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# Experimental examination of pollinator-mediated selection in a sexually deceptive orchid

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• **Background and Aims** Selection exerted by pollinators on flowers is predicted to occur along two distinct axes. While pollinator attraction to flowers is governed by pollinator preferences, pollen transfer efficiency is mediated by the mechanical fit of pollinators to flower morphology. Although pollinator attraction in sexually deceptive orchids is typically underpinned by floral odour, morphological traits are expected to play a vital role in mechanical fit during floral contact with pollinators.
• **Methods**  Here we utilize a comprehensive and novel procedure to test for pollinator-mediated selection through mechanical fit with the flower labellum in the orchid *Chiloglottis trapeziformis*. This approach combines detailed pollinator observations related to plant reproductive fitness with complementary experimental manipulation and phenotypic selection analysis.
• **Key Results** Experiments with virgin flowers revealed that pollen removal occurs only during vigorous pseudocopulation. This behaviour involves male wasps that grasp the insectiform callus structure on the labellum while probing the labellum tip in a forward orientation. Both orientation and duration of pseudocopulation were significant predictors of pollen removal, confirming a direct relationship between pollinator behaviour and plant fitness. Controlled floral manipulation that either shortened or elongated the distance between the callus and the labellum tip detected no change in pollinator attraction. The duration of pseudocopulation, however, was significantly reduced on flowers with shortened or elongated callus–tip distances, consistent with stabilizing selection. Phenotypic selection analysis confirmed this prediction in natural populations by uncovering evidence for stabilizing selection on the distance between the callus and the labellum tip.
• **Conclusions** Our experimental manipulations and selection analysis in natural populations thus demonstrate stabilizing selection on the distance from the callus to the labellum tip, and illustrate the utility of employing multiple approaches to confirm selection exerted by pollinators on floral form.

**Key words:** *Chiloglottis trapeziformis*, floral manipulation, insect behaviour, mechanical fit, mimicry, pollination, pollinator-mediated selection, pseudocopulation, sexual deception, stabilizing selection.

## INTRODUCTION

Pollinators play a crucial role in floral evolution and diversification (Grimaldi, 1999). Evidence for pollinator-mediated selection on floral form has been reported across the angiosperms (Harder and Johnson, 2009), and can be broadly characterized into two main types. Firstly, selection on attractive floral traits can be exerted via the sensory system of pollinators in terms of their ability to detect and prefer a given floral colour (Schemske and Bradshaw, 1999; Newman *et al.*, 2012), floral scent (Shuttleworth and Johnson, 2010; van der Niet *et al.*, 2011) and floral size (Galen, 1989; Schemske and Agren, 1995). Secondly, pollinator morphology can exert strong selection on floral structures via mechanical fit, as reported for flower tubes (Anderson and Johnson, 2008; Pauw *et al.*, 2009) and flower throat length (Cosacov *et al.*, 2014). Although not expected to affect pollinator attraction, mechanical fit between pollinator and floral morphology promotes plant fitness by enhancing the frequency and efficiency of pollen transfer (Newman *et al.*,

2015), making it a regular target of pollinator-mediated selection in specialized plants (Rosas-Guerrero *et al.*, 2011).

Some of the most compelling evidence for the importance of pollinator preferences comes from highly specialized, rewardless plants that mimic sympatric flowers. In these deceptive mimics, flower colour has repeatedly been shown to be a key trait under strong selection by pollinators (Jersáková *et al.*, 2012; Newman *et al.*, 2012). Extensive support for pollinator-mediated selection on floral scent comes from another group of deceptive plants that exclusively attract male pollinators by mimicking female insects. These sexually deceptive (SD) plants are predominantly found in the Orchidaceae (Bohman *et al.*, 2016). Chemical investigations in the orchid genera *Ophrys* (Schiestl *et al.*, 1999), *Chiloglottis* (Schiestl *et al.*, 2003; Peakall *et al.*, 2010*), Drakaea* (Bohman *et al.*, 2014) and *Caladenia* (Bohman *et al.*, 2017; Xu *et al.*, 2017) confirm that pollinator attraction is achieved with the same floral compound(s) as found in the sex pheromone of the pollinator's female.

Although floral scent mimicry is a key factor underpinning pollinator attraction and speciation in SD orchids (Peakall *et al.*, 2010; Ayasse *et al.*, 2011; Peakall and Whitehead, 2014; Bohman *et al.*, 2016), visual and morphological cues are likely also important in sexual mimicry (Kullenberg, 1961; Peakall, 1990; Gaskett, 2011). Comparable experimental evidence for this hypothesis, however, is largely lacking. For example, a phenotypic selection analysis on the South American orchid *Geoblasta pennicillata* found no evidence of increased fitness for flowers with labella that more accurately mimic the shape of its pollinator's female (Benitez-Vieyra *et al.*, 2009). Similarly, an analysis of flower shape across four pollinator-sharing *Cryptostylis* species in Australia (Gaskett, 2012) failed to match orchid shapes with the female insects they putatively mimic. Size and colour matches between female insects and mimicking floral structures, however, have been found in closely related *Chiloglottis* orchids (de Jager and Peakall, 2016).

The paucity of experimental evidence for morphological mimicry in SD orchids may suggest limited pollinator-mediated selection via pollinator preference on floral traits. In contrast, pollinator-mediated selection via mechanical fit to pollinator morphology is expected in these systems, because SD orchids are typically pollinated by a single pollinator species (Peakall *et al.*, 2010; Bohman *et al.*, 2016; Phillips and Peakall, 2017). The evolution of a tight match between an SD orchid's flower morphology and the size and shape of its male pollinator, which governs pollen transfer, is thus expected. Surprisingly few studies on SD orchids have investigated mechanical fit with male pollinators. A correlation between labellum length and male pollinator body length in *Ophrys* has previously been reported (Paulus, 2006) for species where males orientate in the forward position (head pollination), as well as the reverse position (abdominal pollination). A recent experimental study in *Ophrys leochroma* also documented a reduction in the rate of attempted copulation (pseudocopulation) by pollinators when floral parts that males usually grip during visits are removed (Rakosy *et al.*, 2017), indicating that mechanical fit with the labellum is likely important in governing pollinator behaviour in SD orchids.

Sexually deceptive *Chiloglottis* in Australia, which are pollinated by male thynnine wasps, are prime candidates for experimental investigations of pollinator-mediated selection. Pollinators in this system readily attempt to mate with featureless, plastic beads spiked with synthetic copies of the relevant flower's mimetic attractive compounds (Schiestl *et al.*, 2003; Peakall *et al.*, 2010; Peakall and Whitehead, 2014). Because pollinators are sufficiently deceived by olfactory traits, we predict that variation in flower morphology within and among *Chiloglottis* species is under stronger pollinator-mediated selection for mechanical fit than for female mimicry. Nonetheless, experimental evidence for mimicry has recently been found in two morphologically distinct *Chiloglottis* species that employ the same attractive mimetic compound (chiloglottone 1) to attract their primary wasp pollinators (de Jager and Peakall, 2016). These pollinators exhibit contrasting orientations on the flowers (forward versus reverse) during pseudocopulation that match the contrasting floral morphologies of the two orchids. This allowed *a priori* predictions of trait matches between female insects and the floral traits that mimic them, which were confirmed in both species, but more pronounced in *Chiloglottis trapeziformis* (de Jager and Peakall, 2016).

*Chiloglottis trapeziformis* exhibits a small diamond-shaped labellum with a dark insectiform callus structure formed by the



FIG. 1. The male pollinator, *Neozeleboria cryptoides*, exhibiting forward orientation during pseudocopulation on the labellum of *Chiloglottis trapeziformis*. (A) Pollinators typically first contact the stigma, indicated by the white arrow, before (B) dislodging a pollinium. Scale bar = 10 mm. Photographs: Rod Peakall.

fusion of calli that the pollinator grips during pseudocopulation (Fig. 1). This process involves abdominal probing of the distal labellum edge, resulting in the deposition of pollinia on the dorsal thorax while the pollinator is in the forward orientation (Peakall *et al.*, 2010). When the labellum is experimentally shortened, the duration of pseudocopulation is significantly reduced (de Jager and Peakall, 2016). This reduction will likely decrease pollinia removal and deposition, which is typically low in this and many other SD orchids (Weinstein *et al.*, 2016). These findings indicate the potential for strong pollinator-mediated selection in *C. trapeziformis*. In this paper, we comprehensively investigate the hypothesis that pollinators exert selection on *C. trapeziformis* via mechanical fit with its labellum by combining novel pollinator observations, experimental floral manipulation and phenotypic selection analysis in natural populations.

## MATERIALS AND METHODS

For all experiments, fresh flowers were collected from Black Mountain Creek (35°16′37.6″ S, 149°6′6.9″ E), Mulloon Farm (35°14′23.9″ S, 149°36′35.0″ E) and the Australian Botanical Gardens (35°16′36.14″ S, 149°6′35.67″ E).

### *Linking pollinator behaviour and plant fitness*

To confirm that pseudocopulation is a robust proxy for pollination success, we secured virgin *Chiloglottis trapeziformis* flowers to wooden skewers 15 cm above the ground, before exposing them to the pollinator community at Molonglo Gorge (35°16′30.12″ S, 149°5′19.08″ E) during 2015. *Chiloglottis trapeziformis* does not occur at this site, ensuring sufficient pollinator activity for experimentation, since areas with resident *C. trapeziformis* often have low visitation due to learned avoidance by pollinators (Whitehead and Peakall, 2013). We observed the behaviour of all visitors in the field, and recorded the orientation and duration of mating attempts by pseudocopulating *Neozeleboria cryptoides* male wasps on *C. trapeziformis* flowers with a stopwatch. Orientation was scored as the position the pollinator was in when the pollinium became attached to its body, because males exhibiting pseudocopulation for very long periods may move around on the labellum. For every visit, we also recorded whether a pollinium was removed, noting the precise placement of the pollinium on the pollinator's body.

Although it would be ideal to record the above data for pollen deposition as well, this is extremely difficult under experimental and natural conditions for two reasons: the rapid decline in response to experimentally presented flowers at a given location (Whitehead and Peakall, 2013) and the extremely low incidence of resident males carrying *Chiloglottis* pollinia (Bower, 1996; M. L. de Jager, pers. obs.). Nevertheless, pollinator visits resulting in pollinia removal are also expected to contribute to pollen deposition, if the visiting male is carrying *Chiloglottis* pollinia. This is because in *C. trapeziformis* the stigma is located directly below the anther (Fig. 1) and contact with the stigma normally occurs before contact with the anther during pseudocopulation. Indeed, as in other Australian orchids whose pollinia lack a connecting stalk and viscidium, such as the sexually deceptive *Leporella fimbriata*, pollinator contact

with the stigma results in the transfer of stigmatic secretions to the insect's thorax that act as a glue to assist pollinia removal and attachment (Peakall *et al.*, 1987, 1990). Therefore, we treat pollinia removal as a reasonable proxy for pollen deposition. We employed a generalized linear model with a binomial distribution and logit link function to model the effects of orientation (forward versus side), duration of pseudocopulation, and their interaction on pollinia removal.

### *Linking floral manipulation and pollinator-mediated selection*

A key trait proven to be mechanically important during the interaction between *C. trapeziformis* and its pollinator is the distance between the callus and labellum tip (de Jager and Peakall, 2016). Directly manipulating this distance by moving the callus is not possible, because the callus is the site where the pollinator-attracting olfactory compound chiloglottone 1 is produced in *C. trapeziformis* (Falara *et al.*, 2013). Instead, we can manipulate the labellum length, which automatically shortens or elongates the callus–tip distance. These two traits are strongly correlated in natural populations (Supplementary Data Table S1). To explore the effect of shortening the labellum on pollinator behaviour, de Jager and Peakall (2016) cut off a distal portion of the labellum just posterior to the callus, which yielded manipulated flowers with a labellum length of 5.0–5.5 mm (Fig. 2).

To elongate the labellum of *C. trapeziformis* beyond its natural range, we attached the distal portion of a donor flower's labellum to the abaxial side of the recipient flower's labellum with a thin layer of odourless instant-drying superglue. Each donor labellum was selected to match the recipient labellum in colour, as this trait can vary among individuals within populations. Any experimentally elongated flowers that were not properly secured or aligned were discarded. This procedure produced manipulated flowers with a labellum length of 10.0–10.5 mm. In contrast, the mean labellum length of natural *C. trapeziformis* flowers is 7.3 ± 0.5 mm (*n* = 16). The mean length of its pollinator, *N. cryptoides*, by comparison is 9.7 ± 0.9 mm (*n* = 16). Each shortened or elongated flower was paired with a control flower separated by 4 cm and presented 15 cm above the ground in water-filled vials. To restrict the potential effects of volatile release due to flower cutting, control flowers in the shortened experiment had a similar-sized portion of their dorsal sepal removed. Similarly, control flowers in the elongation experiment received an equivalent band of
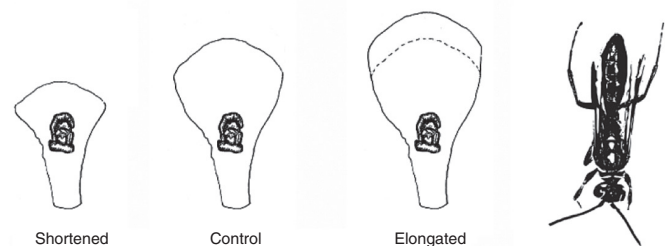


FIG. 2. Labellum manipulations in *C. trapeziformis* to produce shortened and elongated floral morphologies (see Materials and methods for protocol). The pollinator *N. cryptoides* is illustrated for relative scale.

superglue on the abaxial side of its labellum to control for any potential scent effects of glue application.

Because *C. trapeziformis* flowers quickly wilt once pollinated, all control and manipulated flowers were emasculated. Experimental flowers were used within 2 h of manipulation to avoid possible wilting due to cutting or glue application. Manipulation experiments were conducted during 2013 (shortened; de Jager and Peakall, 2016) and 2015 (elongated; this study) at a site on Black Mountain where no *C. trapeziformis* occurs. Responding *N. cryptoides* male wasps were scored as approaching, landing or pseudocopulating. For pseudocopulating males, we also recorded the orientation and duration of pseudocopulation. For each manipulation, we conducted ten replicate experiments. Each experiment comprised four trials of 3 min each. We did not repeat trials at the same location on a given day, and considered pollinator visits independent due to the very low probability of resampling *N. cryptoides* individuals on the same day (Whitehead and Peakall, 2013).

To test whether elongating or shortening the labellum, and consequently the callus–tip distance, impacts pollinator behaviour, we first compared the number of approaches, landings and pseudocopulations between control and manipulated flowers with $\chi^2$ tests. These mutually exclusive behavioural categories were analysed separately for the elongation and shortening experiments. To test whether the duration of pseudocopulation differed between control and manipulated flowers, we used Mann–Whitney *U* tests. If the callus–tip distance is under stabilizing selection, we expect manipulated flowers to receive fewer or shorter pseudocopulations relative to control flowers.

### *Pollinator-mediated selection analysis*

Flowers from Black Mountain Creek (*n* = 71) and Mulloon Farm (*n* = 50) were collected during the 2013–15 flowering seasons to assess the extent of pollinator-mediated selection in *C. trapeziformis*. We randomly picked flowers at least 5 m apart over multiple visits, as most clones in *Chiloglottis* tend to be smaller than 5 m in size (Whitehead *et al.*, 2015). As these are both large populations (>200 m², containing many hundreds of flowers), the likelihood of pseudoreplicating clones is low. We never picked recently opened flowers (identified by their vivid, bright yellow pollinia and light green flower colour) to ensure opportunity for pollinator visitation, and only selected flowers with receptive, wet stigmas to ensure that pollinia removal and deposition was possible. We picked flowers on or after the second day of good weather, because the pollinator, *N. cryptoides*, is mainly active on warm days (>18 °C) without strong wind. Floral traits were measured with digital callipers on the day of picking, and we gave flowers a binary score of 0 (no evidence of either pollinia removal or deposition) or 1 (evidence of pollinia removal or deposition). We selected this approach to explore the floral traits affecting potential pollination (male and/or female fitness), as the rates of pollen deposition were too low for independent statistical analyses. Given the proximity of anthers to the stigma in *C. trapeziformis* and the fact that males typically contact both reproductive parts during pseudocopulation, we deemed this approach justified.

Seven standardized floral traits (mean 0, s.d. 1) relating to labellum and callus morphology (callus–tip distance, callus length, labellum length, callus width, labellum width, callus height and callus–pollinia distance) were used as predictor variables in multivariate regression (Parachnowitsch and Kessler, 2010). This method employs partial regression coefficients to identify the strength of directional selection (*β*) on a given trait, independently of variation in other traits (Lande and Arnold, 1983). We also investigated stabilizing and disruptive selection by estimating quadratic (*γ*) coefficients with multiple linear regression. To estimate parameters, we multiplied the quadratic coefficients by 2, because linear models underestimate stabilizing/disruptive selection gradients by half (Stinchcombe *et al.*, 2008). We calculated linear selection gradients from models including only linear terms, but quadratic gradients from models including both linear and quadratic terms. We did not estimate correlational selection gradients, because models including interactions between all floral traits contained too many parameters for algorithms to converge. Significance was assessed with generalized linear models with a binomial distribution and logit link, as binary response variables tend to have non-normally distributed errors that may violate parametric tests, despite these tests yielding reliable estimates (Lande and Arnold, 1983; Brodie and Janzen, 1996). We estimated the effects of site and year with an analysis of deviance approach by comparing models with and without the effect of interest. All statistics were conducted in R 3.3 (R Development Core Team, 2008).

## RESULTS

### *Linking pollinator behaviour and plant fitness*

Pseudocopulation occurred in all cases of pollinia removal from virgin flowers, thus confirming its use as a reliable proxy for pollination success in *C. trapeziformis*. Furthermore, both the orientation of pseudocopulating pollinators and the duration of pseudocopulation were important factors predicting the probability of pollinia removal (Table 1). Pseudocopulating males were more likely to remove pollinia while in the forward orientation (Fig. 3). Longer pseudocopulation also increased the probability of pollinia removal. We detected no interaction between the orientation of pollinators and the duration of pseudocopulation. All removed pollinia attached to the dorsal thorax of male pollinators, as previously reported for *Chiloglottis* species (Peakall *et al.*, 2010). It is important to note that out of 250 pseudocopulating males, only 32 removed pollinia, indicating that pollination events in *C. trapeziformis* are rare (12.8 %) even when male visitors are sexually stimulated.

TABLE 1. *Logistic regression on the effects of pollinator orientation and duration of pseudocopulation on pollinia removal in unmanipulated* C. trapeziformis *flowers (*n *= 250)*

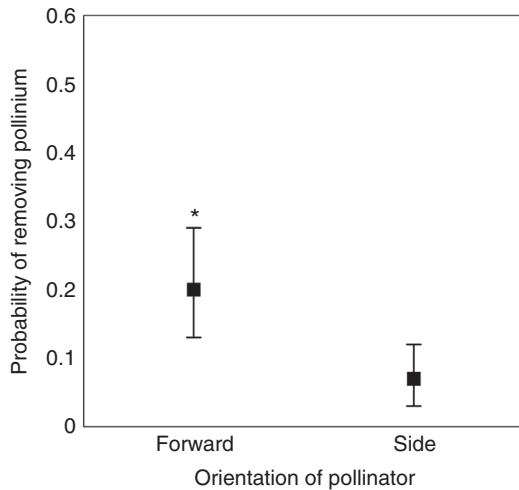|  | *B* | Wald $\chi^2$ | d.f. | *P* |
|---|---|---|---|---|
| Orientation | 1.742 | 8.519 | 1 | 0.004 |
| Duration | 0.650 | 12.695 | 1 | <0.001 |
| Orientation × duration | −0.400 | 2.575 | 1 | 0.109 |

FIG. 3. Probability of male pollinators removing a pollinium from an unmanipulated *C. trapeziformis* flower while pseudocopulating in the forward and side orientations. *$P < 0.005$.

### Linking floral manipulation and pollinator-mediated selection

There was no difference in the number of mutually exclusive approach, land or pseudocopulate responses by pollinators at experimental and control flowers (Table 2), indicating that floral manipulation did not alter the attractiveness of *C. trapeziformis*. The usual forward orientation exhibited by *N. cryptoides* males during pseudocopulation was strongly reduced on flowers with shortened labella, because males were unable to probe the distal labellum edge on shortened flowers. Pseudocopulating males on flowers with elongated labella exhibited no change in the number of forward orientations, because they could still touch the elongated labellum tip with their abdomen while holding on to the callus. The duration of pseudocopulation was significantly reduced on manipulated flowers with both elongated and shortened labella compared with control flowers (Fig. 4), suggesting that stabilizing selection is likely operating on the callus–tip distance.

TABLE 2. *Differences in the number of mutually exclusive behaviours exhibited by male pollinators on manipulated* C. trapeziformis *relative to control flowers*

| Behaviour | Shortening experiment | | Elongation experiment | |
|---|---|---|---|---|
| | Control | Manipulated | Control | Manipulated |
| Approach | 84 | 85 | 173 | 210 |
| Land | 61 | 49 | 44 | 63 |
| Pseudocopulate | 23 | 21 | 24 | 32 |
| Forward orientation | 14 | 2* | 14 | 20 |

Forward orientation refers to the number of pseudocopulating males that exhibited the forward orientation.

Data were analysed with $\chi^2$ tests under an expectation of equal responses between control and manipulated treatments.

*$P < 0.005$.

### Pollinator-mediated selection analysis

As there was no effect of year, we combined the data across years. Site was a significant factor ($\chi^2 = 4.777$, $P = 0.029$) in explaining pollination potential (evidence of either pollinia removal or deposition), driven by the lower rates of pollination at Mulloon Farm (pollinia removal = 16.0 %, pollen deposition = 4.0 %) than at Black Mountain (pollinia removal = 33.8 %, pollen deposition = 12.7 %). Although we detected no evidence of directional selection on any floral traits in *C. trapeziformis* (Table 3), we documented a significant negative quadratic coefficient, indicating stabilizing selection on the distance between the callus and the tip of the labellum (callus–tip).

## DISCUSSION

Our study investigated the effect of variation in flower morphology on pollinator behaviour in *C. trapeziformis*. While sexually deceived pollinators vary in their behaviour at flowers (Peakall, 1990), only pseudocopulatory behaviour is predicted to promote pollination in *C. trapeziformis* (de Jager and Peakall, 2016), as this process brings the male into contact with the reproductive
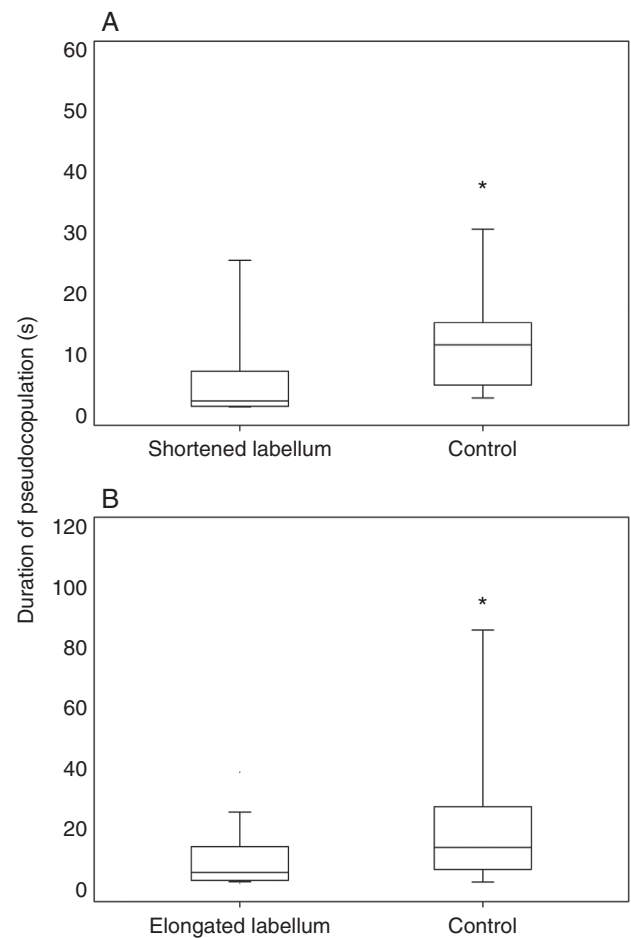


FIG. 4. Duration of pseudocopulation by male *N. cryptoides* on manipulated flowers of *C. trapeziformis* with (A) shortened and (A) elongated labella. Medians and 5th and 95th percentiles are shown. *$P < 0.05$.

TABLE 3. *Selection coefficients ± s.e. for directional (β) and stabilizing (γ) selection on the floral traits of* C. trapeziformis *(n = 121 flowers)*

Potential fitness (pollinia removal and deposition)

| Trait | $\beta$ coefficient | $\gamma$ coefficient |
|---|---|---|
| Callus–tip distance | $0.007 \pm 0.063$ | $-0.191 \pm 0.075*$ |
| Callus length | $0.064 \pm 0.048$ | $0.049 \pm 0.070$ |
| Labellum length | $0.023 \pm 0.072$ | $0.094 \pm 0.089$ |
| Callus width | $0.078 \pm 0.046$ | $-0.058 \pm 0.066$ |
| Labellum width | $0.043 \pm 0.059$ | $0.001 \pm 0.058$ |
| Callus height | $-0.046 \pm 0.049$ | $-0.015 \pm 0.069$ |
| Callus–pollinium distance | $-0.004 \pm 0.044$ | $0.056 \pm 0.063$ |

*$P < 0.05$.

column (Peakall *et al.*, 2010; Peakall and Whitehead, 2014). Our first experiment validates this prediction, by revealing that pollinia removal only occurs during pseudocopulation with the labellum. However, pollinia removal occurred in only a fraction (13%) of these pseudocopulation events. Consistent with expectations from our earlier work (de Jager and Peakall, 2016), pollinia removal in *C. trapeziformis* mainly occurs when the pollinator is positioned in the appropriate forward orientation (Fig. 3).

We also provide the first experimental evidence that the probability of pollinia removal is correlated with the duration of pseudocopulation. A recent study on sexually deceptive *O. leochroma* offers complementary evidence for the importance of pseudocopulation, by revealing an increase in the deposition of massulae with increasing pseudocopulation duration (Rakosy *et al.*, 2017). The frequency and duration of pseudocopulation as a proxy for plant reproductive fitness likely applies broadly across sexually deceptive plants (Ellis and Johnson, 2010; Gaskett, 2011). However, an association between pseudocopulation and pollination is not necessarily expected in all cases (Weinstein *et al.*, 2016). In *Drakaea* orchids, for instance, only the pre-copulatory pollinator behaviour of attempted pick-up of the pseudo-female labellum is essential for pollination, because it brings the pollinator into contact with the column, although pseudocopulation is often also associated with this event (Peakall, 1990; Phillips *et al.*, 2013; Bohman *et al.*, 2014).

Although olfactory cues are crucial for securing pollinator pseudocopulation in *C. trapeziformis* (Schiestl *et al.*, 2003), there is convincing evidence that morphology does matter. Artificially shortening the distance between the callus and the labellum tip, for instance, reduces the duration of pseudocopulation (de Jager and Peakall, 2016). In an extension of that earlier work, here we experimentally tested the effect of extending the callus–tip distance on pollinator behaviour. Our floral manipulations, which purposefully did not alter the callus tissue, where sexual attractants are produced, revealed no change in the response or behavioural repertoire of male visitors, indicating that manipulated flowers had similar long- and short-range attractiveness to control flowers. This suggests a lack of strong pollinator preference for a specific floral shape and size in driving pollinator behaviour in *Chiloglottis*, and corroborates previous results in other sexually deceptive orchids (Benitez-Vieyra *et al.*, 2009; Gaskett, 2012).

In contrast to orchids, studies of sexual deception in the daisy *Gorteria diffusa* reveal that pollinator preference is of prime importance to its floral evolution (Ellis and Johnson, 2010; de Jager and Ellis, 2013). Sexual deception in this daisy differs markedly from sexual deception in orchids, because the mimicry is predominantly visual rather than olfactory (de Jager and Ellis, 2012), and the pollinator is a small dipteran bee fly (Ellis and Johnson, 2009). Beyond the biological idiosyncrasies of these systems, the contrasting findings between sexually deceptive orchids and daisies likely reflect different analytical approaches in studying floral traits that pollinators do not directly interact with (i.e. floral outlines; Gaskett, 2012) versus floral traits clearly associated with pollinator copulatory behaviour (i.e. insectiform structures on the flower; de Jager and Ellis, 2013).

Although we detected no effect of floral manipulation on attractiveness, our experiments revealed the significant impact that manipulation had on the duration of pseudocopulation. Sexually stimulated males that initiated pseudocopulation on flowers with either shortened or elongated labella terminated this behaviour after only a few seconds. Male *N. cryptoides* wasps have slightly longer abdomens than the labella of *C. trapeziformis* (de Jager and Peakall, 2016), and typically arch their abdomens to reach its distal edge during pseudocopulation (Fig. 1), which is difficult, or impossible on manipulated flowers. This suggests that the observed reduction in the duration of pseudocopulation is driven by a lack of mechanical fit with the labellum on manipulated flowers. In *O. leochroma*, a decrease in pseudocopulation duration occurs when the floral components that pollinators functionally interact with during pseudocopulation are removed (Rakosy *et al.*, 2017). In contrast, the removal of floral components with which males infrequently interact during pseudocopulation had little to no effect, leading the authors to class these traits as either mechanically 'active or inactive'.

Considering the evidence for a link between pseudocopulation duration and pollinia removal in *C. trapeziformis*, and independent evidence of a decrease in the duration of pseudocopulation on manipulated flowers, we expect pollinators to exert stabilizing selection on the distance from the callus to the labellum tip. To detect pollinator-mediated selection, limitations on male and female pollination success are required. Across the Orchidaceae this is generally the case (Tremblay *et al.*, 2004), and sexually deceptive orchids are no exception (Weinstein *et al.*, 2016). Indeed, in our study, male and female fitness was low in *C. trapeziformis*, indicating strong potential for pollinator-mediated selection on floral phenotypes that can capitalize on sporadic pollinator visits.

In a final test of pollinator-driven selection on floral traits via mechanical fit, we employed selection analysis. Although phenotypic selection analyses have been applied across a wide range of plants (Harder and Johnson, 2009), this approach has rarely been employed in sexually deceptive species, possibly as a result of a strong research focus on chemistry, which is crucial for securing pollinator attraction. In the only such study to date, Benitez-Vieyra *et al.* (2009) found no evidence for phenotypic selection on floral shape in the sexually deceptive orchid *G. pennicillata*. By contrast, we detected a significant negative quadratic coefficient on the distance from the callus to the labellum tip, indicative of stabilizing selection. This independent result, based on a set of flowers different from that used in our behavioural experiments, corroborates our experimental finding of reduced pseudocopulation duration when this distance is either shortened or elongated. The distance from the

callus structure, which pollinators grip during pseudocopulation, to the distal labellum edge, where they probe with the tip of their abdomen, is thus strongly indicated to be under selection mediated by pollinators.

*Conclusions*

Our results demonstrate that, when combined with a focus on relevant pollinator behaviours, and experimental manipulation of floral form, selection analysis can be a powerful tool in the study of sexually deceptive species. However, without these additional behavioural and experimental components, selection analysis alone cannot provide direct evidence that pollinators are the agents of selection, or identify the mechanism of selection. This is a widespread problem in studies investigating pollinator-mediated selection, which tend to explore either phenotypic selection in natural populations or experimental manipulation of flowers (Harder and Johnson, 2009), but not both. Comparing selection gradients of open-pollinated plants with those of hand-pollinated plants can help identify the component of selection exerted by pollinators (Sandring and Ågren, 2009; Sletvold *et al.*, 2010), although including pollinator observations and floral manipulations offers a more comprehensive analysis of pollinator-mediated selection. In sexually deceptive orchids, this may be especially pertinent due to other potential factors influencing the strength of selection observed in phenotypic selection studies, such as learned avoidance of flower patches by experienced pollinators, low sample size of independent plants due to clonality, and variation in the presence, density and sex ratios of pollinators between periods of study.

Recognizing which floral components influence pollen transfer and plant fitness can help elucidate the evolutionary pathways of different floral traits. This rationale has been successfully applied to studies of floral scent, by identifying behaviourally active and inactive chemical compounds to which pollinators either respond or do not respond (Schiestl *et al.*, 1999; Ayasse *et al.*, 2000; Mant *et al.*, 2005). The analogous extension by Rakosy *et al.* (2017) in identifying active and non-active mechanical traits in sexually deceptive *Ophrys* provides another exemplar. Similarly, studies on deceptive plants that rely on visual mimicry, have uncovered that suites of floral traits involved in female mimicry are under stronger pollinator-mediated selection than suites of traits not involved in mimicry (Ellis *et al.*, 2014). Considering floral structures from this perspective may be instrumental in explaining why floral traits differ in the amount of variation they exhibit (Cresswell, 1998), and can help reveal which traits produce functional divergence and reproductive isolation (Gögler *et al.*, 2015). We suggest that careful consideration of floral traits, in combination with a multi-pronged experimental approach, promises to shed new light on plant–pollinator interactions and the evolution of floral form in deceptive and non-deceptive systems.

## SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: correlation coefficients between the floral traits of *Chiloglottis trapeziformis* investigated in this study.

## ACKNOWLEDGEMENTS

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