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Short communication

Preliminary observations of insect pollination in *Protea punctata* (Proteaceae)

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Abstract

Available information on the phylogeny and pollination systems in *Protea* suggests that bird-pollination is ancestral and occurs in the majority of species, and that there have been several shifts to rodent pollination and a single shift to pollination by cetoniine beetles in this large African genus. Here we report that *Protea punctata* plants in a population in the Swartberg mountains are pollinated primarily by long-proboscid flies and butterflies. The threadlike pollen presenter on an unusually flexible style facilitates insect pollination in this species. The length of the style matches that of the proboscides of its two most common visitors, the nemestrinid fly *Prosoeca longipennis* and the nymphalid butterfly *Aeropetes tulbaghia.* Spectral reflectance of the involucral bracts is similar to that of flowers of other plants visited by long-proboscid flies. *P. punctata* occupies a recently diverged position in a clade (the "white proteas") in which all the other species appear to be bird-pollinated, and may represent a shift to insect pollination in the genus.

Keywords: Aeropetes tulbaghia; Cape flora; Nemestrinidae; Pollinator shift; Prosoeca; Protea; Spectral reflectance; South Africa

1. Introduction

Evolutionary shifts between pollination systems are considered one of the important drivers of floral diversification in flowering plants (Stebbins, 1970; Van der Niet and Johnson, 2012). There is currently much debate about the importance of pollinator-driven diversification in the Cape flora (Schnitzler et al., 2011; Valente et al., 2012; Van der Niet and Johnson, 2009), but this debate is hampered by the fact that pollination systems remain unknown for the vast majority of species in the Cape flora.

Floral diversification in the genus *Protea*, one of the iconic elements of the Cape fynbos vegetation, has traditionally been associated with adaptations for pollination by birds and rodents (Wiens et al., 1983), but recent studies have revealed that there was also at least one shift from bird to beetle pollination in a clade of grassland species (Steenhuisen and Johnson, 2012b). Experiments in which birds have been selectively excluded from inflorescences of "ornithophilous" *Protea* species suggest that small insects can contribute to pollination (Wright et al., 1991), but some of these earlier studies did not control for the contribution of autogamy to seed production and may have therefore overestimated the role of insect pollination (Steenhuisen and Johnson, 2012a). Although specialization for insect pollination is likely in some of the smaller flowered Cape species, actual field studies of these species have not been undertaken.

Our initial observations of *Protea punctata* Meisn. in the Swartberg mountains indicated that its relatively small and vivid pink flowerheads are visited far more commonly by insects than by birds. *P. punctata* is a member of a clade (the "white proteas") that is thought to have diverged from the main *Protea* lineage about 8 million years ago, and the species itself is possibly less than 2 million years old (Schnitzler et al., 2011). Given that this

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clade has previously been considered to be entirely bird-pollinated (Carlson and Holsinger, 2010; Schnitzler et al., 2011) and that bird-pollination is well documented in closely related species, such as *Protea roupelliae* (Hargreaves et al., 2004), we hypothesized that *P. punctata* may represent the outcome of an evolutionary shift from bird to insect pollination. The aim of this study was to conduct further observations of *P. punctata* to establish if insects are potentially effective pollinators and to quantify floral traits that may be important in mediating the interaction between this plant species and its pollinators.

2. Materials and methods

2.1. The study species

P. punctata Meisn. is widely distributed on dry rocky slopes at high altitudes (1200–2000 m) in the Cape floristic region, from the Cederberg in the north west to the start of the Riviersonderend mountains in the south west and then through the Swartberg range as far as the Kammanasie mountains in the south east. It is a shrub up to 4 m tall and has inflorescences ranging from white to brilliant pink in colour. The pollen presenters are unusual in the genus in being threadlike, rather than stiff and knoblike (Rourke, 1980). Flowering occurs mainly in March and April (Rourke, 1980).

2.2. Study site and pollinator observations

We studied a population of several dozen *P. punctata* plants growing at ±1500 m at Tierhoek on the northern slopes of the Swartberg (33°23′S 22°25′E). Observations were conducted on 22 March 2011 and 19–20 March 2012. We camped at the population, allowing a total of 19 h of observations by each of the three observers, commencing from sunrise which is a peak period for bird activity. All insects and birds observed visiting flowerheads of *P. punctata* and making contact with the pollen presenters were recorded. The proboscis length of captured insects was measured and insects examined under a dissecting microscope to establish the location of pollen. Reference slides were made of pollen from *P. punctata*, *Geissorhiza fourcadei* (L. Bolus) G.J. Lewis (Iridaceae) and *Wahlenbergia guthriei* L. Bolus (Campanulaceae) as flowers of these species were observed to be the main sources of nectar for *Prosoeca longipennis* Loew (Nemestrinidae), the most common long-proboscid fly at the study site. No congenerics of these three species were in flower, making pollen identification on the insects straightforward. Voucher specimens for *P. punctata* (*Johnson* s.n.) are deposited in the Bews Herbarium. Insect voucher specimens will be deposited in the Iziko Museum.

2.3. Floral traits

Style length was measured in three florets from each of 10 flowerheads of *P. punctata*. Spectral reflectance measurements were taken from the middle of the outer side of involucral bracts (10 flowerheads), undehisced anthers (five flowerheads) and pollen presenters (one flowerhead) using an Ocean Optics S2000 spectrometer, as described by Johnson and Andersson (2002). Headspace samples of volatile emissions from *P. punctata* plants were taken from a single intact flowerhead in the field and from two cut flowering branches in the laboratory, along with control samples from an intact non-flowering branch in the field and two cut non-flowering branches in the laboratory. Samples were taken for 30 min each and analysed using coupled gas chromatography–mass spectrometry, as described by Shuttleworth and Johnson (2009). Nectar concentration was measured with a Bellingham and Stanley 0–50% refractometer. Small quantities of nectar were collected from nine flowerheads by probing the nectaries of a single flowerhead with a 5 μl micropipette until enough nectar was present (>3 μl) for accurate measurement of concentration.

3. Results

3.1. Pollinator observations

The most common visitors to flowerheads of *P. punctata* during the 3 days of observations were the nemestrinid fly *P. longipennis* Loew (>50 individuals observed feeding from the flower heads) and the nymphalid butterfly *Aeropetes tulbaghia* L. (19 individuals observed). Both of these insect species make extensive contact with the pollen presenters and stigma while feeding on nectar (Fig. 1b–d). We also recorded visits by honeybees *Apis mellifera* (±20 individuals), the solitary bee *Amegilla* (±10 individuals), the carpenter bee *Xylocopa capitata* Smith (two individuals), the cetoniine beetle *Trichostetha signata* (Fabricius) (three individuals), and three other nemestrinid fly species, *Prosoeca westermanni* Wiedemann (three individuals), nemestrinid sp. 1 (two individuals) and nemestrinid sp 2 (±10 individuals). These insects have much shorter proboscides (<20 mm) and were observed to contact the pollen presenters only occasionally when descending through them to feed on nectar. Only three visits by sunbirds (unidentified females) to *P. punctata* flowerheads were observed during the 3 days of observations.

Protea pollen was found on the underside of the thorax and proboscis of individuals of the long-proboscid flies *P. longipennis*, *P. westermanni*, and *Prosoeca* sp 1, the proboscis, head, underside of the thorax, and wings of the butterfly *A. tulbaghia,* and the underside and head of the cetoniine beetle *T. signata* (Table 1). Only *P. longipennis* carried pollen of other plant species. These flies carried a mean (± S.D) of 90.5 ± 176 *Geissorhiza* pollen grains on their undersides, and 3.0 ± 6.0 and 3.0 ± 4.2 *Wahlenbergia* pollen grains on their undersides and heads, respectively.

3.2. Floral traits

The length of the styles of *P. punctata* was 32.7 ± 1.64 mm, which corresponds closely to the length of the proboscides of the main insect flower visitors (Table 1). The average concentration of nectar in *P. punctata* flowerheads was 13.0 ± 1.4%.

We did not detect volatile emissions specific to flowerheads of *P. punctata*. All of the volatiles (various monoterpenes, anisole, and benzaldehyde) recorded in samples taken from flowerheads were also recorded in similar quantities in control samples taken from non-flowering branches, and were thus

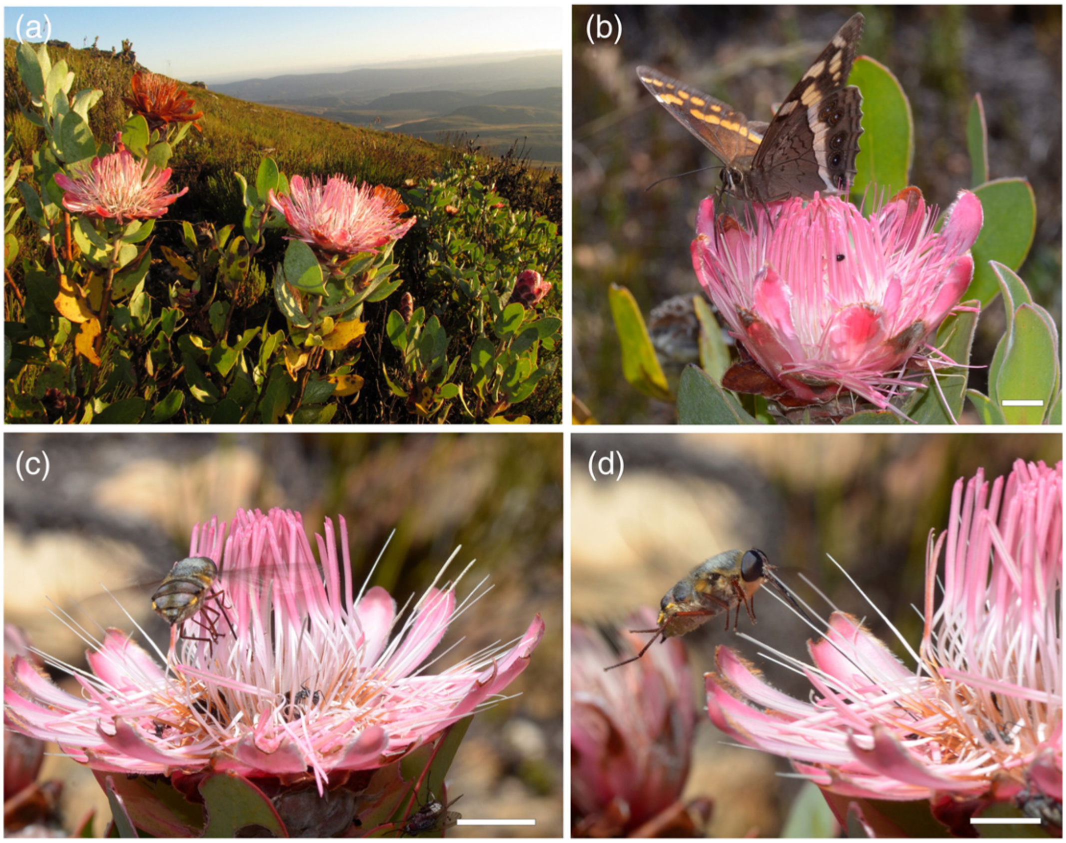

Fig. 1. *Protea punctata* and insect pollinators feeding on nectar from its inflorescences. (a) Flowering *P. punctata* plant in the Swartberg mountains. (b) Mountain pride butterfly (*Aeropetes tulbaghia*). (c,d) Long proboscid tanglewing fly *Prosoeca longipennis*. Scale bars = 10 mm.

considered to be either emitted by leaves or to be environmental contaminants.

Spectral reflectance of the involucral bracts of *P. punctata* was found to be similar to that of flowers of *G. fourcadei* and *W. guthriei* in the 350–600 nm wavelength band used in many insect visual systems (Fig. 2).

4. Discussion

The results of this preliminary study suggest that *P. punctata* is effectively pollinated by insects. We observed an excellent fit between the length of the pollen presenters and the proboscides of the two most frequent visitors, the long-proboscid fly *P. longipennis* and butterfly *A. tulbaghia* (Fig. 1; Table 1). Although the proboscis of *P. longipennis* measures ± 26 mm in its unextended state, it can extend to > 30 mm during feeding, visible in Fig. 1d). Pollen of *P. punctata* was found on most of the captured insect visitors (Table 1). Pollen transfer via the wings of *A. tulbaghia* has been recorded previously for several Cape plants, including the amaryllids *Nerine sarniensis* Herb. and *Brunsvigia marginata* Ait. (Johnson and Bond, 1994). Although *A. tulbaghia* butterflies visiting *P. punctata* carried more pollen than *P. longipennis* flies, the flies had *P. punctata* pollen concentrated on a much smaller body surface that routinely contacted the pollen presenter (Fig. 1c).

The nectar of *P. punctata* is relatively dilute, but still within the range of concentration values reported for plant species pollinated by long-proboscid flies and *Aeropetes* (Goldblatt and Manning, 2000; Johnson and Bond, 1994; Johnson and Steiner, 1997). A previous study showed that the nectar of *P. punctata* is composed

Table 1
The proboscis lengths and pollen loads of insects captured on flowerheads of *Protea punctata.* Values for pollen loads are means ± S.D.

Insect family	Insect species	Sample size		Proboscis length (mm)	Number of *Protea* pollen grains			
		Observed	Captured		Proboscis	Head	Thorax underside	Wings
Nemestrinidae	*Prosoeca longipennis*	50	4	25.7 ± 3.5	4.0 ± 4.2	8.0 ± 7.1	16.8 ± 26.3	0 ± 0
	P. westermanni	3	3	16.8 ± 2.1	18.0 ± 11.3	0 ± 0	39.7 ± 7.7	0 ± 0
	Prosoeca sp. 1	2	2	12.2 ± 0.4	7.5 ± 6.3	0 ± 0	4.5 ± 6.3	0 ± 0
Nymphalidae	*Aeropetes tulbaghia*	19	3	30.2 ± 2.6	139 ± 229	30.0 ± 8.5	44.1 ± 46.7	543 ± 437
Cetoniidae	*Trichostetha signata*	1	1	–	0 ± 0	16 ± 0	20 ± 0	0 ± 0

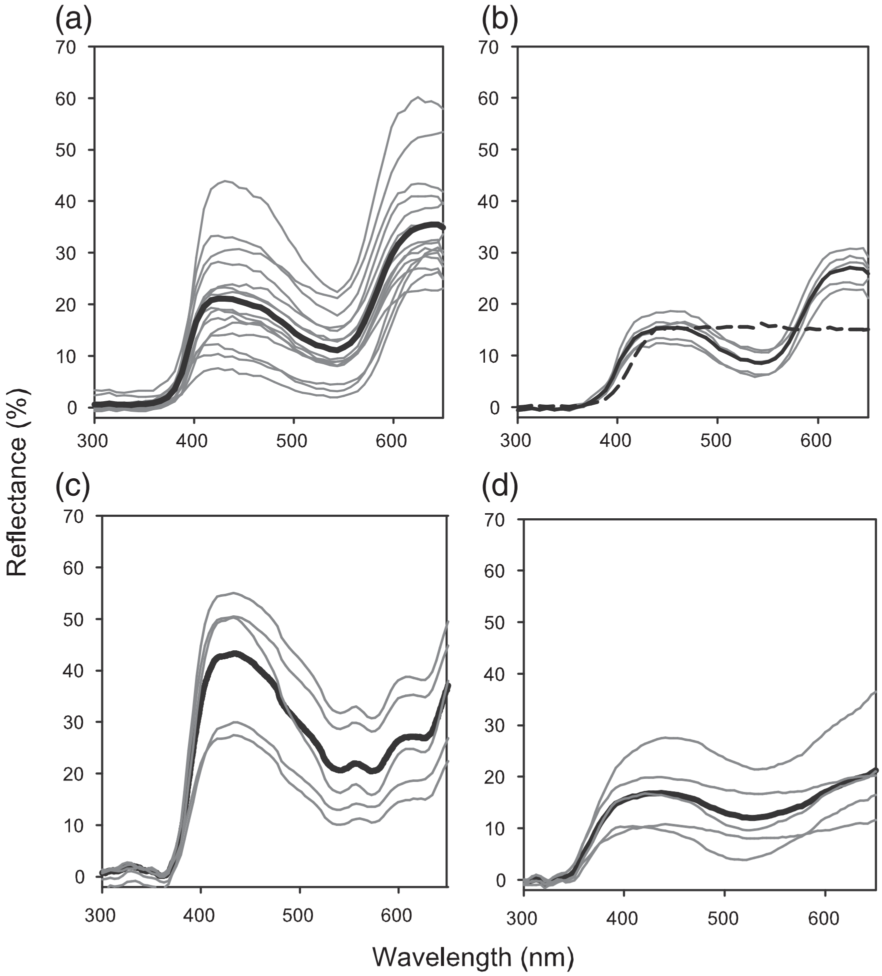

Fig. 2. Spectral reflectance of plant species visited by *P. longipennis* at the Tierhoek site. (a) Inner surface of *Protea punctata* involucral bracts (b) *Protea punctata* perianth (solid lines) and a sample of a pollen-laden presenter (dashed). (c) *Wahlenbergia guthriei*. (d) *Geissorhiza fourcadei*. In all cases individual samples are indicated with grey lines and the mean reflectance with a bold line.

solely of sucrose (Nicolson and Van Wyk, 1998). Nectar sugar composition is highly variable in *Protea*, but most bird-pollinated species, including *P. aurea* and *P. subvestitata* which are closely related to *P. punctata* have nectar dominated by hexose sugars (Nicolson and Van Wyk, 1998). Sugar composition is highly variable in the guilds of plants pollinated by long-proboscid flies (Goldblatt and Manning, 2000) and *Aeropetes* (Johnson and Bond, 1994), suggesting that these insects do not impose strong selection on this trait.

In terms of human perception, involucral bracts of *P. punctata* are very similar in colour to those of many other plants pollinated by long-proboscid flies (Goldblatt and Manning, 2006). Floral spectral reflectance patterns of *P. punctata* (Fig. 2) are similar to those of two sympatric species which were also pollinated by *P. longipennis,* as well as other plant species pollinated by long-proboscid flies (Johnson, 2000). The attraction of mountain pride butterflies *A. tulbaghia* to the pink flowerheads of *P. punctata* was unexpected given that studies elsewhere have shown this butterfly to be most strongly attracted to red and orange flowers (Johnson and Bond, 1994; Newman, et al., 2012). Newman et al. (2012) recently demonstrated geographical variation in the colour preference of *A. tulbaghia* and invoked conditioning by locally abundant nectar plants as one possible explanation.

The butterflies may have become locally conditioned to visit pink flowers at the study site because *P. punctata* is their most abundant source of nectar. Interestingly, there is anecdotal evidence from a photograph in a wildflower guide (Manning, 2004a, 2004b) that *A. tulbaghia* visits *P. punctata* elsewhere in the Swartberg mountains.

In *Protea,* the bird-pollinated species are generally unscented, while the rodent- and beetle-pollinated species are scented (Steenhuisen et al., 2010; Wiens et al., 1983). Our finding that flowerheads of *P. punctata* are effectively unscented suggests that this species has not experienced the same selection for scent emission that occurred during shifts from bird to rodent or beetle pollination in other parts of the *Protea* lineage. Although the full spectrum of pollinators of *P. punctata* has yet to be determined, it does seem plausible that its lack of floral scent emission is related to it being pollinated mostly by long-proboscid flies and the mountain pride butterfly, insects that rely mainly on visual cues for locating flowers (Goldblatt and Manning, 2000; Johnson and Bond, 1994).

This study is the first to implicate long-proboscid flies in the pollination of a *Protea* species. Elsewhere in the Proteaceae, there have been reports of long-proboscid fly pollination in *Leucospermum tottum* (L.) R. Br. in South Africa (Manning, 2004a, 2004b) and *Embothrium coccineum* Forst. in South America (Devoto et al., 2006). Interestingly, *L. tottum* may contain two pollination ecotypes — var. *tottum* pollinated by long-proboscid flies and var. *glabrum* likely to be bird-pollinated (Manning, 2004a, 2004b). Devoto et al. (2006) suggested that bird-pollination in *Embothrium* is recent and is derived from ancestral pollination by nemestrinid flies, which are a much older group of pollinators than hummingbirds in South America.

These findings are preliminary and further work is required to establish whether long-proboscid flies and butterflies are important pollinators in other *P. punctata* populations. While the mountain pride butterfly *Aeropetes* occurs throughout the range of this *Protea* species, *P. longipennis* was known mainly from the southern Cape (Manning and Goldblatt, 1995). However, our discovery of these flies on the northern slopes of the Swartberg suggests that they may have a wider distribution along the margins of the Karoo basin. Given that several long-proboscid fly species were attracted to flowerheads of *P. punctata* at the Swartberg site, we expect that it is fairly generalist in its interactions with insects, and that other long-proboscid fly species, such as *Prosoeca ganglbaueri* and *Moegistorynchus perplexus* (Nemestrinidae) and *Philoliche rostrata* (Tabanidae), could be visitors in populations in other parts of the distribution range.

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