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Experimental evidence for specialized bird pollination in the endangered South African orchid *Satyrium rhodanthum* and analysis of associated floral traits

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The bird pollination syndrome is characterized by red, unscented flowers with dilute nectar in long nectar tubes. However, the extent to which plants with such traits actually depend on birds for seed production is seldom determined experimentally, and traits such as colour and scent production are often assessed only subjectively. We documented bird pollination and quantified floral traits in the critically endangered *Satyrium rhodanthum* (Orchidaceae) from mistbelt grasslands in the summer-rainfall region of South Africa. Direct observations and motion trigger camera footage revealed amethyst sunbirds as the only pollinators, despite the presence of other potential pollinators. Experimental exclusion of sunbirds significantly reduced pollination and fruit set to near zero. Pollination success in naturally pollinated plants was close to 100% in one year, and fruit set varied from 23 to 64% in other years. Pollen transfer efficiency was 5.8%, which is lower than in related insect-pollinated species, probably due to a tendency of birds to wipe pollinaria from their beak. Flowers of *S. rhodanthum* only reflect light in the red range of the spectrum, and they produce only a few aliphatic and monoterpene scent compounds at comparatively low emission rates. Nectar volume and sugar concentration varied between 2.7 and 3.7 μ L and 23.7 and 25.9%, respectively. We conclude that *S. rhodanthum* is highly specialized for pollination by sunbirds. Colour, scent and nectar characteristics differ from insect-pollinated *Satyrium* species and are consistent with those expected for bird-pollinated flowers, and may contribute to lack of visitation by other potential long-tongued pollinators. Habitat loss probably underlies the critically endangered conservation status of *S. rhodanthum*, but the specialization for pollination by a single bird species means that reproduction in this orchid is vulnerable to losses in surrounding communities of plants that subsidize the energetic requirements of sunbirds. © 2014 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, **177**, 141–150.

ADDITIONAL KEYWORDS: bird vision – exclusion experiment – floral scent – flower colour – pollen transfer efficiency – pollination syndrome – sunbird.

INTRODUCTION

‘The lack of reports on orchids which seem obviously bird-pollinated can be attributed to no more than simple failure of observers to note birds visiting orchid flowers. Birds as a rule are wary, furtive and elusive in behaviour and do not stay long while visiting flowers. The few reports which are available result from sheer coincidence or long tedious hours of observations before results are attained.’ (van der Pijl & Dodson, 1966)

Flowers which are specialized for pollination by particular functional pollinator groups tend to exhibit convergence in traits such as flower colour and scent and the positioning of floral rewards and reproductive parts (Vogel, 1954; Faegri & van der Pijl, 1979; Proctor, Yeo & Lack, 1996). These ‘floral syndromes’ may reflect differences among pollinators in their morphology and sensory preferences (Fenster *et al.*, 2004; Schiestl & Johnson, 2013). Flowers of plants specialized for pollination by birds are generally considered to be characterized by bright colours,

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especially red or orange, lack of scent and production of relatively large volumes of dilute nectar in elongated nectar tubes (Vogel, 1954; Faegri & van der Pijl, 1979; Baker & Baker, 1983). However, studies which have investigated specialized bird pollination rarely include an objective analysis of floral traits, despite the biased human perception of colour (e.g. Kevan, Giurfa & Chittka, 1996) and scent (Ohloff, 1994). Furthermore, studies of bird pollination seldom include experimental verification of the pollination importance of birds relative to other visitors (but see Hargreaves, Johnson & Nol, 2004; Botes, Johnson & Cowling, 2009). Indeed, specialized bird pollination is often inferred from general syndrome traits. Inferring pollinators from floral syndromes has been criticized in general (see Waser *et al.*, 1996; Ollerton *et al.*, 2009) and has also been shown to be problematic in the particular case of the bird pollination floral syndrome (Mayfield, Waser & Price, 2001). Species with a characteristic bird pollination syndrome may be less specialized than their traits imply (e.g. Botes *et al.*, 2009; Dalsgaard *et al.*, 2009) or may not be bird-pollinated at all (Marloth, 1895; Porsch, 1926; Johnson & Bond, 1994). A clear understanding of the floral traits that characterize bird-pollinated plants therefore requires studies which combine an objective analysis of traits with a test of pollinator specialization.

Orchids are well known for their highly specialized pollination systems (Johnson & Steiner, 2003; but see Johnson & Hobbhahn, 2010), but in contrast to many other similarly specialized plant families, bird pollination is rare (reviewed by Micheneau, Johnson & Fay, 2009). An exception is the largely African twin-spurred orchid genus *Satyrium* Sw., in which pollination by sunbirds has been observed for five out of *c.* 90 species thus far (Johnson, 1996; Johnson *et al.*, 2011). These discoveries were made relatively recently, probably because bird pollination is notoriously difficult to identify in orchids (van der Pijl & Dodson, 1966). In bird-pollinated orchids pollinaria are glued to the beak, meaning that the structure of their flowers is somewhat different from that of bird-pollinated flowers in which granular pollen is placed on the feathers. However, in keeping with the general floral syndrome, flowers of bird-pollinated *Satyrium* spp. are generally brightly coloured, and seemingly unscented to the human nose. Sunbirds probe the floral spurs to access large quantities of dilute nectar, thereby accumulating pollinaria on their bills and transferring pollen. However, they have also been observed to attempt to wipe pollinaria off on nearby shrubbery (Johnson, 1996), potentially negating their effectiveness as pollinators. Furthermore, Johnson (1996) reported that some of the bird-pollinated *Satyrium* spp. also

received visits by insects, and sunbirds have been observed to visit and remove pollinaria from *Satyrium* spp. that are seemingly specialized for insect pollination (e.g. Rebelo, 1987). Pollinator observations alone should therefore be treated with caution in evaluating claims of specialization for bird pollination. Most insect-pollinated *Satyrium* spp. investigated so far are strongly scented (Johnson, Ellis & Dötterl, 2007; Van der Niet, Hansen & Johnson, 2011; Van der Niet, Juergens & Johnson, in press). *Satyrium* is therefore a suitable candidate for testing whether lack of scent is indeed associated with bird pollination. In this study we investigated *Satyrium rhodanthum* Schltr., a critically endangered orchid species with a narrow distribution in the mistbelt of the summer-rainfall region of South Africa. It is a robust terrestrial orchid with red flowers which are unscented to the human nose. The main aims of this study were to assess the importance and effectiveness of sunbirds as pollinators of *S. rhodanthum* and to quantify floral traits considered characteristic of bird pollination objectively.

MATERIAL AND METHODS

STUDY SPECIES

Satyrium rhodanthum (Fig. 1) is a rare species that flowers from late spring to early summer (October–January) in damp grassland in the mistbelt region of southern KwaZulu-Natal (South Africa). The species is currently known only from three localities (Raimondo *et al.*, 2009). Hall (1982) tentatively classified *S. rhodanthum* as a hybrid between *S. longicauda* Lindl. and *S. neglectum* Schltr. subsp. *woodii* (Schltr.) A.V.Hall, although he stated that further research was needed to confirm its hybrid status. *Satyrium rhodanthum* does not, however, conform to the concept of a hybrid: it occurs in large populations of > 100 plants per site and it does not co-occur with *S. neglectum* subsp. *woodii*. Although it does co-flower with *S. longicauda*, there are sharp trait discontinuities and no indication of a hybrid swarm resulting from backcrossing. Therefore, regardless of whether *S. rhodanthum* could be of hybrid origin, it currently has the attributes of a good species (cf. Linder & Kurzweil, 1999).

Fieldwork was performed during peak flowering at Ixopo over 14 days from October to February between 2005 and 2013 and at Highflats over 5 days during December 2011 and 2013. Detailed information on the fieldwork localities is available on request from the first author. A voucher specimen (CZN092) is deposited at the NU herbarium at the University of KwaZulu-Natal, South Africa.

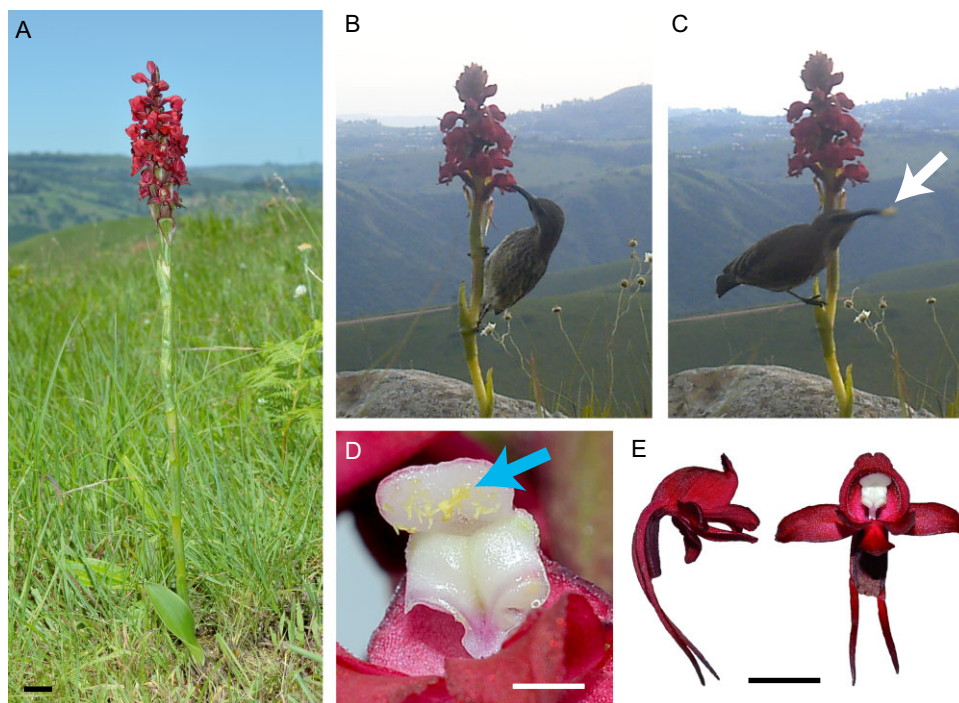


Figure 1. Plant traits and pollination of *Satyrium rhodanthum* at Highflats. A, plant *in situ* (scale bar = 30 mm). B, female amethyst sunbird probing flower of *S. rhodanthum*, photographed with a motion-trigger camera. C, the same bird perched on a stem sheathing leaf. The yellow clump of pollinaria on the tip of the bill is visible (white arrow). D, exposed stigma and rostellum of a flower immediately after a visit by a male amethyst sunbird. Freshly deposited massulae are indicated by the blue arrow. Note the large viscidium on the right is still present, whereas the left viscidium has been removed (scale bar = 1 mm). E, side view and front view of a flower showing the two nectar spurs and large column part including rostellum and stigma (scale bar = 10 mm).

POLLINATION

Pollinator observations

Pollinator observations were continuously carried out during fieldwork at close range (c. 2 m away from plants) to detect potential insect visits and at long range (c. 30 m away from plants) using binoculars to detect potential bird visits. Observations started at dawn and lasted until mid-afternoon. Observations just after dusk (the peak activity time of nocturnal insect pollinators) were also performed. Visitors were identified and checked for presence of the relatively large yellow orchid pollinaria, which can easily be seen with the naked eye [*Satyrium* pollen is aggregated in sectile pollinia, composed of several hundred massulae (Johnson & Edwards, 2000)]. Immediately after visitors departed from plants, stigmas of all flowers were inspected for freshly deposited massulae using a 20× hand-lens. Freshly deposited massulae are bright yellow, have a dry surface and contracted, conical shape. Contact with stigmatic fluid results in decoloration, expansion and softening of massulae.

In 2011 and 2013, we introduced motion trigger cameras to increase chances of recording plant visits by shy pollinators. Up to six Bushnell trophy cams

were set up at a distance of c. 1 m from inflorescences in peak flowering stages at both field sites.

Exclusion experiment

To determine whether plants are dependent on birds for reproduction, we excluded birds by placing plastic mesh cages around plants and compared pollination and fruit set between these caged plants and uncaged control plants. We selected a mesh size of 20 × 24 mm, through which sunbirds are too large to fit but insects such as noctuid moths pass freely (Van der Niet *et al.*, 2014; T. Van der Niet, unpubl. data). Plants with many buds were selected and assigned at random to treatment and control groups. Exclusion experiments were set up at Ixopo on 24 November 2010 and at Highflats on 20 December 2013. Evidence of pollination (deposition of massulae) was recorded after 10 days (Highflats) and fruit set was recorded after 6 weeks (Ixopo). For both experiments, flowers which were already open prior to setting up cages were marked and excluded from analyses, and there were no differences between control and treatment groups in terms of the total number of flowers per inflorescence and the number of flowers included in the

experiment (results not shown). To establish whether caging had a significant effect on the proportion of flowers that were pollinated or set fruit, these data were analysed using generalized linear models with a binomial error structure and logit link function, implemented in SPSS 19. Marginal means and standard errors were back-transformed from the scale used in the link function, resulting in asymmetrical error bars.

Pollination success and pollen transfer efficiency

Pollination success was assessed by inspecting recently wilted flowers for pollinarium removal, which is easily observed from the presence or absence of the large viscidia (Fig. 1), and for deposition of massulae. Thirty-eight wilted flowers, each from a different inflorescence, were randomly sampled during December 2013 at Highflats.

The same data were used to calculate the efficiency of pollen transfer, i.e. the proportion of removed massulae that is deposited on stigmas (Johnson & Brown, 2004). The mean number of massulae per pollinium was estimated by counting individual massulae of pollinaria of nine randomly sampled flowers from different plants, using a compound microscope. The total number of removed massulae was estimated by multiplying the number of removed pollinaria from the 38 wilted flowers by the mean number of massulae per pollinium. The number of massulae deposited on stigmas was similarly counted for the 38 wilted flowers.

Natural fruit set was assessed on randomly selected plants at Ixopo in 2010 ($N = 29$) and 2011 ($N = 10$), at three different patches, by counting developed fruits and wilted flowers with no fruits per plant, approximately 6 weeks after the end of flowering.

FLORAL TRAITS

Colour

To obtain an objective assessment of flower colour, independent of human vision, we measured light reflectance using an Ocean Optics S2000 spectrophotometer and fibre optic reflection probe (UV/VIS 400 μm) held at a fixed distance and at a 45° angle to the object surface, in an enclosed optic holder. We used an Ocean Optics Mini-DT (deuterium–tungsten–halogen) light source with a spectral range of c. 200–1100 nm. Spectra were calibrated using an Ocean Optics WS-1 diffuse reflectance standard and spectra were captured at 0.34-nm intervals using Ocean Optics SpectraSuite software. For analysis we used the range between 300 and 700 nm, which is relevant for most pollinator vision systems. For each measured part we averaged spectra over two measurements

taken along its length and width, respectively. Mean reflectance of the lateral sepals, labella, bracts and sheathing stem leaves was calculated from several individuals. Spectra were analysed and visualized using the package ‘pavo’ (Maia *et al.*, 2013). To optimize noisy spectra, we applied local regression smoothing, with a smoothing parameter (span) of 0.15. Spectra of the lateral sepal and labellum were identical, and variation between plants from Ixopo and Highflats was minimal, so we show only spectra from labella ($N = 16$), bracts ($N = 8$) and sheathing stem leaves ($N = 8$) of flowers from Highflats.

Scent sampling

To characterize composition and emission rates of floral scent of *S. rhodanthum*, we used headspace collection and gas chromatography-mass spectrometry (GC-MS) methods. We sampled scent of two to four individuals at Ixopo (2011 and 2013) and Highflats (2013) during early to mid-afternoon and just after dusk (when crepuscular insects are active). Plants from Ixopo were cut prior to sampling in 2013, which has no effect on scent emission in relatively robust orchid plants such as *Satyrium* spp. (T. Van der Niet, unpubl. data). Polyacetate bags were placed over entire inflorescences of plants that were in peak flower (i.e. inflorescences with wilted flowers, fresh flowers and buds). Air from these bags was immediately pumped through small thermodesorption cartridges filled with 1 mg of Tenax TA 60/80 and 1 mg of Carbotrap TM 20/40 mesh at a flow rate of 50 mL min^{-1} for 20–30 min. Air flow through the bag was facilitated by cutting a small hole in the bag. Plant sampling was always accompanied by sampling of an empty bag to control for volatiles present in the surrounding air. Traps were stored at -20°C until they were analysed.

GC-MS analysis of these samples was carried out using a Varian CP-3800 GC with a 30-m $\times$ 0.25-mm internal diameter and a film thickness of 0.25 μm , and Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode. Thermodesorption cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device (Gordin & Amirav, 2000; Dötterl, Wolfe & Juergens, 2005). The flow of helium carrier gas was 1 mL min^{-1} . The injector was held at 40 $^\circ\text{C}$ for 2 min with a 20:1 split and then increased to 200 $^\circ\text{C}$ at 200 $^\circ\text{C min}^{-1}$ in splitless mode for thermal desorption. After a 3-min hold at 40 $^\circ\text{C}$, the temperature of the GC oven was ramped up to 240 $^\circ\text{C}$ at 10 $^\circ\text{C min}^{-1}$ and held there for 12 min.

Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011; MS search software version 2.0 d)

and verified, where possible, using retention times of authentic standards and published Kovats indices. If a compound could not be identified with certainty, it was scored as unknown, with the six most common mass fragments indicated in decreasing order of quantity. For each compound we calculated its relative emission by dividing the peak surface area (total ion count) for that compound over the cumulative peak surface areas of all compounds, excluding those present in the surrounding air.

Total absolute scent emission rates were calculated using the peak surface area of a known quantity of methyl benzoate as a reference. One nanolitre of methyl benzoate was injected into a thermodesorption cartridge which was run on the GC-MS system using the same programme as was used for the original samples. This procedure was repeated three times. For each run, the peak area was calculated. An average over three runs was calculated to arrive at the average total ion count per nanogram of methyl benzoate. The total ion count of compounds not found in the surrounding air of each sample was subsequently divided by the total ion count per nanogram and by the sampling time in minutes to arrive at the absolute scent emission rate per inflorescence min^{-1} .

Nectar

We measured nectar volume and sugar concentration of flowers at Highflats and Ixopo at midday. At Ixopo, we randomly sampled 14 flowers, each from a different plant, and measured the standing crop using 5- μL glass microcapillary tubes. Sugar concentration (w/w) was measured using a 0–50% Bellingham and Stanley refractometer. At Highflats we selected 12 flowers that had not been pollinated and measured nectar volume and concentration in the same way. We measured total functional nectar spur length as the distance from the rostellum to the tip of the spur, and the distance from the rostellum to the nectar level in the spur, which indicates the minimum length of the mouthpart of a visitor required to consume nectar and can be seen by holding the spur against the light.

RESULTS

POLLINATION

Pollinator observations and exclusion experiment

Direct observations and footage from motion-trigger cameras at Highflats in 2013 revealed that the sole visitor to flowers of *S. rhodanthum* was the amethyst sunbird (*Chalcomitra amethystina*). Nine individuals consisting of five males (three visibly carrying pollinaria), three females (two visibly carrying pollinaria) and one of which gender could not be determined were seen to visit plants over 4 days between 04:57

and 18:24 h. Birds used the stem as a perch and probed several flowers per inflorescence (Fig. 1 and supporting information Video S1). Birds foraged systematically from several plants in a patch before moving to the next patch, including over distances of 50–100 m. During flower visitation pollinaria were extracted and attached on the upper mandible close to the tip of the bill, consistent with insertion of the bill into the galea for a relatively short distance (Fig. 1 and Video S1). We also observed agitated behaviour by birds carrying pollinaria, including repeated attempts to wipe them off on nearby shrubs.

Stigmas of flowers on 21 out of 24 plants in patches that had received sunbird visits had fresh massulae deposited. The mean $\pm$ SD number of flowers per inflorescence ($N = 22$) on which massulae were deposited was 3.2 ± 2.8 . Overall, $35 \pm 24\%$ of flowers on ten inflorescences had fresh massulae deposition (Fig. 1).

No other flower visitors were observed, despite the presence at the sites of long-tongued horseflies (*Philoliche* sp.), butterflies (*Papilio* sp.), wasps (*Hemipepsis* sp.) and bees. No visitors were observed during evening observations, despite the presence of hawkmoths at the site. Malachite sunbirds (*Nectarinia famosa*) were also present at Ixopo, but were not observed visiting plants or carrying pollinaria. Both pollination and fruit set were significantly reduced to close to zero on plants from which birds had been experimentally excluded by cages compared with open pollinated plants (Fig. 2).

Pollination success and pollen transfer efficiency

Of the 38 wilted flowers examined, 36 had one or both pollinaria removed, of which 34 had massulae deposited. The total number of removed pollinaria was 56 and the total number of deposited massulae was 1215. The mean $\pm$ SD number of massulae per pollinium was 375 ± 66 ($N = 9$) and the mean $\pm$ SD number of massulae deposited per flower was 32 ± 30 ($N = 38$). This corresponds to a pollen transfer efficiency of 5.8%. Mean $\pm$ SD fruit set at Ixopo was 0.62 ± 0.31 ($N = 29$) in 2010 and 0.37 ± 0.31 ($N = 10$) in 2011.

FLORAL TRAITS

Colour

Spectral reflectance of flowers showed little reflectance between 300 and 580 nm, followed by a sharp increase in reflectance from 600 nm to approximately 680 nm (Fig. 3). Spectral reflectance of leaves was typical of that for green parts, with a distinct peak at 550 nm, and an increase after 680 nm (Fig. 3). Spectral reflectance of bracts was intermediate, with no clear peak at 550 nm, a slight peak at 630 nm and an

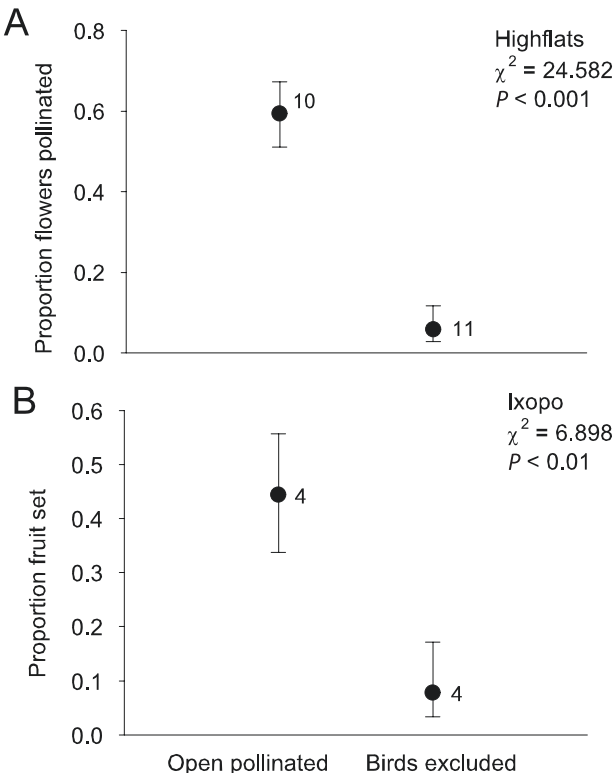


Figure 2. Mean $\pm$ SE pollination and fruit set, respectively, of caged (bird-excluded) and uncaged plants at (A) Highflats (2013) and (B) Ixopo (2010). Numbers indicate sample sizes.

increase after 680 nm (Fig. 3). None of the floral parts showed reflectance in the UV range (Fig. 3).

Scent

We detected a small number of mainly aliphatic and monoterpene compounds in headspace samples of volatiles emitted by *S. rhodanthum* (Table 1; see Table S1 for a detailed list of compounds). The monoterpenes terpinen-4-ol and α -terpineol and benzenoid *o*-cymene were only present in 2-day samples from Highflats. Total emission rates varied between 0.064 and 0.35 ng per inflorescence min⁻¹ during the day; no scent emission was detected during the evening (Table 1).

Table 1. Mean $\pm$ SD number of compounds and emission rates of the scent of *S. rhodanthum* inflorescences at Highflats and Ixopo during the day and evening

Site	Time of day (<i>N</i>)	No. of compounds	Emission rates (ng per inflorescence min ⁻¹)
Highflats	Day (3)	11 $\pm$ 5.2	0.35 $\pm$ 0.24
	Night (2)	0	0
Ixopo	Day (7)	3.6 $\pm$ 0.8	0.064 $\pm$ 0.056
	Night (4)	1 $\pm$ 0.5	0

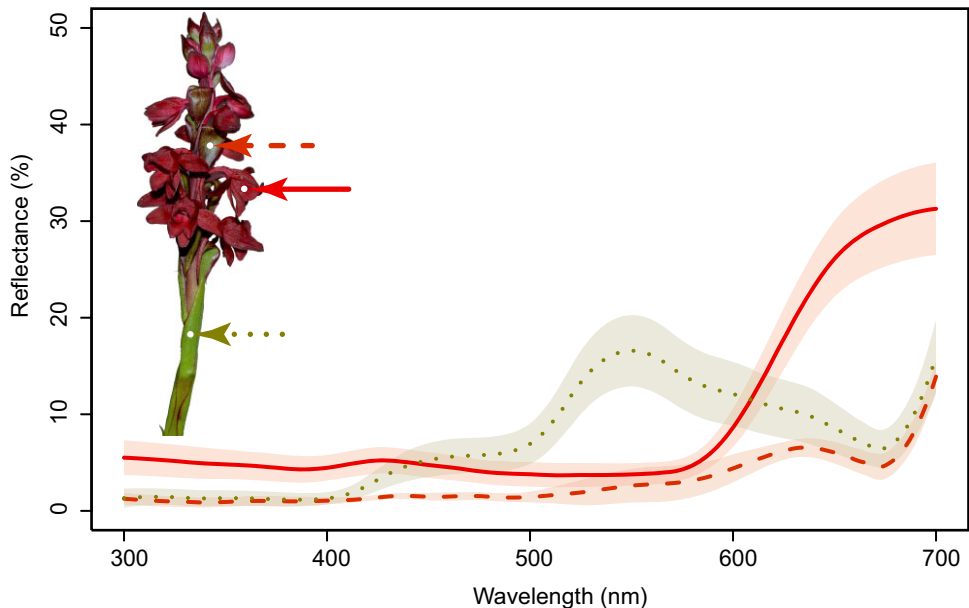


Figure 3. Mean spectral reflectance of leaves, bracts and flowers of *S. rhodanthum* between 300 and 700 nm. Line colours represent the colour of the measured parts, which are indicated by white dots. Shaded areas represent $\pm$ SD.

Table 2. Mean $\pm$ SD spur length, nectar volume, sugar concentration and distance to nectar of *S. rhodanthum* flowers at Ixopo (randomly selected flowers) and Highflats (virgin flowers); sample sizes are given in parentheses

	Spur length (mm)	Volume (μ L)	Sugar concentration (% w/w)	Distance viscidium to top of nectar surface (mm)
Ixopo	29.90 $\pm$ 3.78 (7)	2.7 $\pm$ 1.2 (8)	23.7 $\pm$ 3.0 (14)	–
Highflats	33.87 $\pm$ 2.05 (12)	3.7 $\pm$ 2.0 (12)	25.9 $\pm$ 2.3 (12)	17.6 $\pm$ 3.7 (8)

Nectar

Nectar spurs were slightly longer at Highflats than at Ixopo (Table 2). Nectar standing crop per spur was 2.7 μ L at Ixopo; nectar volume in the spurs of virgin flowers at Highflats was higher (3.7 μ L; Table 2). Sugar concentration was similar at Ixopo (23.7%) and Highflats (25.9%) (Table 2). The mean distance from the rostellum to the nectar surface was 17.6 mm (Table 2).

DISCUSSION

Observations of floral visitors showed that *S. rhodanthum* is exclusively visited and pollinated by sunbirds. Birds use the sturdy stem as a perch while probing the floral spurs for nectar, thereby removing pollinaria and depositing massulae. Experimental exclusion of birds to flowers reduced pollination and fruit set to almost zero, in contrast to relatively high levels of natural pollination and fruit set. *Satyrium rhodanthum* thus appears to be specialized for pollination by sunbirds. Associated floral traits are weakly scented, red flowers, which produce relatively large volumes of nectar. These traits are consistent with those expected for bird-pollinated plants in general.

Pollinator specialization could be overestimated in this study for three reasons. First, birds were observed as visitors during only a single year and at a single site. However, observations were performed extensively over the larger range of *S. rhodanthum* during several years and no other floral visitors were observed. We therefore exclude potential spatial and temporal variation in pollination systems in this system (e.g. Herrera, 1988; Fenster & Dudash, 2001). Secondly, some (< 10%) pollination occurred in plants from which birds were experimentally excluded. We think that incidental pollination may be caused by florivores such as grasshoppers which could export pollen by chance. Indeed, occasional evidence of florivory was observed. Finally, the mesh size used in the exclusion experiment not only excludes birds, but also some larger insect visitors such as hawkmoths (T. Van der Niet, unpubl. data). Hawkmoths were, however, not observed visiting plants during evening observations at one of the field sites, despite their presence. We therefore think that our inference of

pollinator specialization is not an overestimate. Although visitors were never directly observed at Ixopo, we infer that plants at this site are also pollinated by sunbirds because a sunbird captured at the site readily visited *S. rhodanthum* plants and pollinated flowers in an aviary (T. Van der Niet, unpubl. data) and, secondly, because results from the exclusion experiment at this site are consistent with bird pollination.

The nectar reward provided for floral visitors by *S. rhodanthum* is comparable in volume and concentration to that of other specialized bird-pollinated plant species in general (Johnson & Nicolson, 2008) and to other bird-pollinated *Satyrium* spp. in particular (Johnson 1996; Johnson *et al.*, 2011). In comparison with *S. rhodanthum*, insect-pollinated *Satyrium* spp. tend to produce smaller volumes of nectar (Johnson, 1997; Johnson *et al.*, 2011; Van der Niet *et al.*, in press). Despite the presence of other potential visitors that are capable of reaching the relatively large nectar reward hidden in the long spurs, specialization for bird pollination occurs in *S. rhodanthum*. Potential long-tongued visitors present at the field sites included butterflies, long-tongued flies and hawkmoths. The lack of visitation by these insects, which are specialized pollinators of other South African plants (Johnson, 2010), is probably due to the specific attractant traits of *S. rhodanthum*. Flower colour of *S. rhodanthum* may not be attractive to other local potential visitors. Indeed, long-tongued flies from the summer-rainfall region typically visit flowers with a peak in spectral reflectance of 300–400 nm (Jersáková *et al.*, 2012; Newman, Manning & Anderson, 2014). However, butterflies (*Papilio* sp.) from the region are attracted to nectar-producing red flowers in other plant species (Goldblatt & Manning, 2006). More research is needed to link the visual perception of local butterflies with pollination. The absence of visitation by hawkmoths can be explained by both flower colour and floral scent of *S. rhodanthum*. Moth pollination in *Satyrium* is associated with white flowers and scent production during the evening (Van der Niet *et al.*, 2014). Although floral scent was not completely absent in *S. rhodanthum*, it is only produced during the day, and in quantities that are orders of magnitude smaller than those of

insect-pollinated *Satyrium* spp.: mean emission rate of the bird-pollinated *S. rhodanthum* is 12.55 ng per inflorescence h⁻¹ (Table S1), whereas emission rates of insect-pollinated *Satyrium* spp. range between 150 and 108 000 ng per inflorescence h⁻¹ (Johnson *et al.*, 2007; Van der Niet *et al.*, 2011, in press). Observed scent production is also similar to that in other weakly scented plant species pollinated primarily by birds (Knudsen *et al.*, 2004; Steenhuisen, Raguso & Johnson, 2012; Van der Niet *et al.*, 2014).

Our quantification of flower colour in *S. rhodanthum* allows evaluation of the prediction from Shrestha *et al.* (2013), that the colour of sunbird-pollinated flowers might be differentiated from those pollinated by hummingbirds and honey-eaters, due to differences in the visual capabilities of pollinators. Sunbirds have a UV-sensitive visual system, with an optimum around 557 nm, whereas hummingbirds and honey-eaters have a violet-sensitive visual system with an optimum around 600 nm (Odeen & Hastad, 2010). However, the difference in visual optima is not associated with differences in spectral reflectance, as the pattern observed in *S. rhodanthum* is similar to that of a large group of Australian flowers which are predominantly pollinated by honey-eaters (Shrestha *et al.*, 2013).

The site of pollinarium placement at the tip of the bill of the bird implies that the bird does not insert its entire bill into the relatively narrow floral spur. Instead, they probably use their slender extendable tongues to lick up nectar in the spurs (cf. Schlamowitz, Hainsworth & Wolf, 1976; Johnson, 1996; Downs, 2004). Selection for optimal matching of nectar tubes and mandibles found in other bird pollination systems (e.g. Geerts & Pauw, 2009; Van der Niet *et al.*, 2014) and in several specialized long-proboscid insect-pollination systems (e.g. Nilsson, 1988; Anderson & Johnson, 2009; Pauw, Stofberg & Waterman, 2009) may be less likely to occur. Indeed, spur length varies both among bird-pollinated *Satyrium* spp. and between the two populations of *S. rhodanthum*.

Pollen transfer efficiency (PTE) was lower than that currently known for any insect-pollinated *Satyrium* spp. (6.0–17.6%, median 9.0%; Harder & Johnson, 2008; Van der Niet *et al.*, 2011). This could be due to birds occasionally succeeding in brushing pollinaria off their bill by wiping it on branches of nearby shrubs, thereby reducing PTE. In other plant genera, PTE differences between pollinators have been proposed to drive evolutionary shifts in pollination systems (cf. Stebbins, 1970; Castellanos, Wilson & Thomson, 2003; Muchhala & Thomson, 2010). *Satyrium* is characterized by a large number of different pollination systems (Johnson, 1997; Johnson *et al.*, 2011). An analysis of evolutionary shifts

between pollination systems in *Satyrium*, using PTE and pollinator data in a phylogenetic framework, could be used to test whether shifts usually occur away from bird pollination towards more efficient insect pollinators. Further research into variation in PTE, both among bird-pollinated *Satyrium* spp. and among species with different pollination systems, is required.

Plant species with highly specialized pollination systems are vulnerable to extinction (Pauw, 2007). Indeed, *S. rhodanthum* is a highly threatened orchid species, known from only a few sites. It seems unlikely, however, that specialization for sunbird pollination explains its current narrow distribution, as its pollinator, the amethyst sunbird, is widely distributed in South Africa [South Africa Bird Atlas Project II (sabap2.adu.org.za/index.php)]. Instead, we think that extensive habitat transformation as a result of intensive agriculture in the range of *S. rhodanthum* probably explains its rarity. It is, however, at risk of reproductive failure if transformation of surrounding plant communities results in a situation where there are insufficient nectar resources to attract sunbirds to the site.

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REFERENCES

- Anderson B, Johnson SD. 2009. Geographical covariation and local convergence of flower depth in a guild of fly-pollinated plants. *New Phytologist* **182**: 533–540.
- Baker HG, Baker I. 1983. Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ, eds. *Handbook of experimental pollination biology*. New York: Van Nostrand Reinhold Co., 117–141.
- Botes C, Johnson SD, Cowling RM. 2009. The birds and the bees: using selective exclusion to identify effective pollinators of African tree aloes. *International Journal of Plant Sciences* **170**: 151–156.
- Castellanos MC, Wilson P, Thomson JD. 2003. Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution* **57**: 2742–2752.

- Dalsgaard B, Gonzalez AMM, Olesen JM, Ollerton J, Timmermann A, Andersen LH, Tossas AG. 2009. Plant–hummingbird interactions in the West Indies: floral specialisation gradients associated with environment and hummingbird size. *Oecologia* **159**: 757–766.
- Dötterl S, Wolfe LM, Juergens A. 2005. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. *Phytochemistry* **66**: 203–213.
- Downs CT. 2004. Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. *Ostrich* **75**: 169–175.
- Faegri K, van der Pijl L. 1979. *The principles of pollination ecology*. Oxford: Pergamon.
- Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialisation. *Annual Review of Ecology Evolution and Systematics* **35**: 375–403.
- Fenster CB, Dudash MR. 2001. Spatiotemporal variation in the role of hummingbirds as pollinators of *Silene virginica*. *Ecology* **82**: 844–851.
- Geerts S, Pauw A. 2009. Hyper-specialisation for long-billed bird pollination in a guild of South African plants: the malachite sunbird pollination syndrome. *South African Journal of Botany* **75**: 699–706.
- Goldblatt P, Manning JC. 2006. Radiation of pollination systems in the Iridaceae of sub-Saharan Africa. *Annals of Botany* **97**: 317–344.
- Gordin A, Amirav A. 2000. SniffProbe: new method and device for vapor and gas sampling. *Journal of Chromatography* **903**: 155–172.
- Hall AV. 1982. A revision of the southern African species of *Satyrium*. *Contributions from the Bolus Herbarium* **10**: 1–137.
- Harder LD, Johnson SD. 2008. Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Sciences* **169**: 59–78.
- Hargreaves AL, Johnson SD, Nol E. 2004. Do floral syndromes predict specialisation in plant pollination systems? An experimental test in an 'ornithophilous' African *Protea*. *Oecologia* **140**: 295–301.
- Herrera CM. 1988. Variation in mutualisms – the spatio-temporal mosaic of a pollinator assemblage. *Biological Journal of the Linnean Society* **35**: 95–125.
- Jersáková J, Jürgens A, Šmilauer P, Johnson SD. 2012. The evolution of floral mimicry: identifying traits that visually attract pollinators. *Functional Ecology* **26**: 1381–1389.
- Johnson SD. 1996. Bird pollination in south African species of *Satyrium* (Orchidaceae). *Plant Systematics and Evolution* **203**: 91–98.
- Johnson SD. 1997. Insect pollination and floral mechanisms in South African species of *Satyrium* (Orchidaceae). *Plant Systematics and Evolution* **204**: 195–206.
- Johnson SD. 2010. The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society B-Biological Sciences* **365**: 499–516.
- Johnson SD, Bond WJ. 1994. Red flowers and butterfly pollination in the fynbos of South Africa. In: Arianoutsou M, Groves RH, eds. *Plant–animal interactions in Mediterranean-type ecosystems*. Dordrecht: Kluwer Academic Publishers, 137–148.
- Johnson SD, Brown M. 2004. Transfer of pollinaria on birds' feet: a new pollination system in orchids. *Plant Systematics and Evolution* **244**: 181–188.
- Johnson SD, Edwards TJ. 2000. The structure and function of orchid pollinaria. *Plant Systematics and Evolution* **222**: 243–269.
- Johnson SD, Ellis A, Dötterl S. 2007. Specialisation for pollination by beetles and wasps: the role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). *American Journal of Botany* **94**: 47–55.
- Johnson SD, Hobbhahn N. 2010. Generalized pollination, floral scent chemistry, and a possible case of hybridization in the African orchid *Disa fragrans*. *South African Journal of Botany* **76**: 739–748.
- Johnson SD, Nicolson SW. 2008. Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biology Letters* **4**: 49–52.
- Johnson SD, Peter CI, Ellis AG, Boberg E, Botes C, Van der Niet T. 2011. Diverse pollination systems of the twin-spurred orchid genus *Satyrium* in African grasslands. *Plant Systematics and Evolution* **292**: 95–103.
- Johnson SD, Steiner KE. 2003. Specialised pollination systems in southern Africa. *South African Journal of Science* **99**: 345–348.
- Kevan P, Giurfa M, Chittka L. 1996. Why are there so many and so few white flowers? *Trends in Plant Science* **1**: 280–284.
- Knudsen JT, Tollsten L, Groth I, Bergstrom G, Raguso RA. 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society* **146**: 191–199.
- Linder HP, Kurzweil H. 1999. *Orchids of southern Africa*. Balkema: Rotterdam.
- Maia R, Eliason CM, Bitton PP, Doucet SM, Shawkey MD. 2013. pavo: an R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution* **4**: 906–913.
- Marloth R. 1895. The fertilization of *Disa uniflora* Berg. by insects. *Transactions of the South African Philosophical Society* **7**: 74–88.
- Mayfield MM, Waser NM, Price MV. 2001. Exploring the 'most effective pollinator principle' with complex flowers: bumblebees and *Ipomopsis aggregata*. *Annals of Botany* **88**: 591–596.
- Micheneau C, Johnson SD, Fay MF. 2009. Orchid pollination: from Darwin to the present day. *Botanical Journal of the Linnean Society* **161**: 1–19.
- Muchhala N, Thomson JD. 2010. Fur versus feathers: pollen delivery by bats and hummingbirds and consequences for pollen production. *American Naturalist* **175**: 717–726.
- Newman E, Manning J, Anderson B. 2014. Matching floral and pollinator traits through guild convergence and pollinator ecotype formation. *Annals of Botany* **113**: 373–384.

- Nilsson LA. 1988.** The evolution of flowers with deep corolla tubes. *Nature* **334**: 147–149.
- Odeen A, Hastad O. 2010.** Pollinating birds differ in spectral sensitivity. *Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology* **196**: 91–96.
- Ohloff G. 1994.** *Scent and fragrances: the fascination of odours and their chemical perspectives*. Berlin: Springer-Verlag.
- Ollerton J, Alarcon R, Waser NM, Price MV, Watts S, Cranmer L, Hingston A, Peter CI, Rotenberry J. 2009.** A global test of the pollination syndrome hypothesis. *Annals of Botany* **103**: 1471–1480.
- Pauw A. 2007.** Collapse of a pollination web in small conservation areas. *Ecology* **88**: 1759–1769.
- Pauw A, Stoffberg J, Waterman RJ. 2009.** Flies and flowers in Darwin's race. *Evolution* **63**: 268–279.
- van der Pijl L, Dodson CH. 1966.** *Orchid flowers: their pollination and evolution*. Coral Gables, FL: University of Miami Press.
- Porsch O. 1926.** Vogelblütige Orchideen. I. *Biologia Generalis* **2/1**: 107–136.
- Proctor M, Yeo P, Lack A. 1996.** *The natural history of pollination*. London: Collins.
- Raimondo D, von Staden L, Foden W, Victor JE, Helme NA, Turner RC, Kamundi DA, Manyama PA. 2009.** *Red List of South African plants*. Pretoria: South African National Biodiversity Institute.
- Rebelo AG. 1987.** Sunbird feeding at *Satyrium odorum* Sond. flowers. *Ostrich* **58**: 185–186.
- Schiestl FP, Johnson SD. 2013.** Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution* **28**: 307–315.
- Schlamowitz R, Hainsworth FR, Wolf LL. 1976.** On the tongues of sunbirds. *Condor* **78**: 104–107.
- Shrestha M, Dyer AG, Boyd-Gerny S, Wong BBM, Burd M. 2013.** Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytologist* **198**: 301–310.
- Stebbins GL. 1970.** Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* **1**: 307–326.
- Steenhuisen SL, Raguso RA, Johnson SD. 2012.** Floral scent in bird- and beetle-pollinated *Protea* species (Proteaceae): chemistry, emission rates and function. *Phytochemistry* **84**: 78–87.
- Van der Niet T, Hansen DM, Johnson SD. 2011.** Carrion mimicry in a South African orchid: flowers attract a narrow subset of the fly assemblage on animal carcasses. *Annals of Botany* **107**: 981–992.
- Van der Niet T, Juergens A, Johnson SD. in press.** Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species? *Plant Biology*. doi: 10.1111/plb.12196.
- Van der Niet T, Pirie MD, Shuttleworth A, Johnson SD, Midgley JJ. 2014.** Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub *Erica plukenetii*? *Annals of Botany* **113**: 301–315.
- Vogel S. 1954.** *Blütenbiologische Typen als Elemente der Sippengliederung, dargestellt anhand der Flora Südafrikas*. Jena: Fischer.
- Waser NM, Chittka L, Price MV, Williams NM, Ollerton J. 1996.** Generalization in pollination systems, and why it matters. *Ecology* **77**: 1043–1060.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of scent compounds detected in *S. rhodanthum* samples from Ixopo and Highflats, sampled during the day and evening. Kovats retention indices for these compounds are based on comparison to a set of alkanes run on the same GC-MS machine as the *S. rhodanthum* samples. Compound identifications are provided if the Kovats retention indices matched those published in the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011; MS search software version 2.0d) and if retention indices could be verified, where possible, using those of authentic standards. For identified compounds the CAS (Chemical Abstracts Service) number is provided; for unknown compounds the six most frequent mass fragments are given. The percentage of each compound in the headspace of respective samples is provided. Red-coloured cells indicate presence of a particular compound. Emission rates are given at the bottom of each column.

Video S1. Two clips recorded with a motion-trigger camera of amethyst sunbirds feeding on *S. rhodanthum* at Highflats. In both cases it is clearly visible that the bird inserts its bill into several flowers to lick up nectar. Pollinaria can be seen as yellow masses near the tip of the upper mandible in both clips.