology matter in sexual deception? We address this question through a series of experiments where we compare attempted copulation frequency between two morphologically distinct *Chiloglottis* species and between morphologically manipulated conspecific flowers. Secondly, we ask, is there morphological evidence for mechanical fit with male pollinators in labellum or floral reproductive structures and is there evidence for female mimicry? We answer this by comparing orchid morphological traits of size and colour with male and female wasp morphology. We then evaluate our findings against *a priori* predictions that take into account the distinct orientations of attempted copulation at the flowers, allowing us to predict separate morphological responses for each orchid species under both the mechanical fit and mimicry hypotheses.

Materials and methods

BACKGROUND

Orchid study species

Chiloglottis are small, colony-forming orchids endemic to south-eastern Australia and Tasmania. They are found in moist forest habitats and, except for one selfing species, are all pollinated by sexually attracted male thynnine wasps (Peakall *et al.* 2010). The flowers of *Chiloglottis* are dull-coloured and characterized by labella containing complex calli structures that male wasp pollinators grip during attempted copulation (Figs 1 and S1). Despite molecular evidence for the existence of three clades (Mant, Peakall & Weston 2005; Peakall *et al.* 2010), labellum morphology across the genus may be subdivided into just two morphotypes (Mant, Peakall & Weston 2005). Our principal component analysis (PCA) of floral measurements from across all three major clades confirmed this conclusion, with some species bearing small (6–10 mm) diamond-shaped labella containing clustered insectiform calli and



Fig. 1. (a) *Chiloglottis trapeziformis* and (b) its pollinator *N. cryptoides* indicating the forward orientation and probing at the distal labellum tip during attempted copulation (arrow). (c) *C. valida* and (d) its pollinator *N. monticola* indicating the reverse orientation and probing at the proximal part of the labellum (arrow). Important floral traits are indicated. Scale bar = 10 mm.

other species bearing larger (8–17 mm) cordate-shaped labella containing less insectiform and less dense calli (Fig. S2a). PCA also confirmed that *C. trapeziformis* and *C. valida* are good representatives of these two labellum shapes, which are clearly separated by size and shape in morphospace (Fig. S2b). Mean trait values of *C. trapeziformis* were significantly smaller (Table S1). *C. trapeziformis* flowers were collected from naturally occurring plants at Mulloon farm (S35 14 23.9, E149 36 35.0) and the Australian Botanical Gardens (S35 16 36.14 E149 06 35.67). *C. valida* flowers were sourced from Tallaganda State Forest (S35 30 27.0 E149 32 03.5).

Wasp study species

As overlapping populations of *N. cryptoides* and *N. monticola* are very rare, it was necessary to conduct separate collections and experiments for each wasp species. Experiments with male *Neozeleboria cryptoides* (pollinator of *C. trapeziformis*) were conducted at Black Mountain (S35 16 31.91 E149 05 17.92) and Molonglo Gorge (S35 16 30.12 E149 05 19.08), while experiments with male *N. monticola* (pollinator of *C. valida*) were conducted at Tallaganda State Forest, adjacent to the *C. valida* study population. PCA of pollinator morphology revealed some overlap in size between the two wasp species (Fig. S3b). However, mean trait values were significantly smaller in *N. cryptoides* when compared with *N. monticola* (Table S2). Analysis of pairs of mating *Neozeleboria* wasps confirmed sexual dimorphism for length (t -test for dependant samples: $t = -7.74$, d.f. = 18, $P < 0.001$) and height ($t = -9.61$, d.f. = 18, $P < 0.001$), but not width ($t = 0.00$, d.f. = 18, $P = 0.99$). Males were nearly twice as large as females (Fig. 2), and PCA revealed no overlap between conspecific males and females (Fig. S4).

Pollinators of diamond-shaped species, such as *C. trapeziformis*, orientate with their heads towards the reproductive column and

probe the distal labellum tip with genital claspers on the tip of their abdomen, with pollen transfer occurring in this position (Figs 1 and S1, Video S1). Pollinators of cordate-shaped species, such as *C. valida*, frequently turn around on the labellum and probe the proximal part of the labellum instead (i.e. at 180° rotation compared to pollinators of diamond-shaped species), with pollination occurring in this reverse orientation (Figs 1 and S1, Video S1).

EXPERIMENTS – DOES MORPHOLOGY MATTER IN SEXUAL DECEPTION?

Reciprocal pollinator choice tests between orchids

Choice experiments involving the presentation of a single flower of *C. trapeziformis* and *C. valida* separated by 4 cm were conducted independently within *N. cryptoides* and *N. monticola* wasp populations. To match the average floral height of the orchid typically pollinated by each wasp species (Peakall & Handel 1993; Schiestl & Peakall 2005), flowers were presented at 15 cm above the soil surface for *N. cryptoides* and 5 cm for *N. monticola*. Pollinator responses to flowers were recorded as mutually exclusive approaches or landings or attempted copulations. We also recorded the landing place of the wasp, as well as the duration and orientation of copulation attempts.

The tendency of pollinators to attempt copulation with flowers will likely impact orchid plant fitness. For example, a meta-analysis of the pollination rates of sexually deceptive orchids indicates that reproductive success is higher in species that elicit more intense sexual behaviour from their male pollinators (Gaskett, Winnick & Herberstein 2008). Similarly, in sexually deceptive daisies, reproductive success is highest in morphotypes that elicit more intense sexual behaviour (Ellis & Johnson 2010). We thus

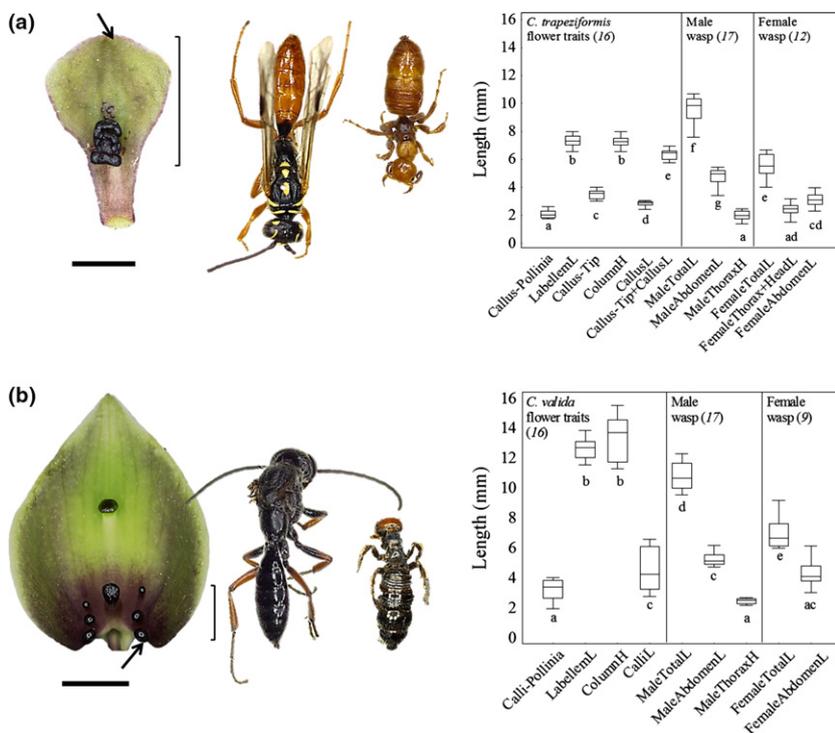


Fig. 2. (a) *Chiloglottis trapeziformis* labelium with its male *N. cryptoides* pollinator and its female, illustrating the forward orientation of pollinators attempting copulation. (b) *C. valida* labelium with its male *N. monticola* pollinator and its female, illustrating the reverse orientation. Next to each are box plots with the median and 95th and 5th percentile of important morphological traits. Different letters indicate significant differences between traits as investigated with one-way ANOVA ($P < 0.05$). Arrows point to the probing point of respective pollinating males during copulation attempts and brackets indicate the labellum trait proposed to be mimicking the respective female wasp. Scale bar = 3 mm.

analysed the frequency and duration of attempted copulation as a potential proxy for orchid reproductive fitness. In total 8–10 replicate experiments per pollinator species were completed, each consisting of four 3-min trials conducted at least five metres from the previous trial. We never reused a position on the same day and treated pollinator visits within a trial as independent due to the very low probability of pseudoreplication (Whitehead & Peakall 2012). New flowers were used after every fourth experiment in order to sample multiple phenotypes.

Labellum manipulation experiments

In a variant of the choice experiments described above, the response of *N. cryptoides* to a *C. trapeziformis* flower with a shortened labellum vs. a control *C. trapeziformis* flower with an intact labellum was compared. For the control flower, a section of its dorsal sepal similar in size to that removed from the labellum of the manipulated flower was taken to control for any potential effects of plant volatile release due to cutting of flowers. As no chiloglottone production has been detected in the dorsal sepals or non-calli containing parts of the labellum in *Chiloglottis* (Falara *et al.* 2013), this manipulation did not alter chemical attraction. To further minimize any potential effects of plant volatile release on pollinator behaviour, we conducted experiments within an hour of cutting the flowers. *N. monticola* wasps were similarly exposed to a choice test between a *C. valida* flower with a shortened labellum and a control *C. valida* flower with its labellum intact. For both orchids, we recorded the same behavioural data as described above.

Statistical analysis of choice tests

We pooled data across experiments within a choice test and used G-tests to investigate differences between observed and expected number of approaches, landings or copulation attempts to each species (expected numbers representing no difference between presented flowers = 1 : 1). We analysed differences in the duration of

copulation attempts in seconds with Mann–Whitney *U*-tests. All stats were conducted in SPSS version 21 (SPSS Inc., Chicago, IL, USA).

IS THERE MORPHOLOGICAL EVIDENCE FOR MECHANICAL FIT AND/OR FEMALE MIMICRY?

Morphological measurements

Measurements of seven floral traits (Table S1) were made from fresh *C. trapeziformis* and *C. valida* flowers (see Fig. 2 for sample sizes). We also measured seven size traits (Table S2) of their respective pollinators, *N. cryptoides* and *N. monticola* male wasps, from ethanol-preserved samples. Dimensions of the head, thorax and abdomen of *N. cryptoides* and *N. monticola* females were also measured. These females were obtained from pairs in copula either caught directly in the field or housed in the Australian National Insect Collection (Canberra), as pairs of these two species are rarely seen in the field. Given that many *Neozeleboria* species are morphologically cryptic, identification of field-caught pairs was confirmed by DNA sequencing at the CO1 gene following the methods of Griffiths *et al.* (2011).

Colour measurements

Spectral reflectance measurements of *C. trapeziformis* and *C. valida* labella, and *N. cryptoides* and *N. monticola* female wasps, were made using a UV/VIS preconfigured JAZ-EL200 portable spectrometer (Ocean Optics, Florida, USA). A 400- μ m fibre reflection probe (R400-7-UV-VIS) was connected to the internal deuterium tungsten halogen light source (200–850 nm) of this instrument. Reference spectra were taken using a WS-1 Diffuse Reflectance Standard. For labellum measurements, leaves were used as background colour. The results were averaged over three independent measurements before plotting as points in a colour hexagon (Chittka 1992) to model colours in hymenopteran visual space.

The closer the points in the hexagon are to each other, the more similar they are perceived by the wasps that pollinate *Chiloglottis*.

Results

DOES MORPHOLOGY MATTER IN SEXUAL DECEPTION?

Reciprocal pollinator choice tests between orchids

In Table 1, we report a summary of the behavioural differences that were evident between the two wasp species during our experiments in terms of preferred landing place, tendency to attempt copulations and orientation during copulation attempts. The number of responding wasps (Table 1) comprises all wasps that responded to a specific species. Similar proportions of responding *N. cryptoides* ($G = 2.622$, $P = 0.105$) and *N. monticola* ($G = 1.778$, $P = 0.673$) landed on each orchid species, likely indicating similar long-range attractiveness of the two orchids. However, when analysing the total number of each mutually exclusive response to each orchid, both pollinator species exhibited significantly more copulation attempts on the orchid that they normally pollinate (Fig. 3c,f, *N. cryptoides* on *C. trapeziformis*: $G = 10.978$, $P < 0.001$; *N. monticola* on *C. valida*: $G = 3.900$, $P = 0.048$). Copulation attempt duration also tended to be longer on these species (Fig. 4a, *N. cryptoides*: $U = 163.500$, $P = 0.098$; *N. monticola*: $U = 5.00$, $P = 0.044$).

Labellum manipulation experiments

No differences in the number of responses for either pollinator were detected between the shortened labellum

treatment and the control, indicating that cutting the flowers did not affect attractiveness. However, a twofold reduction was observed in the duration of *N. cryptoides*' copulation attempts on artificially shortened *C. trapeziformis* labella ($U = 91.500$, $P < 0.001$; Fig. 4b). A corresponding significant decrease in forward orientation (Table 1) was observed on flowers with shortened labella (GLM with binomial distribution and logit link: *Wald chi-square* = 4.454, d.f. = 1, $P = 0.002$). For *N. monticola*, which does not probe the distal labellum tip, no difference in copulation attempt duration was observed when the labella of *C. valida* were shortened (Fig. 4b). The proportion of reverse orientations (*Wald chi-square* = 0.247, d.f. = 1, $P = 0.601$) was also unchanged during attempted copulation with flowers that had shortened labella (Table 1).

IS THERE MORPHOLOGICAL EVIDENCE FOR MECHANICAL FIT AND/OR FEMALE MIMICRY?

In Table 2, we summarize our *a priori* predictions for trait matches between flowers and wasps. We subdivide trait matches by the two hypotheses of mechanical fit and female mimicry. Under mechanical fit, we recognize traits involved in fit to the labellum that are important during copulation attempts, and fit to the reproductive structures that promotes pollen transfer. Given the different pollinator orientations at the two study species, there are contrasting expectations for the fit to the labellum during attempted copulation. The forward orientating male pollinator of *C. trapeziformis* strongly curves its abdomen while probing the distal labellum tip during attempted copulation (Fig. 1b, Video S1). We thus predicted that labellum length (LabellumL), and the correlated distance

Table 1. Pollinator behavioural responses to diamond-shaped *Chiloglottis trapeziformis* and the cordate-shaped *C. valida* flowers, exhibiting the same semiochemical chiloglottone 1, across experiments. Wasps responding are the total number of wasps that responded to an orchid, including all approaches, landings and attempted copulations

Pollinator	<i>N. cryptoides</i> (pollinates <i>C. trap.</i>)				<i>N. monticola</i> (pollinates <i>C. val.</i>)			
	<i>C. trap.</i> Intact labellum	<i>C. val.</i> Intact labellum	<i>C. trap.</i> Shortened labellum	<i>C. trap.</i> Intact labellum	<i>C. trap.</i> Intact labellum	<i>C. val.</i> Intact labellum	<i>C. val.</i> Shortened labellum	<i>C. val.</i> Intact labellum
Wasps responding	200	307	168	155	389	218	264	214
Responding wasps that landed (%)	55.0	48.9	50.0	45.2	64.8	41.3	65.9	59.8
On labellum (%)	44.5	35.3	50.0	63.1	14.7	51.1	18.5	29.1
On dorsal sepal (%)	7.3	44.7	7.1	6.0	2.8	14.4	7.5	5.5
On lateral sepal (%)	1.8	17.3	2.9	3.6	0.4	1.1	16.2	10.2
On pedicel (%)	46.4	2.7	40.0	27.4	31.9	4.4	27.4	21.3
On ground (%)	0.0	0.0	0.0	0.0	50.2	28.9	30.1	33.9
Landing wasps that copulated (%)	40.0	7.3	27.4	30.0	1.2	15.6	10.9	10.2
Forward orientation (%)	81.8	54.5	9.5	60.9				
Reverse orientation (%)					0.0	61.5	31.6	23.1

For each pollinator species, similar shaded columns indicate a direct choice test (i.e. flowers presented at the same time at the same place). Although formal statistical tests between choice tests are not possible (as conducted at different times in different sites), responses can be compared across columns using percentages as this removes the variability in the number of responses.

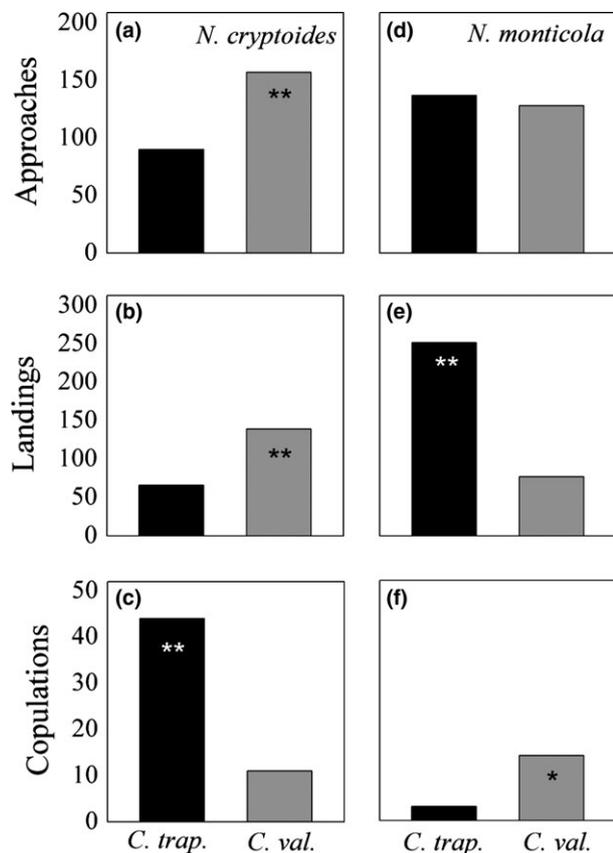


Fig. 3. Number of mutually exclusive (a, d) approaches, (b, e) landings or (c, f) copulation attempts on *Chiloglottis trapeziformis* and *C. valida* by *N. cryptoides* (primary pollinator of *C. trapeziformis*) and *N. monticola* (primary pollinator of *C. valida*), respectively. Significant differences between the expected and observed numbers are indicated with * $P < 0.05$ and ** $P < 0.005$.

between the callus and labellum tip (Callus-Tip), will be equal to or less than the total pollinator length (MaleTotalL) and pollinator abdomen length (MaleAbdomenL), respectively. Evidence against the importance of mechanical fit might be obtained from the opposite pattern (e.g. LabellumL and Callus-Tip being longer than MaleTotalL and MaleAbdomenL). By contrast, for *C. valida*, whose male pollinators exhibit the reverse orientation while probing the proximal point of the calli with their slightly curved abdomen during copulation attempts (Fig. 1d), we predicted that calli length (CalliL) will be equal to or less than the male abdomen length. Our findings broadly matched these predictions (Fig. 2, Table 2).

For fit with the reproductive structures, we investigated the gap between the calli and the pollinia, leading to the expectation of an overlap in size between the Calli-Pollinia distance and height of the male wasp thorax. This was found for both species (Fig. 2). Another potentially important trait for fit with reproductive structures is column height. In this case, we predicted that column height would be larger in *C. valida* than in *C. trapeziformis*, given that the reverse orientation requires male wasp alignment with the column during pollination. In *C. valida*, we thus

expected an overlap in size between column height and *N. monticola* male total length. At first, it appeared that no obvious match was found (Fig. 2, Table 2). However, because *N. monticola* grips the labellum and not the column (see Fig. 1d), pollination in the reverse orientation seems to only require that the column is not shorter than the male wasp. By contrast, in *C. trapeziformis*, column height is much shorter than *N. cryptoides* male total length (Fig. 2a), suggesting pollination via the reverse orientation in this species might not even be possible (and has not been observed by us). This observation highlights the fact that both attempted copulation and the correct orientation for pollen removal are required for the successful pollination of the two morphologically contrasting types of *Chiloglottis* (see Video S1). Thus, the predicted direction of the differences between the two orchids is supported, even though we did not always find a precise match in size.

Under the mimicry hypothesis, we investigated two orchid calli traits of interest: length and colour. Note that while the size measurements differed between the two orchids, because in *C. trapeziformis* the calli are fused into the callus rather than composed of distinct calli, the matches with female wasp traits were predicted to be common to both orchids. Total female size was larger than orchid calli in both species, but with some overlap between individual *C. valida* and *N. monticola* females (Fig. 2b). The combined thorax and head length of *N. cryptoides* females (FemaleThorax+HeadL), however, overlapped in size with callus length in *C. trapeziformis*. Taking into account the copulatory probing with the distal labellum tip in *C. trapeziformis*, this suggests that the Callus-Tip length mimics the female abdomen, while the callus length is mimicking the female thorax and head. These traits also overlapped in size, as did the combined callus length and Callus-Tip length with the total *N. cryptoides* female length (Table 2, Fig. 2), further providing evidence for female morphological mimicry in *C. trapeziformis*.

Spectral reflectance analysed in hymenopteran visual space revealed that female wasp abdomen and calli were very close to each other in the colour hexagon (Fig. S5), with colour contrasts of 0.11 and 0.05 for *C. trapeziformis* callus/*N. cryptoides* females and *C. valida* calli/*N. monticola* females, respectively. The threshold for colour discrimination by hymenopterans has been calculated as 0.06 (Dyer & Chittka 2004), indicating great similarity between the labellum calli and female wasps. Both calli and females also fell within the very centre of the hexagon and are thus both perceived as uncoloured, or achromatic by the hymenopteran pollinators (Chittka *et al.* 1994).

Discussion

DOES MORPHOLOGY MATTER IN SEXUAL DECEPTION?

Notwithstanding the well-established primary role of semiochemicals for securing long-range pollinator attraction in sexually deceptive orchids (Schiestl *et al.* 1999,

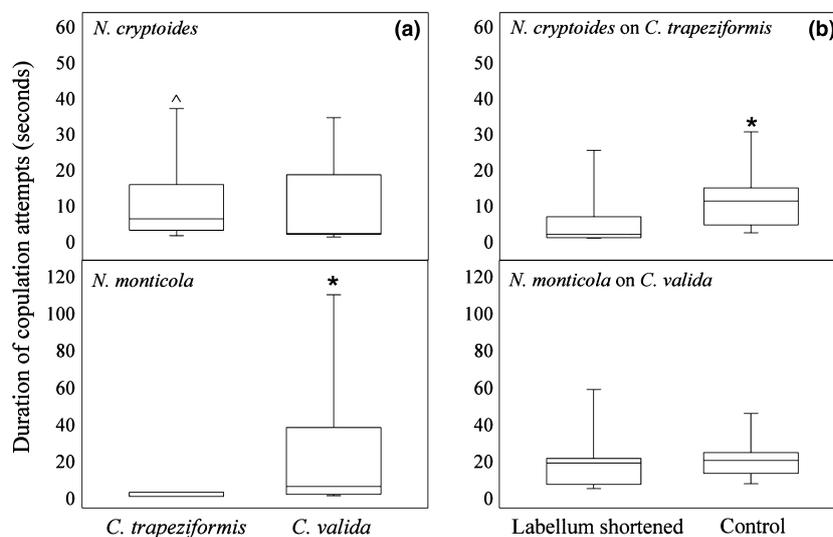


Fig. 4. (a) Duration of copulation attempts on *Chiloglottis trapeziformis* and *C. valida* by *N. cryptoides* (primary pollinator of *C. trapeziformis*) and *N. monticola* (primary pollinator of *C. valida*). (b) Duration of copulation attempts on flowers with experimentally shortened labella and control flowers with intact labella. Medians are indicated by lines, and *denotes significant differences at $P < 0.001$ and ^ near significant differences at $P < 0.1$.

Table 2. Summary of *a priori* predictions for trait matches between flowers and wasps, taking into account the different pollinator orientations

Floral trait	Expected wasp match	Observed wasp match
<i>Chiloglottis trapeziformis</i> (Forward orientation)		
Mechanical fit		
LabellumL*	≤MaleTotalL	<MaleTotalL
Callus-Tip*	≤MaleAbdomenL	<MaleAbdomenL
Callus-Pollinia†	MaleThoraxH	MaleThoraxH
Female mimicry		
CallusL	FemaleThorax+ HeadL	FemaleThorax+ HeadL
Callus-Tip	FemaleAbdomenL	FemaleAbdomenL
Callus-Tip+CallusL	FemaleTotalL	FemaleTotalL
CallusColour	FemaleColour	FemaleColour
<i>C. valida</i> (Reverse orientation)		
Mechanical fit		
CalliL*	≤MaleAbdomenL	MaleAbdomenL
Calli-Pollinia†	MaleThoraxH	MaleThoraxH
ColumnH†	MaleTotalL	>MaleTotalL
Female mimicry		
CalliL	FemaleTotalL	<FemaleTotalL
CalliColour	FemaleColour	FemaleColour

L, length; H, height.

For Mechanical fit, *indicates fit to the labellum during attempted copulation, †indicates fit to the floral reproductive structures.

2003; Ayasse, Stökl & Francke 2011; Bohman *et al.* 2014), we present here compelling new experimental evidence that morphology also matters. Both *C. trapeziformis* and *C. valida* elicited more and longer copulation attempts from their primary pollinator despite using the same chiloglottone for pollinator attraction. This finding suggests that the two orchids are likely morphologically adapted to their primary pollinators. Although previous studies have also reported higher rates of attempted copulation for primary pollinators in congeneric sexually deceptive orchids (Peakall *et al.* 2010; Whitehead & Peakall

2014), these studies always focussed on taxa with divergent odour chemistry.

While it is potentially possible that *C. trapeziformis* and *C. valida* differ in their rate of chiloglottone 1 emission, chiloglottones are emitted in such low amounts that unfortunately neither standard headspace nor SPME (solid-phase microextraction) techniques can detect them (Falara *et al.* 2013; Amarasinghe *et al.* 2015). However, even if systematic variation in chiloglottone emission rates did exist between the two orchids, it cannot explain the consistent pattern of behavioural responses we uncovered. These include reciprocally stronger responses to the orchid that each wasp pollinates, and differences in landing place, orientation and probing point. The relative percentages of wasps that landed on the two species were also broadly overlapping, suggesting that these fundamental differences in sexual responses to *C. trapeziformis* and *C. valida* are unlikely to be chemical.

This is further supported by results from our labellum manipulation experiments, which avoided any systematic emission rate differences by manipulating non-chiloglottone-producing tissues within the same species. In these experiments, we thus retained common chemistry while specifically manipulating labellum size. Consistent with our *a priori* expectation that *C. trapeziformis* labellum size overlaps with pollinator size (and the correlated Callus-Tip overlaps with MaleAbdomenL; see Table 2), manipulations in this orchid yielded a significant reduction in the duration of attempted copulation (Fig. 4b). Conversely, there was no significant change in *C. valida* (Fig. 4b), consistent with the *a priori* expectation that labellum size is less important given the reverse orientation of the pollinator.

Seed set is strongly pollinator-limited in both *C. valida* (Peakall & Whitehead 2014) and *C. trapeziformis* (Schiestl 2004). Under this norm of pollinator limitation, *Chiloglottis* flowers with morphological traits that elicit more frequent and prolonged copulation attempts are predicted to exhibit higher reproductive success in the field, as found in other sexually deceptive species (Gaskett, Winnick &

Herberstein 2008; Ellis & Johnson 2010). Conducting field experiments to directly test this prediction in *Chiloglottis*, while not impossible, would be difficult as extensive replications across multiple populations would be required. With these constraints in mind, we suggest that the frequency and duration of attempted copulation measured in this study likely provides a reasonable proxy for plant reproductive fitness.

Furthermore, in the case of diamond-shaped *Chiloglottis*, our experimental morphological manipulations suggest that flowers with maladapted morphology will be at a significant fitness disadvantage under pollinator limitation, indicating the likelihood of pollinator-mediated selection in the wild for morphological traits such as labellum size. Thus, we conclude that morphology does indeed matter in sexual deception.

IS THERE MORPHOLOGICAL EVIDENCE FOR MECHANICAL FIT AND/OR FEMALE MIMICRY?

Our strategic focal orchid species were chosen both because they represented contrasting floral morphologies and because they were broadly representative of the two floral shapes across the genus. *C. valida* is larger and showed little overlap in size with *C. trapeziformis* in any traits (Fig. 2). Similarly, there was no overlap in multivariate morphometric space between the two orchids (Fig. S2b). By contrast, the differences in size between *N. monticola* and *N. cryptoides* were smaller (Fig. 2), with considerable overlap in multivariate space (Fig. S3b), although trait means differed significantly for most measured traits (Table S2).

From the outset of the study, we knew that pollinator orientation differed between the two focal species and that these orientation differences hold true across the genus for the two flower morphologies (Fig. S1). In the light of these behavioural differences, we predicted there would be two labellum traits for mechanical fit to copulating males in *C. trapeziformis*, length (LabellumL) and callus to tip length (Callus-Tip), which would not be relevant in *C. valida* (Table 2). As expected, these two traits showed overlap in size with male pollinator total length (MaleTotalL) and abdomen length (MaleAbdomenL) in *C. trapeziformis* (Fig. 2). The labellum-shortening experiments further revealed that the duration of copulation attempts on *C. trapeziformis* was severely disrupted (Fig. 4b), likely due to the breakup of mechanical fit between Callus-Tip and male abdomen length.

In the light of this interesting finding, we investigated the correlation between mean Callus-Tip length and mean male pollinator abdomen length for all *Chiloglottis* species and pollinators measured in this study (see Table S3). A significantly positive relationship was found for diamond-shaped species (Pearson's product-moment correlation: $r = 0.839$, $P = 0.037$, Fig S6), indicating that mechanical fit between these traits may be common in all diamond-shaped *Chiloglottis*.

By contrast, in the cordate-shaped *C. valida* whose pollinator attempts copulation in the reverse orientation, we found no match between pollinator and labellum size. Nor did experimentally shortening the labellum length alter pollinator behaviour. We also found no relationship between the mean Calli-Tip length and mean male pollinator abdomen length for cordate-shaped species ($r = -0.241$, $P = 0.603$, Fig. S6). Other mechanical fit traits may also be associated with the fit between the male pollinator and the floral reproductive structures where pollination occurs. One such floral trait was the distance between the calli and the pollinia. As predicted, we found overlapping sizes between pollinator thorax height and Calli-Pollinia distance (Fig. 2) in both *C. trapeziformis* and *C. valida*.

We defined female mimicry as floral traits such as size and colour that match the female of the pollinator. Here, we explicitly excluded sex pheromone mimicry, which is already clearly established in our study system (Schiestl *et al.* 2003; Schiestl & Peakall 2005). The role of such morphological traits is to enhance both attraction and the intensity of sexual responses of the male pollinator beyond that achieved by chemical mimicry alone. The implicit assumption is that such traits contribute to the false perception by the male that he has found a female. Directly testing this assumption is challenging. Furthermore, when specific floral structures, such as the calli, are both the known source of the chemical mimicry and putative morphological mimics, experimental floral manipulations may not be feasible.

Mimicry traits must enhance the reproductive fitness of the plant; thus, our fitness proxy of attempted copulation holds true for both mechanical fit and mimicry traits. We postulated in the introduction that the sexual dimorphism in our wasp study system of large winged males vs. small wingless females might provide a unique opportunity to help tease apart traits for mechanical fit from traits for female mimicry. In *C. trapeziformis*, all of our *a priori* predictions for trait matches with female wasp morphology were confirmed. In *C. valida*, we also found overlapping sizes between orchid structures and female components consistent with the mimicry hypothesis.

Additional evidence for mimicry was found in the colour similarity of calli and female abdomen. The spectral reflectance of both fell within the very centre of the colour hexagon representing hymenopteran vision (Chittka 1992), effectively making them both achromatic (Chittka *et al.* 1994). Interestingly, a recent experimental study revealed that achromatic flowers might exclude hymenopteran visitors because they are difficult to detect (Lunau *et al.* 2011). In this case, both labellum calli and female wasps may thus be similarly cryptic in colour.

MORPHOLOGICAL ADAPTATION IN SEXUAL DECEPTION BEYOND CHILOGLOTTIS?

Other systems also provide evidence for morphological adaptation in sexual deception. For example, in the Australian fungus gnat pollinated sexually deceptive orchid,

Pterostylis sanguinea, experimental rotation of the labelum generated a significant reduction in the copulation attempts of the pollinator (Phillips *et al.* 2014). In the sexually deceptive African daisy, *Gorteria diffusa*, floral morphology, rather than semiochemicals, is the main form of attraction (De Jager & Ellis 2012). The male bee fly pollinators of this species are attracted to complex spots on *G. diffusa*'s ray florets and grip these spots when exhibiting copulation behaviour (Ellis & Johnson 2010), much like male wasps grip calli on *Chiloglottis* labella. Floral forms of *G. diffusa* differing in deceptive spot morphology also elicit different behaviours from their shared pollinator, including where they land and the likelihood and duration of attempted copulation (De Jager & Ellis 2013), confirming the potential for deceptive morphology to strongly influence pollinator behaviour (De Jager & Ellis 2014b).

In conclusion, our results indicate that there is considerable scope for pollinator-mediated selection to operate on floral morphology in sexually deceptive plants. We found evidence for both mechanical fit to male pollinators and mimicry of female wasps. We also discovered that pollinator behavioural differences, such as orientation during copulations attempts, are correlated with contrasting floral types, suggesting a key role for pollinator behaviour in shaping floral morphology. Thus, despite the critical and long-distance role of chemical cues in securing pollinator behaviour, we offer a new and compelling case for the importance of floral morphology in sexual deception. We recommend that future studies experimentally investigate the role of pollinator behaviour as a driver of floral morphological trait variation that is likely under pollinator-mediated selection.

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Author's contributions

MDJ and RP designed the study, MDJ carried out data collection and analyses. MDJ and RP wrote the paper. We have no competing interests to declare. Animals were only observed, and this study conforms to the legal requirements of Australia.

Data accessibility

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.bm29v> (De Jager & Peakall 2015).

References

- Amarasinghe, R., Poldy, J., Matsuba, Y., Barrow, R.A., Hemmi, J.M., Pichersky, E. *et al.* (2015) UV-B light contributes directly to the synthesis of chiloglottone floral volatiles. *Annals of Botany*, **115**, 693–703.
- Amiet, F., Herrmann, M., Müller, A. & Neumeyer, R. (2010) *Apidae 6 - Andrena, Melitturga, Panurginus, Panurgus*. *Fauna Helvetica* 26. CSCF & SEG: Neuchâtel, Switzerland.
- Anderson, B. & Johnson, S.D. (2008) The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution*, **62**, 220–225.
- Ayasse, M., Stöckl, J. & Francke, W. (2011) Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry*, **72**, 1667–1677.
- Bohman, B., Phillips, R.D., Menz, M.H.M., Berntsson, B.W., Flematti, G.R., Barrow, R.A. *et al.* (2014) Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception. *New Phytologist*, **203**, 939–952.
- Bower, C. (1996) Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of *Chiloglottis* (Orchidaceae: Caladeniinae). *Australian Journal of Botany*, **44**, 15.
- Bradshaw, H.D. & Schemske, D.W. (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*, **426**, 176–178.
- Chittka, L. (1992) The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *Journal of Comparative Physiology A*, **170**, 533–543.
- Chittka, L., Shmida, A., Troje, N. & Menzel, R. (1994) Ultraviolet as a component of flower reflections, and the colour perception of hymenoptera. *Vision Research*, **34**, 1489–1508.
- De Jager, M.L. & Ellis, A.G. (2012) Gender-specific pollinator preference for floral traits. *Functional Ecology*, **26**, 1197–1204.
- De Jager, M.L. & Ellis, A.G. (2013) The influence of pollinator phylogeography and mate preference on floral divergence in a sexually deceptive daisy. *Evolution*, **67**, 1706–1714.
- De Jager, M.L. & Ellis, A.G. (2014a) Floral polymorphism and the fitness implications of attracting pollinating and florivorous insects. *Annals of Botany*, **113**, 213–222.
- De Jager, M.L. & Ellis, A.G. (2014b) Costs of deception and learned resistance in deceptive interactions. *Proceedings. Biological sciences/The Royal Society*, **281**, 20132861.
- De Jager, M.L. & Peakall, R. (2015) Data from [Does morphology matter? An explicit assessment of floral morphology in sexual deception]. *Dryad Digital Repository*, <http://dx.doi.org/10.5061/dryad.bm29v>.
- Dyer, A. & Chittka, L. (2004) Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *Journal of Comparative Physiology A*, **190**, 105–114.
- Ellis, A.G. & Johnson, S.D. (2010) Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *The American Naturalist*, **176**, E143–E151.
- Falara, V., Amarasinghe, R., Poldy, J., Pichersky, E., Barrow, R.A. & Peakall, R. (2013) The production of a key floral volatile is dependent on UV light in a sexually deceptive orchid. *Annals of Botany*, **111**, 21–30.
- Franke, S., Ibarra, F., Schulz, C.M., Twele, R., Poldy, J., Barrow, R.A. *et al.* (2009) The discovery as a new class of natural products. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 8877–8882.
- Gaskett, A.C. & Herberstein, M.E. (2010) Colour mimicry and sexual deception by Tongue orchids (*Cryptostylis*). *Die Naturwissenschaften*, **97**, 97–102.
- Gaskett, A.C., Winnick, C.G. & Herberstein, M.E. (2008) Orchid sexual deceit provokes ejaculation. *The American Naturalist*, **171**, E206–E212.
- Griffiths, K.E., Trueman, J.W.H., Brown, G.R. & Peakall, R. (2011) Molecular genetic analysis and ecological evidence reveals multiple cryptic species among thynnine wasp pollinators of sexually deceptive orchids. *Molecular Phylogenetics and Evolution*, **59**, 195–205.
- Kullenberg, B. (1961) Studies in *Ophrys* pollination. *Zoologiska Bidrag från Uppsala*, **34**, 1–340.
- Lunau, K., Papiorek, S., Eltz, T. & Sazima, M. (2011) Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *The Journal of Experimental Biology*, **214**, 1607–1612.
- Mant, J., Peakall, R. & Weston, P.H. (2005) Specific pollinator attraction and the diversification of sexually deceptive *Chiloglottis* (Orchidaceae). *Plant Systematics and Evolution*, **253**, 185–200.

- Muchhala, N. (2007) Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *The American Naturalist*, **169**, 494–504.
- Newman, E., Anderson, B. & Johnson, S.D. (2012) Flower colour adaptation in a mimetic orchid. *Proceedings. Biological sciences/The Royal Society*, **279**, 2309–2313.
- Paulus, H.F. (2006) Deceived males – Pollination biology of the Mediterranean orchid genus *Ophrys* (Orchidaceae). *Journal Europäischer Orchideen*, **38**, 303–351.
- Paulus, H.F. & Gack, C. (1990) Pollination of *Ophrys* (Orchidaceae) in Cyprus. *Plant Systematics and Evolution*, **169**, 177–207.
- Pauw, A., Stofberg, J. & Waterman, R.J. (2009) Flies and flowers in Darwin's race. *Evolution*, **63**, 268–279.
- Peakall, R. (1990) Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. *Functional Ecology*, **4**, 159–167.
- Peakall, R. & Handel, S.N. (1993) Pollinators discriminate among floral heights of a sexually deceptive orchid: implications for selection. *Evolution*, **47**, 1681–1687.
- Peakall, R. & Whitehead, M.R. (2014) Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids. *Annals of Botany*, **113**, 341–355.
- Peakall, R., Bower, C.C., Logan, A.E. & Nicol, H.I. (1997) Confirmation of the hybrid origin of *Chiloglottis pescottiana* (Orchidaceae:Diurideae). 1. Genetic and morphometric evidence. *Australian Journal of Botany*, **45**, 839–855.
- Peakall, R., Jones, L., Bower, C.C. & Mackey, B.G. (2002) Bioclimatic assessment of the geographic and climatic limits to hybridisation in a sexually deceptive orchid system. *Australian Journal of Botany*, **50**, 21–30.
- Peakall, R., Ebert, D., Poldy, J., Barrow, R.A., Francke, W., Bower, C.C. *et al.* (2010) Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. *New Phytologist*, **188**, 437–450.
- Peter, C.I. & Johnson, S.D. (2008) Mimics and magnets: the importance of color and ecological facilitation in floral deception. *Ecology*, **89**, 1583–1595.
- Phillips, R.D., Scaccabarozzi, D., Retter, B.A., Hayes, C., Brown, G.R., Dixon, K.W. *et al.* (2014) Caught in the act: pollination of sexually deceptive trap-flowers by fungus gnats in *Pterostylis* (Orchidaceae). *Annals of Botany*, **113**, 629–641.
- Ridsdill Smith, T.J. (1970) The behaviour of *Hemithynnus hyalznatus* (Hymenoptera: Tiphidae), with notes on some other thynninae. *Journal of the Australian Entomological Society*, **9**, 196–208.
- Schiestl, F.P. (2004) Floral evolution and pollinator mate choice in a sexually deceptive orchid. *Journal of Evolutionary Biology*, **17**, 67–75.
- Schiestl, F.P. & Peakall, R. (2005) Two orchids attract different pollinators with the same floral odour compound: ecological and evolutionary implications. *Functional Ecology*, **19**, 674–680.
- Schiestl, F.P., Ayasse, M., Paulus, H.F., Lofstedt, C., Hansson, B.S., Ibarra, F. *et al.* (1999) Orchid pollination by sexual swindle. *Nature*, **399**, 421–423.
- Schiestl, F.P., Peakall, R., Mant, J.G., Ibarra, F., Schulz, C., Franke, S. *et al.* (2003) The chemistry of sexual deception in an orchid-wasp pollination system. *Science*, **302**, 437–438.
- Shuttleworth, A. & Johnson, S.D. (2010) The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems. *Proceedings. Biological sciences/The Royal Society*, **277**, 2811–2819.
- Steiner, K.E. & Whitehead, V.B. (1990) Pollinator adaptation to oil-secreting flowers-*Rediviva* and *Diascia*. *Evolution*, **44**, 1701–1707.
- Streinzer, M., Paulus, H.F. & Spaethe, J. (2009) Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator. *The Journal of Experimental Biology*, **212**, 1365–1370.
- Whitehead, M.R. & Peakall, R. (2012) Short-term but not long-term patch avoidance in an orchid-pollinating solitary wasp. *Behavioral Ecology*, **24**, 162–168.
- Whitehead, M.R. & Peakall, R. (2014) Pollinator specificity drives strong pre-pollination reproductive isolation in sympatric sexually deceptive orchids. *Evolution*, **68**, 1561–1575.
- Xu, S., Schlüter, P.M., Scopece, G., Breitkopf, H., Gross, K., Cozzolino, S. *et al.* (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution*, **65**, 2606–2620.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Table S1. Table with the measured floral traits of *C. trapeziformis* and *C. valida*.

Table S2. Table with the measured traits of *N. cryptoides* and *N. monticola*.

Table S3. Table with the orchid and wasp species investigated in this study.

Figure S1. Figure showing the Forward and Reverse orientations of pollinators on diamond- and cordate-shaped *Chiloglottis* species, respectively.

Figure S2. Figure showing the PCA plots of all measured *Chiloglottis* species.

Figure S3. Figure showing the PCA plots of all measured pollinator species.

Figure S4. Figure showing the extreme sexual dimorphism found within thynnine wasps.

Figure S5. Figure showing the position of *C. trapeziformis* and *C. valida* labellum calli, as well as the females of their respective pollinators, in hymenopteran visual space.

Figure S6. Figure showing the correlation between Calli-Tip distance and male pollinator abdomen length for diamond- and cordate-shaped *Chiloglottis* species.

Video S1. Video showing the behaviour of sexually attracted male thynnine wasps on diamond- and cordate-shaped *Chiloglottis*.