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Reproductive isolation between *Zaluzianskya* species: the influence of volatiles and flower orientation on hawkmoth foraging choices

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Summary

- Floral trait differences between related species may play a key role in reproductive isolation imposed by pollinators. Volatile emissions can influence pollinator choice, but how they act in combination with traits such as flower orientation is rarely studied.
- We compared flower-opening patterns, morphology, colour, orientation and volatile emissions for two closely related species of *Zaluzianskya* and their natural hybrids. Hawkmoth pollinators were tested for preference between flowers of the two species, and between flowers with manipulations of volatiles or orientation.
- Flowers of *Z. natalensis* and *Z. microsiphon* open at night and day, respectively, but they overlap during early evening, when hawkmoths showed a strong preference for *Z. natalensis*. The species have similar flower size and colour, but *Z. natalensis* emits more floral volatiles in the evening and presents flowers vertically face-up as opposed to horizontally in *Z. microsiphon*, whereas natural hybrids are intermediate. Adding methyl benzoate and linalool to flowers of *Z. microsiphon* did not increase hawkmoth attraction, but re-orientation of flowers to face vertically increased attraction when scent cues were present, whereas re-orientation of *Z. natalensis* flowers to face horizontally decreased attraction.
- This study highlights the importance of flower orientation in imposing reproductive isolation.

Introduction

Reproductive isolation is a key element in the formation of new species (Dobzhansky, 1937). In plants, pollinator behaviour is often a large contributor to reproductive isolation (Lowry *et al.*, 2008). Indeed pollinators are thought to be critically important to the remarkable diversification of the angiosperms. Traits that can influence pollinator behaviour include size and shape, petal colour, rewards, angle of orientation and scent. If related plant species receive flower visits from different pollinators as a result of behavioural responses to these traits, then reproductive isolation is enforced. This form of reproductive isolation was described in the 1940s as ethological isolation (Grant, 1949). Most subsequent studies on floral trait responses and ethological isolation by pollinators have focused on size, shape or rewards (Harder & Johnson, 2009). Much less is known about the roles played by floral scent (Van der Niet *et al.*, 2014) and flower orientation in the process of ethological isolation (but see Fulton & Hodges, 1999).

Over 1700 volatile organic compounds (VOCs) that contribute to scent have been identified from flowers (Knudsen *et al.*, 2006). A variety of insects are known to respond to many of these compounds in laboratory settings (Raguso, 2008; Klahre *et al.*, 2011; Byers *et al.*, 2014), but the behaviour of animal pollinators

in response to specific VOCs has rarely been studied in natural settings (Dudareva *et al.*, 2013). In the few recent examples, blocked expression of benzyl acetone reduced hawkmoth visitation to *Nicotiana* (Kessler *et al.*, 2008), application of sulphur compounds to a wasp-pollinated species of *Eucomis* caused a shift to fly visitation (Shuttleworth & Johnson, 2010), and application of indole to a hummingbird-pollinated species of *Ipomopsis* caused hawkmoths to approach and hover at the plants (Bischoff *et al.*, 2015).

Related plant species can also differ in the orientation of flowers relative to the ground (Fulton & Hodges, 1999), suggesting that orientation could also cause a shift in pollinators. A relatively early study suggested that hawkmoths visit only upright flowers but did not use manipulations to separate the effect of that trait from a confounded change in flower colour (Eisikowitch & Rotem, 1987). Some more recent studies have examined the effect of altering orientation on pollinator behaviour or pollen transfer (Tadey & Aizen, 2001; Fenster *et al.*, 2009, 2015; Ushimaru *et al.*, 2009; Wang *et al.*, 2014). Most of these studies, however, focused on a single plant species and were not done in the context of understanding ethological isolation. In the exceptional case of *Aquilegia*, altering orientation from upright to pendant reduced hawkmoth visitation (Fulton & Hodges, 1999), but the

reverse manipulation in the hummingbird-pollinated species did not increase hawkmoth visitation to the level observed in the other species (Hodges *et al.*, 2002). Thus, we still lack examples where a shift in floral orientation by itself is sufficient to cause a shift in pollinators. Furthermore, most related plant species differ in multiple floral traits, and whether traits such as scent and orientation act in an independent, redundant or synergistic way to influence pollinators (Hebets & Papaj, 2005) is poorly known. There has been some work on the combined effect of colour and scent on pollinator attraction in the laboratory (Raguso & Willis, 2005; Klahre *et al.*, 2011; Dell'Olivo & Kuhlemeier, 2013) and field (Bischoff *et al.*, 2015). However, the relative importance of flower orientation compared to floral scent in pollinator specificity, is unknown as previous studies have not investigated both traits in the same system.

A full understanding of the role of floral traits in reproductive isolation also requires consideration of how hybrids compare to the parental species. Even if some F_1 hybrids are formed between the two species and can survive to flowering, pollinators might avoid visiting hybrids and thereby reduce backcrossing or formation of second generation hybrids. How natural hybrids compare with their parental species in floral scent is largely unstudied except in some food-deceptive orchids (Cortis *et al.*, 2009; Vereecken *et al.*, 2010; but see Bischoff *et al.*, 2015). Studies of floral orientation in natural hybrids are also scarce, although F_2 hybrids were compared with the parental species in *Aquilegia* for this trait (Hodges *et al.*, 2002).

We studied floral scents, orientation and other traits, as well as hawkmoth responses to floral traits, in a system of two species of *Zaluzianskya* and their natural hybrids in South Africa. *Zaluzianskya microsiphon* is a day-flowering species, primarily pollinated by long-proboscid flies, that evolved within a night-flowering clade that includes the very closely related hawkmoth-pollinated *Zaluzianskya natalensis* (Johnson *et al.*, 2002). Earlier reports suggested that these two species are isolated mainly by patterns of flower opening (Archibald *et al.*, 2004), but more detailed recent observations by the authors indicated that there is a period in the early evening when flowers of both species are open and thus liable to interspecific pollination by hawkmoths. This provides an opportunity to investigate the effects of floral scent and orientation on hawkmoth foraging choices. We therefore asked three questions. (1) How do flowers of the two species and their natural hybrids compare in emission of volatile scent compounds, flower-opening patterns, morphology, colour and seed production? (2) To what extent do hawkmoths discriminate between the species? (3) Are hawkmoth foraging choices influenced by flower orientation and/or scent emissions? We examined how hawkmoths responded to addition of scent and to both gain and loss of upright flowers.

Materials and Methods

Study system

The genus *Zaluzianskya* (Scrophulariaceae) occurs mainly in South Africa. Both *Z. microsiphon* (O. Kuntze) K. Schum.

(Fig. 1a) and *Z. natalensis* Krauss (Fig. 1b) belong to the section *Nycterinia*, which consists of 20 species in total. *Zaluzianskya microsiphon* is the only day-flowering species in that section. Our study site was at Mt Gilboa (29°19′S, 30°17′E) in the Karkloof Mt Range, KwaZulu-Natal province, South Africa. At that site there are extensive intermingled patches of the two species of *Zaluzianskya* and of their natural hybrids, making a mosaic hybrid zone as demonstrated by molecular markers (Archibald *et al.*, 2004). Both species and their hybrids have long-tubed flowers with petals that are white on the upper surface and deep red on the outside of the lobes, which makes the flowers inconspicuous when they are closed. During the daytime *Z. microsiphon* flowers are visited primarily by long-proboscid flies of the genus *Prosoeca* (Nemestrinidae) (Johnson *et al.*, 2002) (Fig. 1a). At night-time hawkmoths (mostly *Hippotion celerio* and *Basiothia schenki*, with *Agrius convolvuli* less commonly) probe flowers of *Z. natalensis* (Fig. 1b). Both long-tongued flies and hawkmoths have been shown to carry *Zaluzianskya* pollen (Johnson *et al.*, 2002). We also observed anthophorid bees collecting pollen from the anthers of *Z. natalensis* during periods (17:45 h to 18:25 h) when day-time *Z. microsiphon* flowers were usually still open. There has been a single observation of a long-proboscid fly visiting flowers of *Z. natalensis* as they open just before dusk (Johnson *et al.*, 2002), but usually flowers of those species are not open when the flies are active. Nectar standing crop at the time of pollinator activity is similar in the two species (Johnson *et al.*, 2002).

Question 1: comparisons of the two species and natural hybrids with respect to floral traits

Flower presentation patterns Although the two species of *Zaluzianskya* are primarily day- or night-blooming, there is some overlap in flower presentation. We quantified the pattern of overlap for both species and natural hybrids by recording the number of flowers on the inflorescence that were fully open during daytime (15:00–15:40 h), early transition time (17:00–17:10 h for *Z. natalensis* only), late transition time (17:20–17:45 h) and night-time (20:30–22:20 h), and dividing by the total number that were open during any of those periods. We surveyed 10 plants of *Z. natalensis*, 11 plants of *Z. microsiphon* and 18 hybrids, that were also measured for scent and morphological traits, on 27 January 2014. The total number of flowers open was similar for *Z. natalensis*, *Z. microsiphon* and hybrids (mean ± SE = 7.8 ± 1.0, 6.0 ± 0.4 and 6.2 ± 0.5).

Scent collection We took both day-time and night-time scent samples from the 39 plants of *Z. natalensis*, *Z. microsiphon* and hybrids. Samples were collected using dynamic headspace extraction methods followed by direct thermodesorption of scent samples using a Chromatoprobe device (Amirav & Dagan, 1997). The entire inflorescence of a plant was enclosed in a scentless Nalophan® oven bag (Kalle GmbH, Wiesbaden, Germany). Scent was allowed to accumulate for 15 min, and then the air in the bag was pumped for 5 min through a scent trap. The scent trap consisted of a glass micro vial (Gordin & Amirav, 2000)

Fig. 1 Study species, pollinators and flower manipulations. (a) *Zaluzianskya microsiphon* visited by a long-proboscid *Prosoeca* fly. (b) *Zaluzianskya natalensis* visited by the hawkmoth *Hippotion celerio*. Note pollen deposited on the proboscis. (c) A choice test stick used in Expt 3 with two inflorescences of *Z. natalensis*. The one on the left has flower orientation manipulated to resemble *Z. microsiphon*. (d) Detail of modified *Z. natalensis* inflorescence. (e) Control *Z. natalensis* inflorescence. (f) Modification of *Z. microsiphon,* on the left, so that its flower orientation resembles that of *Z. natalensis* on the right.

filled with 1 mg of Carbotrap® and 1 mg of Tenax® and plugs of silanised quartz wool to keep the adsorbent in place. Pumping was accomplished with a micro air sampler (Spectrex PAS-500; Redwood City, CA, USA) with a realized flow rate of 150 ml min^{-1}. An air control was taken during day and night from an empty Nalophan® bag enclosing air and pumped for the same duration. Day samples were collected between 11:30 and 14:30 h when the long-proboscid flies are active. Night samples were collected between 19:00 and 22:00 h when the hawkmoths are active. To determine whether night-time compounds are also present shortly before dusk, we took an additional transition time sample between 17:50 h and 18:20 h for five of the plants. In total, we accumulated 77 samples (plus air controls) from seven categories of plants: *microsiphon* day, *microsiphon* night, hybrid day, hybrid night, *natalensis* day, *natalensis* night, *natalensis* transition. The number of flowers (open or closed) was recorded also at the time of scent collection.

Gas chromatography-mass spectrometry (GC-MS) analysis of floral scent Scent samples were analysed using coupled gas chromatography and mass spectrometry (GC-MS). We used a Varian CP-3800 GC (Varian, Palo Alto, CA, USA), with an Alltech EC-WAX column (30 m × 0.25 mm internal diameter, film thickness 0.25 μm), coupled to a Varian 1200 quadrupole mass spectrometer in electron-ionization mode (70 eV). Scent traps were placed in a Varian 1079 injector equipped with a Chromatoprobe (Amirav & Dagan, 1997) for thermal desorption. The flow of helium carrier gas was 1 ml min^{-1}. The injector was held at 40°C for 3 min with a 20 : 1 split and then increased to 200°C at 200°C min^{-1} in splitless mode for thermal desorption. After a 3 min hold at 40°C, the temperature of the GC oven was ramped up to 240°C at 10°C min^{-1} and, after reaching 240°C, held there for 12 min.

Volatile compounds were tentatively identified using the Varian Workstation software (MS Data Review v.6.8) with the NIST 11 mass spectral library (v.2.0g), and were verified with retention times of authentic standards and published retention time indices wherever possible. Compounds present at similar abundance in ambient air samples were considered to be contaminants and were excluded from the statistical analysis. To obtain the floral scent emission rate per inflorescence per hour, known amounts of standard compounds were analysed using the same instrument settings. Eleven standard compounds (α-pinene, limonene, (*E*)-ocimene, (*E*)-3-hexen-1-yl acetate, (*Z*)-hex-3-en-1-ol, benzaldehyde, linalool, β-caryophyllene, methyl benzoate, methyl salicylate, benzyl alcohol) diluted in hexane were injected into scent traps that were then thermally desorbed using the same methods used for floral scent samples.

Statistical analyses of scent data Before analysis of the scent data, unknown sesquiterpenes were lumped into one variable. Four other unknowns were present only in trace quantities in 1 or 2 plants, and we lumped these also into one variable. After collapsing the unknowns, we had 28 scent variables, each corresponding to ng emitted per inflorescence per hour for a different compound (Supporting Information Table S1). To reduce the skew in the data, we analysed log-transformed values after adding 1. Then the seven types of plants were compared using canonical analysis of principal coordinates (Anderson & Willis, 2003; Pratt *et al.*, 2014). The advantage of this approach is that it does a constrained analysis (looking for differences among the plant types rather than unconstrained variability across the entire dataset as in nonmetric dimensional scaling) while allowing use of any distance measure rather than assuming a Euclidean distance (Anderson & Willis, 2003). The analysis was implemented with the capscale function in the Vegan package of R (R Development Core Team, 2014), using Bray distances. To determine if hybrids were intermediate between the two species in key species-specific compounds (methyl benzoate and linalool; see the Results section), we used a two-way ANOVA on the raw values with the factors of species (*Z. microsiphon* vs *Z. natalensis* vs hybrid) and time of sampling (day vs night). The null hypothesis for hybrid intermediacy was specified using a contrast statement in Procedure GLM of SAS (v.9.2) comparing the natural hybrids with the mean over the two species.

Floral morphology and spectral reflectance Several morphological traits were measured on these same plants before bagging them for scent sampling: plant height, inflorescence height (for description see Archibald *et al.*, 2004), diameter of the flower face, petal angle between the corolla tube and upper petal lobes, and the tube to inflorescence angle. A single flower from each plant was returned to the lab to measure corolla tube length and flower colour using an Ocean Optics S2000 Spectrometer (Ocean Optics, Dunedin, FL, USA) coupled to an Ocean Optics Mini-D2T light source. We measured spectra of adaxial and abaxial surfaces at a 45 degree angle along the length.

Morphological traits (all of the above except colour) were compared between the two species using MANOVA and canonical discriminant analysis. The first canonical function was the linear function that explained the greatest variance between species. That function was then applied to the hybrids to see how they compared in traits that separate the species. The analysis was implemented with procedure Candisc in SAS (v.9.2; SAS Institute Inc., Cary, NC, USA). We also used contrast statements in ANOVA to test the null hypothesis of hybrid intermediacy for tube and petal angles.

In order to establish the hybrid status of the hybrids we sampled, we used MANOVA to compare their morphological traits with hybrids previously genotyped from the same localities by Archibald *et al.* (2004). That previous study used genetic data to confirm hybridization based on inter-simple sequent repeat (ISSR) markers, and the hybrids had intermediate band frequencies for the majority of the loci.

Seed production Although the primary emphasis of our study was on prezygotic ethological isolation, another potential form of reproductive isolation between these species would be low reproductive success of hybrids. To assess how the hybrids compared with the parental species in seed production, all of the plants were revisited in March to determine the number of fruits made and count seeds in a sample of fruits. The total number of seeds was calculated by multiplying seeds per fruit by the number of fruits not eaten by seed predators. Seed production was compared across the three types of plants (*microsiphon*, *natalensis* and hybrids) using a generalized linear model with the factor of plant type and a covariate of flower number, along with the interaction. Because the interaction was significant, we followed this analysis with a model using the factor of plant type and the covariate of flower number nested within plant type to obtain separate slopes for each type. Proc Genmod in SAS (v.9.2) was used, employing a normal distribution, as it returned a much lower AIC value than other potential distributions.

Questions 2 and 3: hawkmoth discrimination

We examined hawkmoth choices using the interview method in which two types of inflorescences were matched by number of open flowers and then mounted 60 cm apart on a T-shaped stick 150 cm in length (Fig. 1c). An observer held the end of the interview stick in the path of a flying hawkmoth such that the two inflorescences were at equal distances from the insect and recorded whether the moth probed flowers on one choice, the alternate choice, probed both or ignored both. We used red LED bulbs on headlamps to facilitate observations without disturbing the hawkmoths. For all choice tests combined, moths were observed by three observers on four warm, clear nights during 28 January to 11 February 2014 and by one observer for two additional nights during 3–4 February 2015. Observations were conducted for the entire time that hawkmoths were active (between 18:50 h and 20:00 h). We recorded moth identity (species and individual) for each moth responding to the interview stick, whether or not the moths participated in a trial (i.e visited at least one of the offered flowers) and the first choice that it made. The position (left or right) of a particular treatment was rotated between moths. All choice tests were conducted within a dense (*c.* 500 plants per hectare) natural population of *Z. natalensis* so that scents characteristic of the latter species were always in the background. Because the *Z. microsiphon* population and the hybrids always occur within the more extensive population of *Z. natalensis*, we deemed this locality for the choices to be more biologically realistic than other localities.

We performed four types of experiments. First, to determine whether moths discriminate among open flowers of *Z. natalensis* and *Z. microsiphon*, we offered moths a choice between inflorescences of these two species. To examine responses to specific traits that differ between the plant species, we performed three additional manipulative experiments. We tested whether adding night-time scent compounds emitted by *Z. natalensis* attract hawkmoths to *Z. microsiphon*. We presented choices between an

inflorescence of *Z. microsiphon* with a vial containing a scent mixture of the compounds linalool and methyl benzoate (diluted in paraffin oil) attached to the end of the interview stick directly below the inflorescence and an inflorescence of *Z. microsiphon* with a vial containing paraffin oil only as a control in the corresponding location. The dilution of the two standards in paraffin oil was adjusted to approximate the emissions from inflorescences of *Z. natalensis*. For adjusting the emission rate, scent was collected from vials with the two standards in paraffin oil, using the same sampling protocol as for collecting the scent of the field samples. The selected dilution, comparable in its emission to that of *Z. natalensis*, contained 2 µl linalool and 1 µl methyl benzoate in 2 ml paraffin oil.

In the remaining two experiments, we tested how hawkmoth behaviour is influenced by flower orientation. In Expt 3, we simulated loss of upright orientation by offering choices between *Z. natalensis* and *Z. natalensis* with flowers tied at a 90 degree angle (i.e. horizontal) to resemble the presentation of *Z. microsiphon* flowers (Fig. 1d). Those flowers have a tube angle averaging 45 degrees downward from vertical, and also a petal angle averaging 149 degrees (see the Results section), making them average just 4 degrees off from facing directly out from the inflorescence. To eliminate the possibility that the material used to tie flowers (green electrical wire) itself affected moth behaviour, we tied a similar amount of the wire onto the control (otherwise unmanipulated) inflorescences (Fig. 1e). Expt 4 simulated the gain of upright orientation and was conducted the following year. Because we saw a strong effect of orientation in Expt 3, we made the *a priori* prediction that moths would prefer *Z. microsiphon* with flowers presented vertically (Fig. 1f) to resemble the orientation of *Z. natalensis* flowers over the normally horizontally presented flowers of *Z. microsiphon*.

In order to assess spontaneous preference in each of the four experiments, we analysed the type of inflorescence visited first for each moth trial in which the moth probed at least one flower. Because some individual moths participated in more than one trial and those responses could be correlated, the type of inflorescence visited was analysed with a generalized estimating equation (GEE), including individual moth identity as a subject to account for the repeated trials and using a binomial distribution, logit link and an exchangeable correlation matrix. This analysis was implemented in Proc Genmod of SAS (v.9.2) using the repeated statement to specify the subject and an intercept-only model to compare the incidence of first visits to the null hypothesis of equal frequency to both choices. For Expts 1 and 4, all probes were made to a single type of inflorescence yielding an estimated variance of zero. In those cases, we simply compared the proportion of first visits in a moth trial with the null expectation of equal frequency using an exact binomial test.

We also compared the proportion of moths that participated in a trial (probed at least one flower on either test inflorescence) between the experimental manipulations and the control Expt 1 to assess whether a particular manipulation of one species fully restored moth responses similar to that seen with unmanipulated flowers of the two species. Participation (yes or no) as a function of experiment was analysed using a generalized estimating

equation with binomial distribution, exchangeable correlation matrix and including individual moth identity as a subject. A contrast statement was used in Proc Genmod to specifically contrast each of the experimental manipulations (Expts 2–4) with Expt 1 in which flowers of the two species were presented. The results need to be interpreted with caution, as differences in participation rate between experiments could reflect true differences in response to the plant choices offered or other factors that affect propensity to probe flowers, given that experiments were not run simultaneously.

Results

Question 1: comparisons of the two species and natural hybrids with respect to floral traits

Flower-opening During the mid-afternoon only flowers of *Z. microsiphon* and hybrids were fully open, with all flowers of the night-blooming *Z. natalensis* closed (Fig. 2). During the transition time of 17:00–17:40 both species and the hybrids presented some open flowers. All three types also overlapped to some extent at night, with 13% of flowers of the normally day-flowering *Z. microsiphon* still fully open at 21:50 h and hybrid flowers remaining open to a greater extent than those of the day-bloomer (Fig. 2). All flowers of *Z. natalensis* were fully open at
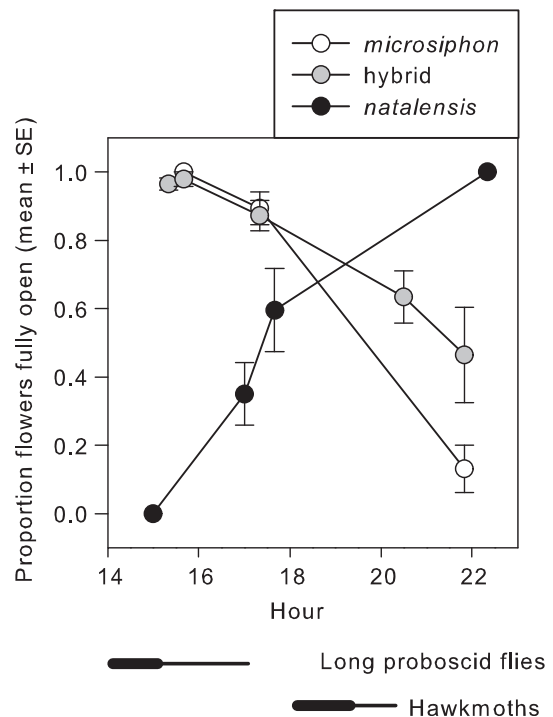

Fig. 2 Timing of flower opening. The proportion of flowers (*Zaluzianskya microsiphon*, *Z. natalensis* and hybrids) fully open are shown over time. Two groups of hybrids that were sampled > 20 min apart are shown separately. Means and standard errors across inflorescences are shown. Activity bars are overlaid for the two major types of flower visitors. The thickest part of the bar shows the major period of activity. Long proboscid flies start foraging *c.* 07:00 h in the morning (not shown). Activities are based on observations made in the current study and by Johnson *et al.* (2002).

that time. Thus, although backcrossing was potentially possible at all times, pollinator movements between flowers of the two species were potentially possible only during the evening transition time and night-time when hawkmoths and the occasional bee, but not day-flying long-proboscid flies, forage (Fig. 2).

Volatiles The most abundant volatile scent compounds on average were (in order starting with the most abundant): β-myrcene, (*E*)-ocimene, limonene, linalool, α-pinene and methyl benzoate (Table S1). Scent mixtures differed across the seven types of plants mainly in methyl benzoate and linalool. The first constrained principal coordinate (CAP1) explained 74% of the variance and contributed significantly to differences among plant types (permutation test using ANOVA function on the results of the function capscale in the vegan package of R, $P = 0.005$). None of the other CAPs showed significant differences (all $P > 0.05$). Methyl benzoate and linalool had by far the highest scores on CAP1, and were primarily responsible for separating the night-time *Z. natalensis* samples from all other types (Fig. 3). Emission of these compounds showed a rapid increase around dusk. Only two of the five *Z. natalensis* plants sampled during the transition time of 17:00 h to 17:40 h emitted any methyl benzoate or linalool at all, and yet between 19:00 h to 19:30 h, 10 out of 10 plants did so (Table S2). In *Z. natalensis*, overall scent emission increased at night, not only for the whole inflorescence, but also on a per-flower basis (Table S1). Samples other than those from night-time *Z. natalensis* differed mainly along CAP2, on which β-myrcene had the highest positive score and (*E*)-

ocimene had the highest negative score. Hybrids were generally more similar in scent to *Z. microsiphon* (Fig. 3). For the critical compounds of methyl benzoate and linalool, the hybrids either resembled *Z. microsiphon* in not emitting these compounds at all or emitted generally smaller amounts than *Z. natalensis* (Table S1). Although the amounts emitted by hybrids were usually small (Fig. 3), contrast statements in two-way ANOVAs could not detect a difference between the natural hybrids and the parental mid-point ($F_{1,66} = 3.51$, $P = 0.0653$ for methyl benzoate and $F_{1,66} = 3.20$, $P = 0.0783$ for linalool). Two compounds were found uniquely in hybrids (myrtenal and γ-muurolene), but each compound was found in only one sample, so these rare occurrences could represent sampling error.

Morphology and colour The two species differed in morphological traits (MANOVA, $F_{7,13} = 35.16$, $P < 0.0001$), with *Z. microsiphon* having greater plant height, inflorescence height, petal angle and tube angle, along with a smaller distance between the upper petals (Fig. 4) that makes them appear more zygomorphic. The canonical discriminant function was highly correlated with each of those five traits ($r = 0.59–0.92$). Hybrids were generally intermediate in the function separating the species, but some overlapped with *Z. microsiphon* (Fig. S1). Hybrids had tube angles and petal angles that departed significantly from the parental mean in the direction more similar to *Z. microsiphon* (contrast $P = 0.0047$ and 0.0493, respectively). Hybrids were particularly variable in inflorescence height (Fig. 4d). Flowers of the two species and hybrids had similar patterns of spectral reflectance, except that the upper side of *Z. natalensis* petals showed slightly higher reflectance in the blue compared to *Z. microsiphon,* (Fig. S2).

The hybrids studied here were morphologically indistinguishable from the genotyped hybrids in Archibald *et al.* (2004), based on MANOVA for the traits of inflorescence height, tube length, and petal angle that were measured in both studies (Wilks $\Lambda = 0.848$, $F_{3,20} = 1.19$, $P = 0.3374$). Our hybrids were morphologically different from the genotyped individuals of the two pure species in that previous study (MANOVA, $P = 0.0002$ for comparison with *Z. microsiphon* and $P < 0.0001$ for *Z. natalensis*). Thus, the individuals that we classified as hybrids were genetically as well as morphologically distinct from the two parental species.

Seed production Plants of *Z. microsiphon* made the most seeds (mean = 472 seeds per plant), *Z. natalensis* made the least (mean = 59) and hybrids were intermediate (mean = 279). The increase in seeds with flower number differed across the three types (likelihood ratio $\chi^2 = 21.48$, df = 2, $P < 0.0001$). Nesting the slopes within plant type indicated that *Z. microsiphon*, hybrids and *Z. natalensis* made 51, 34 and 12 seeds per additional flower, respectively. Thus, the natural hybrids reproduced at least as well as the average of the two parental species.

Questions 2 and 3: hawkmoth discrimination

We observed visits by the hawkmoths *Hippotion celerio, Basiothia schenki* and *Agrius convolvuli*. In choices between *Z. natalensis*
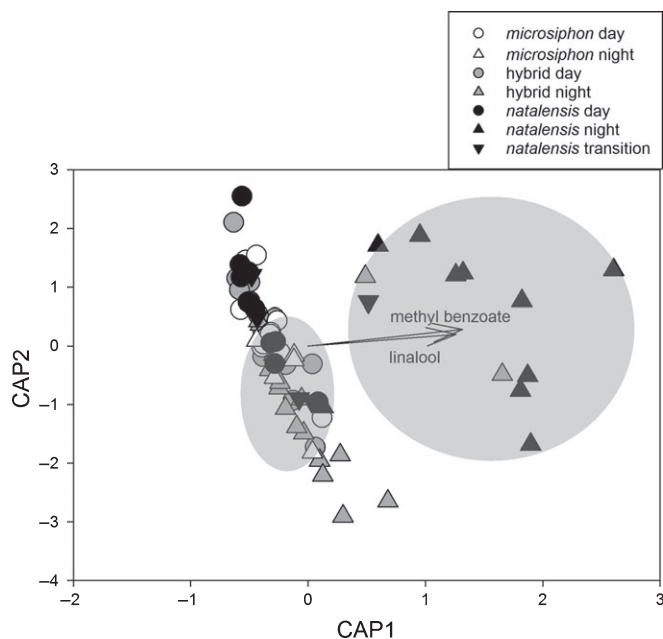

Fig. 3 The seven types of plants plotted as a function of the first two axes separating scent profiles in a canonical analysis of principal coordinates. The night-time *Zaluzianskya natalensis* scent samples separate out from the other groups primarily along the first axis (CAP1). Methyl benzoate and linalool have the highest scores on this axis (1.32 and 1.26, see arrows), with other compounds having scores with absolute values ranging from 0.01–0.52. The two ellipses enclose the night-time samples for *Z. natalensis* and *Z. microsiphon*.
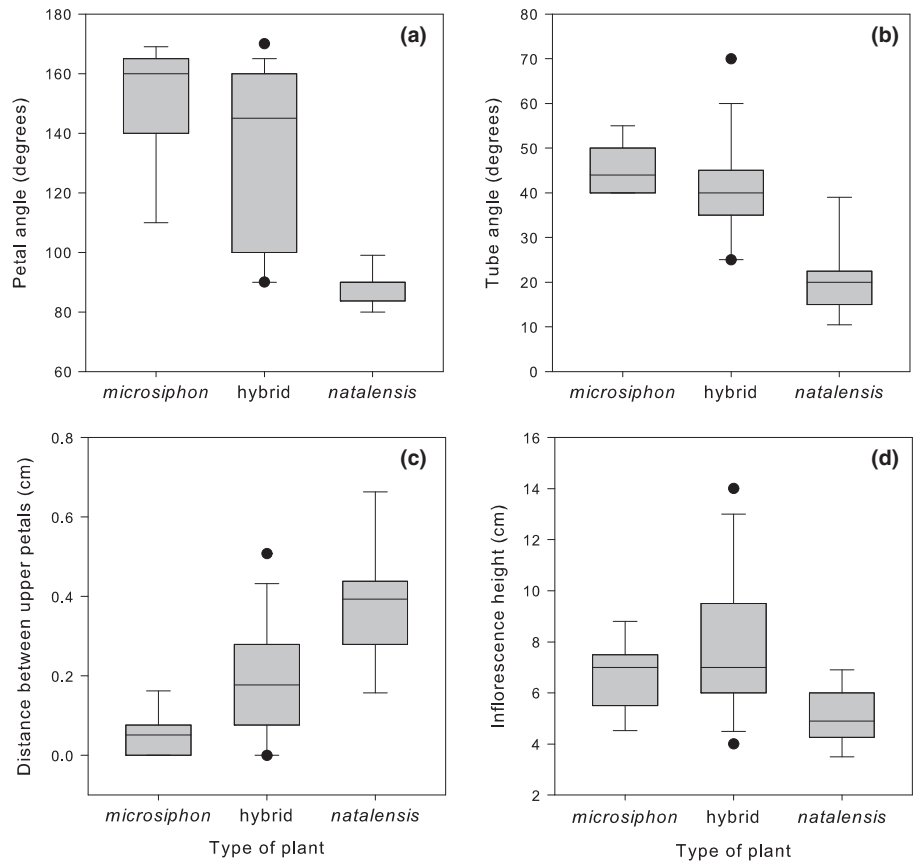
Fig. 4 Morphological traits of the two species (*Zaluzianskya microsiphon* and *Z. natalensis*) and hybrids. The box plots show the median, 25th and 75th percentiles, 10th and 90th percentiles and 5th and 95th percentiles (as outliers). The four traits shown in (a)–(d) and plant height all differed significantly between the species (univariate ANOVAs $P < 0.007$ equivalent to $P < 0.05$ with Bonferroni correction for 7 traits).

and *Z. microsiphon* with the same number of open flowers, 77% of moth trials (10 out of 13) resulted in the moth probing at least one flower (Fig. 5, Expt 1). All of the moths that participated probed *Z. natalensis* first ($P = 0.0019$ with exact binomial test) and none went on to probe *Z. microsiphon*. Thus, the moths showed spontaneous preference for *Z. natalensis*.

In a choice between *Z. microsiphon* and *Z. microsiphon* with a scent vial emitting linalool and methyl benzoate, only one moth probed any flower (on the scented treatment) and 10 different moths ignored (or only approached) both choices. The rate of moth participation was lower than the rate seen in the test between the two species (contrast in GEE model, $\chi^2 = 5.35$, $P = 0.0208$). Adding the scent mixture by itself did not cause moths to visit *Z. microsiphon*.

In a choice between *Z. natalensis* (which has upright flowers facing up) and *Z. natalensis* with flowers presented horizontally, 22 out of 27 moths probed at least one flower. All but two of those moths were involved in only one foraging trial. The moths showed a very strong spontaneous preference for the vertical orientation (GEE model; $P = 0.003$; Fig. 5, Expt 3). The number of probes per flower presented was also higher for the control treatment than the horizontal treatment (mean = 0.42 vs 0.05; randomized block ANOVA with a moth foraging bout as a block, $P < 0.0001$). The rate of moth participation in this experiment did not differ significantly from that seen in the test between two species (GEE model with individual moth id as the subject, $\chi^2 = 0.43$, $P = 0.43$), consistent with moths treating the

horizontally-presented *Z. natalensis* flowers no differently from those of *Z. microsiphon*.

When the flower orientation was manipulated for *Z. microsiphon*, 14 moths came to the interview stick, with six moths participating in more than one trial and five moths probing flowers, in each case on only one foraging bout. In all 5 of those cases, the moth visited just the *Z. microsiphon* with flowers manipulated into a vertical orientation, a pattern that differs from the null expectation of no preference (exact binomial with a one-tailed test based on our *a priori* prediction, $P = 0.03$). The rate of moth participation was lower but not significantly different from that seen in the test between two species (Fig. 5, Expt 4 vs 1; contrast in GEE model with moth id as the subject, $\chi^2 = 1.88$, $P = 0.1708$), suggesting that imposing an unright orientation could cause a shift to hawkmoth visitation similar to that seen in the normally moth-pollinated species.

Discussion

The results of this study show that a single floral morphological trait (flower orientation in this instance) can contribute strongly to the reproductive isolation of closely related plant species through its effects on pollinator behaviour. The strong signalling function for flower orientation in our study system was unexpected, as previous studies have tended to emphasize classical signalling traits, such as colour and scent, in reproductive isolation (Waelti *et al.*, 2008; Dell'Olivo & Kuhlemeier, 2013).
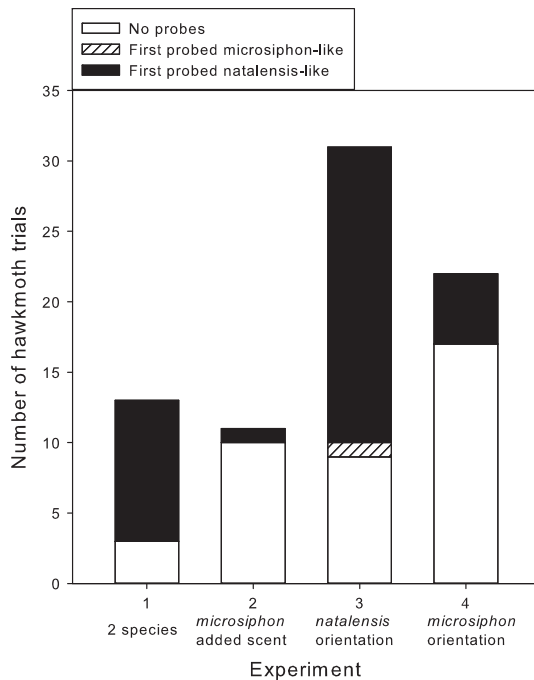
Fig. 5 Responses of hawkmoths in four binary choice field experiments with an interview stick. In total we observed 77 choice trials by 60 hawkmoths. Although each trial is shown here, statistical analysis took into account the clustering of multiple trials by the same moth.

Zaluzianskya microsiphon and *Z. natalensis* are isolated in part by timing of flower opening, but our study showed that they have overlapping flower presentation during early evening when hawkmoths forage for nectar (Fig. 2), making it critical to understand the potential for ethological isolation due to hawkmoth discrimination. Flowers of the two species differ in volatile scent composition, orientation generated by angles of the flower tube and the petals, and the distance between the upper petals (Figs 3, 4). By manipulating scent and flower orientation we were able to examine the roles of these particular traits in generating ethological isolation (Fig. 5).

The two closely related species differed in scent. Flowers of both species emitted large amounts of β-myrcene and (*E*)-ocimene, with the two species differing primarily in the large amounts of linalool and methyl benzoate emitted by flowers of *Z. natalensis*. Hawkmoths show antennal responses to both linalool and methyl benzoate (Raguso *et al.*, 1996), and both compounds are frequently found in moth-pollinated plants (Knudsen & Tollsten, 1993), so it is not surprising to find them in flowers of *Z. natalensis*. Overall there was less scent in the species pollinated by long-tongued flies, a group that has been comparatively little studied with respect to volatiles. The natural hybrids were generally intermediate in scent or more similar to *Z. microsiphon*. Because we don't know the precise genetic composition of the natural hybrids, it is unknown whether that bias towards *Z. microsiphon* reflects only genetics or is also influenced by microhabitat effects on the scent traits (Majetic *et al.*, 2009). *Zaluzianskya natalensis* was strongly preferred by hawkmoths, with no visits recorded to unmanipulated flowers of

Z. microsiphon (Fig. 5). Although another hawkmoth species preferred scented *Petunia* containing methyl benzoate over a scentless form (Klahre *et al.*, 2011), in our study addition of methyl benzoate and linalool at the base of the inflorescence of *Z. microsiphon* did not induce hawkmoths to visit (Fig. 5). One likely explanation is that scent alone does not make the flowers attractive and that other floral traits are also required for visitation. Indeed, our manipulations of flower orientation showed that vertically-oriented flowers are necessary for hawkmoths to visit *Z. natalensis* (Fig. 5). We cannot rule out at least two other explanations, however. Because the experiment was conducted in a dense population of *Z. natalensis*, sufficient quantities of scent may already have been perceived by the hawkmoths, and they may not exhibit dosage dependent responses above that level. Another species of hawkmoth, *Hyles lineata* did not exhibit dosage-dependent responses to indole at levels beyond those occurring naturally in a favoured nectar source (Bischoff *et al.*, 2015), but in general little is known about dosage-dependent effects of floral volatiles (Galen *et al.*, 2011). In addition, other components of the scent mixture besides linalool and methyl benzoate may be required in particular ratios to induce visitation.

Whereas the role of scent compounds in reproductive isolation between the study species requires additional study, we have demonstrated that flower orientation is a critical trait required for hawkmoth visitation to *Zaluzianskya*. Loss of vertical orientation in *Z. natalensis* caused hawkmoths to avoid visiting (Fig. 5), thus treating the inflorescence as if it were *Z. microsiphon*. Conversely, gain of vertical orientation in *Z. microsiphon* induced hawkmoth visitation to these normally long-proboscis fly-pollinated flowers (Fig. 5). This system represents the first demonstration that a change in flower orientation by itself can cause a shift both away from and to hawkmoth pollinators to the extent that occurs naturally in comparisons of closely related species. The overall rate of participation for hawkmoths was similar regardless of whether they were presented with a choice between the two species or between the normally horizontal flowers of *Z. microsiphon* and *Z. microsiphon* with flowers presented upright. Furthermore, in all trials where hawkmoths were presented with a choice between upright and horizontal flowers, only once did a hawkmoth visit horizontal flowers. The combined results indicate that changing orientation alone can induce hawkmoth visitation. Interestingly, the change in flower orientation influences whether or not hawkmoth attempt to probe flowers, suggesting that the change in orientation is perceived visually, perhaps because the horizontal flowers present the outer colour of human-red and vertical flowers present human-white to a hawkmoth flying above.

The only other case in which manipulating orientation reduced visitation by hawkmoth pollinators involved a species with hawkmoth-pollinated upright and hummingbird-pollinated pendent flowers (Fulton & Hodges, 1999), rather than two long proboscid insects as in *Zaluzianskya*. That system also contrasts with ours in that gain of upright orientation had only a small effect on hawkmoth visitation and did not restore the level to that seen in the moth-pollinated species, indicating that other traits had to be involved in ethological isolation (Hodges *et al.*, 2002). For *Zaluzianskya,* the strong hawkmoth responses to both gain and

loss of the upright orientation indicate for the first time the important role that floral orientation can play in ethological isolation.

This trait of orientation is achieved by a combination of altering the angle of the flower tube compared to the stem and the angle of the petals compared to the tube. These two underlying traits are not correlated in the natural hybrids ($r = -0.07$, $N = 19$). Assuming that some of those hybrids are second generation in which correlations due to linkage have started to break down, this lack of correlation suggests the traits may be due to different mutations during the evolution of this genus. It remains unknown whether the response to orientation requires a background of scent from *Z. natalensis* or not. In theory, this possibility could be tested in an area away from natural *Z. natalensis* by offering inflorescences with flowers of *Z. microsiphon* manipulated into a vertical orientation, either alone or with flowers of *Z. natalensis* hidden in green bags next to the inflorescence to provide the scent cue but not a visual cue. However, it is very difficult to locate flying hawkmoths away from populations of their food plants, making this experiment likely impossible. Even more importantly, it would represent a biologically unrealistic situation, as hybridization between the two species occurs only at scented populations of *Z. natalensis* where hawkmoths are abundant.

It is possible that other floral traits also influence pre-zygotic reproductive isolation between these two species of *Zaluzianskya*. Flowers of *Z. microsiphon* have a shorter distance between the upper petals, which increases zygomorphy of the horizontally-pointing flowers. This difference in morphology could conceivably affect hawkmoth behaviour, but more likely influences the effectiveness of pollen transfer or visitation by long-proboscid flies. Previous studies have shown that day-flying pollinators can respond to the visual outline of flowers (Herrera, 1993; Johnson & Dafni, 1998). Horizontal orientation of zygomorphic flowers in *Commelina communis* enhances visitation by syrphid flies, presumably by facilitating recognition of the flowers (as all horizontal ones would present the same positioning) and enhances pollen transfer by ensuring a legitimate landing with contact of stigmas and anthers (Ushimaru *et al.*, 2009). The long-proboscid fly visitors of *Z. microsiphon* hover at flowers, but horizontal orientation could in principle still enhance recognition or ensure contact between the flower reproductive parts and the face and underside of the proboscis (Johnson *et al.*, 2002) Flowers of the two species are similar in spectral reflectance patterns (Fig. S2). Although some hawkmoths can distinguish between monochromatic lights as similar as 440 and 470 nm (Kelber, 1997), it is unclear whether or not they could separate the subtly different spectra of the two *Zaluzianskya* species. At this point we cannot exclude the possibility that variation in colour also influences discrimination, but our manipulations of flower orientation that induced hawkmoths to visit the usually fly-pollinated *Z. microsiphon* suggest that any floral colour differences between the species are not the key determinant of hawkmoth foraging decisions.

Conclusions

We demonstrated that the night-blooming, hawkmoth-pollinated *Z. natalensis* emits more floral volatiles than its sympatric, largely day-blooming and long-proboscid fly-pollinated congener *Z. microsiphon*, including linalool and methyl benzoate, compounds often found in moth-pollinated flowers. Furthermore, the two species differ in flower orientation, and natural hybrids are intermediate or more similar to *Z. microsiphon* in floral morphology, orientation and volatiles. Our manipulative experiments indicate that flower orientation is a critical trait enforcing hawkmoth specificity in the presence of floral scent, as loss of vertical orientation in *Z. natalensis* virtually eliminated hawkmoth visitation and gain of vertical orientation in *Z. microsiphon* induced it. Through its effect on ethological isolation, flower orientation thus plays a key role in limiting hybridization between these highly inter-fertile and sympatric species.

Author contributions

D.R.C., A.J. and S.D.J. planned and designed the research. D.R.C., A.J. and S.D.J. conducted fieldwork. A.J. and S.D.J. conducted lab work. D.R.C. analysed data. D.R.C. wrote the manuscript that was improved by A.J. and S.D.J.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Canonical discriminant analysis of morphological traits.

Fig. S2 Reflectance spectra for flowers.

Table S1 Means of absolute emission rates (ng per inflorescence h^{-1}) for VOCs by plant type and time of sampling

Table S2 Relative emission rates (percentage amount per flower per hour) for VOCs by plant type and time of sampling

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