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RESEARCH ARTICLE

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Flower mites steal *Protea neriifolia* pollen and nectar

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Flower mites are well-known nectar and pollen thieves of hummingbird-pollinated plants in the Americas, where they may reduce seed set and alter host population dynamics. They use hummingbirds for transport and are pollinators of some plants. Among African ornithophilous *Protea* shrubs, the hummingbird-pollination niche is occupied by sugarbirds and sunbirds that often carry substantial numbers of flower mites. The role of these mites in *Protea* pollination and seed set is unknown. We investigated the role of flower mites as pollinators of ornithophilous *Protea neriifolia* in South Africa using field-based exclusion experiments. Their role as pollen and nectar consumers was quantified using laboratory-based feeding studies. We demonstrate that even though they consume pollen and nectar, flower mites are not pollinators of *P. neriifolia*. Quantification of nectar consumption rates indicated that these mites likely have little effect on nectar availability for pollinating birds. However, flower mites may consume more than 50% of available *P. neriifolia* pollen when mite numbers peak. Flower mites on African ornithophilous *Protea* may therefore significantly decrease *Protea* male fitness and significantly impact *Protea* population dynamics.

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INTRODUCTION

Many flowering plant species rely on animals for pollination and, in turn, may provide nectar and pollen rewards for this service. However, flowers often also host organisms that exploit these resources without providing pollination services. These are considered nectar and pollen robbers (Colwell 1973; Guerra et al. 2010; Inouye 1980) that can have substantial ecological and evolutionary consequences as they affect host plant population dynamics (Hargreaves et al. 2009, 2010; Irwin et al. 2001, 2010).

A particularly well-studied multipartite robber system involves associations between hummingbirds, plants, and flower mites (Acari: Mesostigmata: Melicharidae). In this system, flowers that are adapted for hummingbird pollination are also occupied by flower mites (Irwin et al. 2001; Lara and Ornelas 2002a, 2002b; Maloof and Inouye 2000) that disperse to new flowers either on the beaks or within the nostrils of the birds (Colwell 1973, 1995; Proctor and Owens 2000). These mites consume large quantities of pollen and nectar (Colwell 1973, 1995; Paciorek et al. 1995), which leads to a decrease in the number of male gametes available for pollination and may decrease reproductive success (Burkle et al. 2007; Irwin et al. 2001; Maloof and Inouye 2000). However, hummingbird-associated flower mites may also act as pollinators, at least for some self-compatible, non-autogamous plant species (Dobkin 1984, 1987, 1990; Lara and Ornelas 2002a, 2002b).

Although flower mite-bird-plant interactions are well-studied in the Americas, similar systems have received no attention in the rest of the world, despite the near-global distribution of flower mite genera in the family Melicharidae (Eliaderani et al. 2013; Halliday et al. 1998; Krantz and Walter 2009). In South Africa, for example, certain members of the plant genus *Protea* L. (Proteaceae) are primarily pollinated by sugarbirds (Promeropidae) and sunbirds (Nectariniidae) (Gideon et al. 1980; Nicolson and Flemming 2003). The inflorescences and infructescences of these *Protea* species also house numerous mite species (Ryke 1964; Roets et al. 2007, 2009; Theron 2011; Theron et al. 2012; Theron-de Bruin et al. 2018). The flower mite *Proctolaelaps vandenbergi* Ryke (Melicharidae) often attains particularly high numbers (upwards of 60,000 per inflorescence) in *Protea* inflorescences (Myburgh et al. 1973). Even though various insects can vector these mites (Roets et al. 2007, 2009), *Protea*-pollinating birds are their main vectors (Theron-De Bruin et al. 2018). Like other flower-associated members of the genus, *P. vandenbergi* may also principally feed on nectar and pollen (Colwell and Naeem 1994; Dobkin 1984; Krantz and Lindquist 1979; Krantz and Walter 2009; Paciorek et al. 1995; Royce and Krantz 1989) and may therefore be nectar and pollen thieves (Colwell 1995; Hargreaves et al. 2009; Paciorek et al. 1995).

Natural *Protea* seed set is usually low, with infructescences containing only 1 - 30 % fertile seeds (Collins and Rebelo 1987; Rebelo and Rourke 1986). This low seed set has been ascribed to various factors, including a shortage of pollinators, inadequate pollen transfers or resource shortages (Littlejohn 2001; Rebelo and Rourke 1986). As was found in the hummingbird system, consumption of pollen and/or nectar by large numbers of flower mites may offer an additional explanation for the unusually low seed set in *Protea* (Colwell 1995; Paciorek et al. 1995). Conversely, flower mites may act as pollinators of *Protea*. This can either be through the transfer of pollen from one plant to the next (outcrossing) enabled through phoresy on birds (Theron-de Bruin et al. 2018), or when moving around within inflorescences (inbreeding). *Protea* flowers are protandrous

(anthers mature before the stigma) and have a modified style to which pollen is attached laterally (Collins and Rebelo 1987; Van der Walt and Littlejohn 1996a, 1996b). Stigmas become receptive (opening of a narrow split) *ca*. 48 hours after pollen release (Ramsey and Vaughton 1991). The maturation of sexually active flowers progresses from the outer ring of the inflorescence towards the centre. This difference in maturing time, to some extent, prevents self-pollination. However, as *Protea* species are generally self-compatible (Nottebrock 2016; Steenhuisen et al. 2012; Steenhuisen and Johnson 2012; Van der Walt and Littlejohn 1996a), the transfer of pollen from another flower within the same inflorescence may still lead to fertilization. Therefore, as mites move around within inflorescences, they may deposit pollen inside mature stigmatic grooves and enhance fertilization (Kaufmane and Rumpunen 2002). This kind of self-fertilization can lead to inbreeding depression, with the associated reduced flowering and survival of later generations (Charlesworth and Willis 2009; Forrest et al. 2011; Robertson et al. 2011).

Here we investigate the role of flower mites on a bird-pollinated African ornithophilous *Protea* species. We hypothesised that *Protea*-associated flower mites may act as pollinators. We postulated that, as with other members of the genus, *P. vandenbergi* mites feed on pollen and nectar of *Protea* and can reproduce using only these resources (Heyneman et al. 1991; Krantz and Walter 2009). As an alternative hypothesis we, propose that, as in the hummingbird system, flower mites may remove significant amounts of nectar and pollen, potentially hampering *Protea* pollination.

MATERIALS AND METHODS

Study species

Protea neriifolia (Figure 1A) is a widely distributed shrub species in the Cape Floristic Region of South Africa, globally recognised as a biodiversity hotspot (Myers et al. 2000). It often dominates fynbos plant communities (Cambell and Van der Meulen 1980; Rebelo 2001; Van Wilgen and McDonald 1992) and is widely cultivated for the flower export market (Leonhardt and Criley 1999; Littlejohn 2001). It produces large and colourful inflorescences during most of the year (Feb. to Nov.) (Coetzee et al. 2007; Rebelo 2001) and is primarily pollinated by birds (*Promerops cafer* and *Anthobaphes violacea*), although insects such as beetles may also play a minor role (Wright et al. 1991; Wright and Saunderson 1995). This species also houses particularly large numbers of inflorescence-associated mites such as *Proctolaelaps vandenbergi* (Roets et al. 2009, 2013; Theron et al. 2012) that use the flower visitors as vectors to new inflorescences (Theron-de Bruin et al. 2018). We determined whether flower mites play a role in the pollination of *P. neriifolia* by determining whether they can carry pollen grains and by conducting field-based pollinator exclusion experiments.

Flower mites as carriers of Protea *pollen*

During Oct. 2014, 20 *P. neriifolia* inflorescences, each from a different tree, at mid flowering stage (~40–60% of flowers open) were collected from Jonkershoek Nature Reserve, Stellenbosch (33°59′24.5″ S, 18°57′25.2″ E). Inflorescences were transported

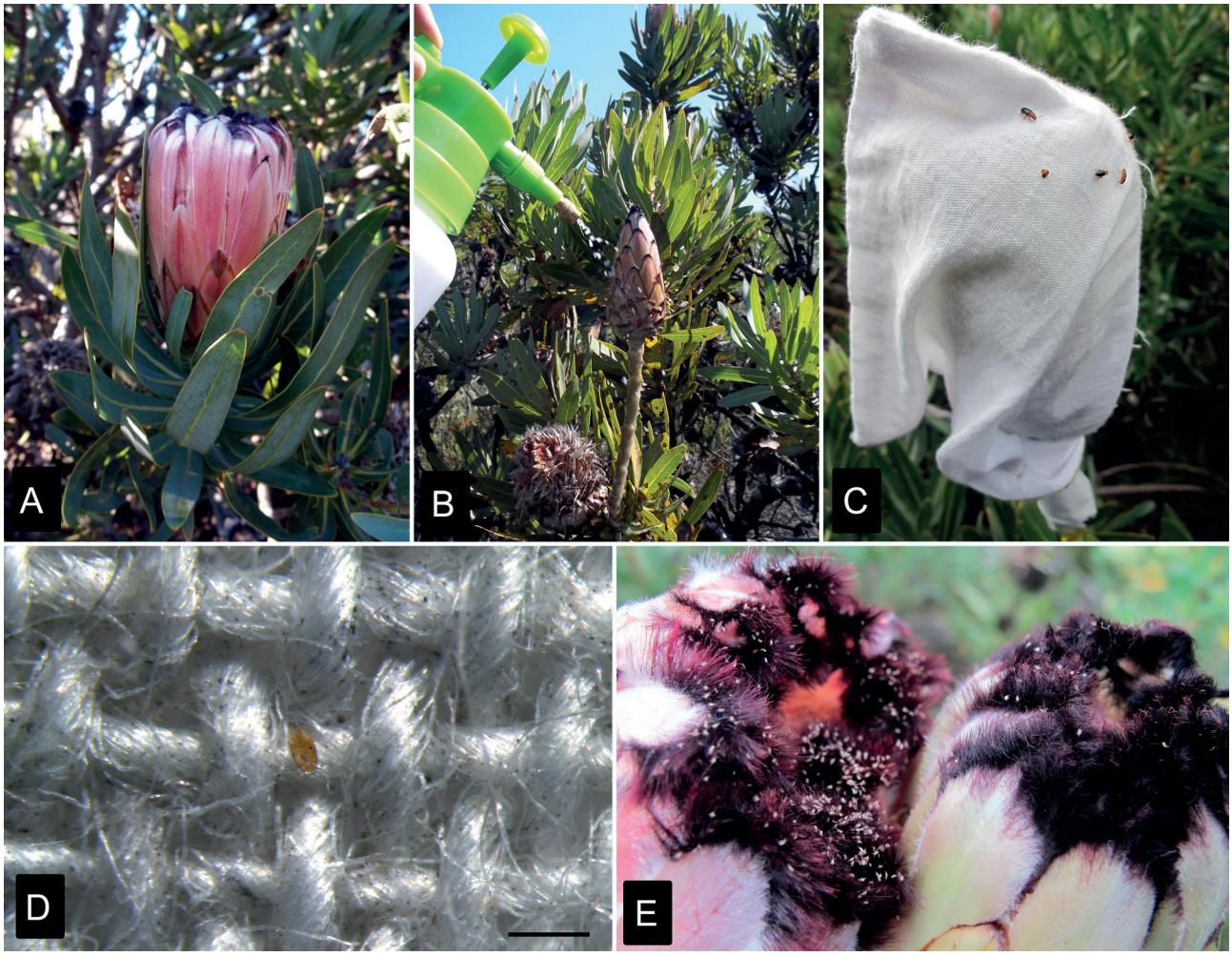

Figure 1. (A) *Protea neriifolia* inflorescence; (B) Applying EKO-spray to experimental *Protea neriifolia* bud; (C) *Protea neriifolia* inflorescence covered by material bag to exclude insect and bird visitors such as *Chirodica chalcoptera* (Chrysomelidae) beetles depicted; (D) Close-up of *Tarsonemus* sp. mite on material bag (Scale bar = 0.04 mm); (E) Transfer of *Proctolaelaps vandenbergi* mites to uncolonized *Protea neriifolia* inflorescence (see Methods section: Flower mites as pollinators).

upright in a bucket with added water to keep them fresh. Mites that were disturbed in the process of transport often accumulated at the tops of inflorescences but soon retreated into the structures after translocation to the laboratory. In the laboratory, inflorescences were individually placed in water-filled vases and re-visited after two days when flower-associated mites started to accumulate at the top of the inflorescences in anticipation of the arrival of pollinators for transport (Theron-de Bruin et al. 2018). Mites were collected from these structures following methods described in Theron-de Bruin et al. (2018). Broadly, this entailed collecting mites for 40 seconds from the top of inflorescences using adhesive tape strips (one strip per inflorescence). A hundred, randomly chosen mites per adhesive strip were examined for the presence of pollen. Mites were only counted as positive for carrying pollen when pollen grains were stuck to their integument (Dobkin 1984). Data were recorded as presence/absence only as, when present, pollen grains were often innumerable. Numbers of mite individuals that carried *Protea* pollen were compared between mite species using generalised linear mixed models (GLMM) in R (R Development Core Team 2020) and the *lme4* package (Bates and Sarkar 2008). The percentage data was fitted to a binomial curve (with Laplace approximations) and the specific inflorescence from which mites were collected was included as a random variable (random intercept model) as this improved the model significantly as judged by the Akaike Information Criterion (AIC = 210.28 vs. AIC = 867.47). A Tukey post-hoc test in the R package *multcomp* was used to determine pairwise differences in percentages of mites by taxa that carried pollen (Horthawn et al. 2020). Significant differences were reported when $P \leq 0.05$.

Flower mites as Protea *pollinators*

Four exclusion experiments were conducted in three natural *P. neriifolia* populations: Du Toits Kloof Pass (33°41′45.2″ S, 19°05′14.2″ E), Jonkershoek Nature Reserve and Franschoek Pass (33°55′10.2″ S, 19°09′42.0″ E), during March 2014 in the Western Cape province, South Africa. At each site, 90 young inflorescences (before flowers opened), each on a different *P. neriifolia* plant, were treated with organic SK ECO oil spray (Makhro-Agro, SA (Pty) Ltd), an environmentally friendly acaricide and insecticide to eliminate all arthropods even before they had access to the flowers. SK ECO oil spray (diluted 1:100 water) was applied using a plastic gardening spray bottle until the young inflorescence was thoroughly drenched (Figure 1B). The top 15 cm of leaves on the stem under each young inflorescence were removed to create a smooth surface and the bud was enclosed in a cotton voile muslin fabric bag (Neal and Anderson 2004) to prevent arthropods and birds from visiting (Figure 1C). This material was fine enough to exclude larger arthropods including *Proctolaelaps* mites, but not very small mites such as a *Tarsonemus* sp. (Figure 1D). Each bag was sealed around the stem using durable adhesive tape (duct tape - Sellotape, Henkel limited, UK). These sites were revisited 6 weeks later for experimental treatment once the inflorescences had opened.

The first treatment (All-access) involved the permanent removal of 25 bags per site to allow unhindered flower visitors access to the inflorescences from this stage onwards. The second treatment (Mites added) involved the introduction of mites to 25 pre-treated inflorescences. For these, untreated inflorescences in full flower that contained high abundances of mites on their surface waiting for vectors (Figure 1E) were collected from neighbouring plants. Mites from these untreated inflorescences were allowed to move freely across to the treated inflorescence (Figure 1E). To minimise accidental transferring of pollen to treated inflorescences, untreated inflorescences were brought into contact with treated inflorescences such that the longest bracts of the untreated inflorescence were at least 1 cm below the rim of the open untreated inflorescence. Mites were allowed to self-disperse from untreated inflorescences to the treated inflorescences for 2 min, where after the inflorescences were closed in their bags again. We thus did not standardise for the number of mites per transfer but for mite transfer time. As a negative control (no access = negative control) and to eliminate any interference from flower visitors (to judge levels of autogamy), bags were removed from 25 inflorescences, SK ECO oil was re-applied, and the inflorescences were closed off again (i.e. these were devoid of arthropods throughout the entire flowering stage). For control of the treatment effect (no treatment = positive control), 25 young inflorescences were initially marked at the same time as the bagged ones, but they were never enclosed in a bag at any stage during flowering. After seed set in Mar. 2015 (Van Staden 1978; Wright 1994), all infructescences except those damaged by baboons and/or arthropods were collected to determine the number of viable seeds in each.

Each seed within each infructescence was cut open with a scalpel to determine its fertility. Fertile seeds display clear white cotyledons and a developing embryo when cut horizontally, while non-fertile seeds are woody with a hollow centre (Rebelo 2006). In addition, infructescences were examined for any signs of seed predation by boring insects. If present, these infructescences were excluded from analyses. Seed-set was calculated as the percentage of fertile seeds per intact infructescence (Nottebrock et al. 2013).

As *Protea* species are protandrous, it was necessary to establish the number of stigmas that were available to receive pollen from the stage when mites were introduced onward. Assuming that only this proportion of potential flowers could be pollinated by the transferred mites and that *Protea neriifolia* is self-compatible (Coetzee et al. 2007) and that mite would be able to assist in geitonogamous pollen movement, this would give an upper limit for the percentage of fertile seeds produced because of the actions of the added mites. Therefore, twenty inflorescences at the same flowering stage as that of the experimental inflorescences were collected from the same study sites. They were dissected and individual flowers were separated into open (open stigmatic groove) and already closed flowers (past the pollen-receptive stage) using a dissecting microscope. Seed set results for final analyses for the treatment where mites were added were adjusted by subtracting the mean percentage of flowers that were past the pollen-receptive stage from the total number of flowers within these inflorescences.

The percentage of fertile seeds per inflorescence was compared between treatments using a generalised linear mixed model (GLMM) using R software. The percentage data were fitted to a binomial curve (with Laplace approximations) and the experimental site was included as a random variable (random intercept model) as this improved the model significantly as judged by the Akaike Information Criterion (AIC = 2605.5 vs. AIC = 4412.7). A Tukey post-hoc test was used to determine pairwise differences in percentages of mites that carried pollen.

Flower mites as nectar and pollen robbers

Pollen and nectar availability in *Protea neriifolia* inflorescences

Total pollen availability within inflorescences was estimated for three flowering stages of *P. neriifolia*, defined using percentage of flowers open: stage 1 ~ 30% of flowers open, stage 2 ~ 60% of flowers open and stage 3 ~ 100% of flowers open. Twenty inflorescences per stage, each from a different tree, were collected during Jul. 2017 in Stettynskloof pass (33°47′48.7″ S, 19°19′14.4″ E), Rawsonville and transported to the laboratory in water-filled buckets to keep them fresh. The average pollen load on the pollen presenter per flower was calculated from the total mass of pollen removed from ten randomly selected pollen presenters (using a scalpel blade and weighed on a Lab1st 500 g × 1 mg Analytical Balance) from freshly dehisced flowers that had no evidence of pollen removal, from each inflorescence. The total number of

flowers in each of the collected inflorescences was counted and used to determine the total mass of pollen available on average per inflorescence at each of the three flowering stages when no pollen had been removed.

Flowers within inflorescences mature from the outside inwards. We, therefore, calculated the average daily rate of opening of flowers within *P. neriifolia* inflorescences to estimate the total mass of pollen that becomes available for mites to feed on per day. Ten inflorescences (each from a different *P. neriifolia* individual) at flowering stage 1 (30% flowers open) in the Jonkershoek Nature Reserve were monitored for 5 days. The number of open flowers was counted daily for each of these days and the mean number of newly opened flowers per inflorescence per day was calculated. These data were used to estimate the mean mass of pollen that became exposed per day in an inflorescence during the flowering period.

The volume of nectar available in each of the inflorescences collected from Rawsonville was established for each of the flowering stages. The top half of the inflorescence was removed using pruning shears. We assumed that this action would remove minimal available nectar and that the volume of nectar removed would be consistent between treatments. The inflorescence was then placed inside a clean, re-sealable plastic bag and sealed around the exerted stem. The bagged inflorescences were swung in a circular motion for 15 seconds at a constant speed to produce enough centrifugal force to expel nectar from them (Armstrong and Paton 1990). The nectar that accumulated at the bottom of the bag was collected with a pipette, filtered and quantified (µl) using pipettes. This method captures about 70% of the total volume of nectar produced (Armstrong and Paton 1990). All collected nectar was stored at 4˚C in a sterilized container for later use in feeding studies. Nectar volume was compared between different flowering stages using linear modelling (*lm* function) in R, after square root transformation of the data to ensure normal distribution as determined by a Shapiro-Wilk test in the *nortest* package (Stephens 1986).

Numbers of *P. vandenbergi* mites within inflorescences

The tops of inflorescences that were removed for the quantification of nectar (where *P. vandenbergi* typically gather), were used to establish the numbers of *P. vandenbergi* mites at each flowering stage. These flower parts were placed in separate containers for each inflorescence and then frozen for 2 days to euthanize the mites. The material was dried in an oven at 30˚C for one day, and then shaken by hand for 1 minute to loosen dead and dry mites from the plant material. The material was sieved with a kitchen four sifter to separate mites from larger plant material, where after mites could easily be counted using a dissecting microscope. Data on *P. vandenbergi* numbers were compared between the three stages using a general linear model with Poisson distribution (as this is count data) in R. In addition, we compared our data to data from a previous study on mites associated with *P. neriifolia* inflorescences (Theron-de Bruin et al. 2018) for comparative purposes. In the study of Theron-de Bruin et al. (2018), mites were sampled from the top surface of inflorescences collected during Oct. 2014 in Jonkershoek Nature Reserve (33°59′24.5″ S, 18° 57′25.2″ E), Stellenbosch, when 30–50% of flowers within the inflorescences were open. These data were included as they represented a different season (spring as opposed to winter), which may affect mite numbers within inflorescences. Importantly, immature stages of *P. vandenbergi* are not phoretic. Therefore, due to the collection method used, data from Theron-de Bruin et al. (2018) only included mature mites that were awaiting pollinators for transport to new inflorescences.

Pollen and nectar as a food source for P. vandenbergi *mites*

The feeding, survival and reproduction of mites were tested on a diet of pollen, nectar and a combination of the two. *Proctolaelaps vandenbergii* mites were collected from *P. neriifolia* inflorescences from Stettynskloof Pass in Jul. 2017 and placed in artificial feeding chambers (n = 5, fully grown females per tube) using a fine paintbrush. Feeding chambers consisted of 100 µl Eppendorf tubes (20 replicates per treatment) containing either: 1) 5 µl nectar with pollen-free pollen presenter, 2) 5 µl ddH_2O with pollen-free pollen presenter, 3) 5 µl nectar with pollen-laden pollen presenter and 4) 5 µl water with pollen-laden pollen presenter. Tubes were kept in the dark at room temperature (~ 22°C) for 10 days after which numbers of mites (including eggs and larvae) in each tube were counted. Data were used to calculate and compare the survival rate (as a percentage) of adults and the numbers of eggs, larvae and adults in each tube after 6 days and the population growth (as a percentage) after 10 days. Consumed pollen resources could be enumerated by determining the percentage of pollen removed (visual scoring) from each pollen presenter after 10 days and calculating its mass as a proportion of the mean of mass available per pollen presenter. We were unable to account for any pollen that may have fallen off the pollen presenters rather than having been eaten by the mites and therefore assume that most pollen removed by the mites via feeding or other means would be "lost". Due to the actions of mites within the tubes containing pollen on pollen presenters, it was not possible to determine the amount of nectar consumed in all experimental units. However, fluid consumed in the treatments that contained only nectar or water and no pollen could be determined by pipetting. Data on the percentage of mites that survived after 6 days were analysed using a general linear model with binomial distribution. This model had an AIC value of 230.85 and residual deviance of 171.56 on 57 degrees of freedom. Count data on the numbers of eggs and larvae after 6 days, and population growth after 10 days, were analysed using a Chi-Square test for goodness of fit in R. Data on the number of adults after 6 days were analysed using a general linear model for the non-parametric data (AIC = 230, residual deviance of 142.1 on 57 degrees of freedom). Data on pollen and nectar consumed after 10 days were parametric as determined by a Shapiro-Wilk test and were subsequently analysed using a linear model (*lm*) in R.

RESULTS

Flower mites as carriers of Protea *pollen*

Three mite species were collected from the top of *P. neriifolia* inflorescences at the mid-flowering stage. These included a hypopus of an undetermined mite species, *P. vandenbergi*, and a *Tarsonemus* species. Very few individuals of the *Tarsonemus* and the hypopus carried *Protea* pollen grains (Figure 2A). Significantly more *P. vandenbergi* mites carried *Protea* pollen than either the hypopus or the *Tarsonemus* species ($Z = 9.87$, SE = 0.5021, $p < 0.001$ and $Z = 9.30$, SE = 0.6670; $p < 0.001$, respectively), even though numbers were still low (median = 12%). Similar numbers of the hypopus and the *Tarsonemus* species carried *Protea* pollen ($Z = 0.34$, SE = 0.4505, $p = 0.94$).

Flower mites as Protea *pollinators*

In the pollinator exclusion experiments, an average of 44% of flowers were receptive to pollen (open stigmatic groove) at the time of mite transfer and afterwards. Seed set results for this treatment were therefore adjusted to reflect this before statistical analyses were conducted (*i.e.* the initial number of available flowers was adjusted to 44% of the total number of flowers within inflorescences). Inflorescences from the no-access treatment that were kept closed throughout the experimental period failed to produce any viable seeds, indicating that this species is not autogamous and that our exclusion experiment prevented access by all pollinators. However, these florets may still be fertilized by geitonogamous pollen transfer. Inflorescences to which mites were added also mostly failed to produce viable seeds (Figure 2B). Only seven of these inflorescences contained

viable seeds, but the seed set was always extremely low (max < 2%), which was significantly less than viable seeds produced in the positive control (Z = 14.23, p < 0.001) and the all-access treatment (Z = 10.84, p < 0.001). Inflorescences from the positive control had significantly higher seed set than either the all-access inflorescences (Z = 19.46, p < 0.001) or the ones to which mites were added (Figure 2B).

Flower mites as pollen and nectar robbers

Pollen and nectar availability within inflorescences

Based on calculations of mean pollen mass per intact pollen presenter (0.431 ± 0.112 µg) and total number of pollen presenters in *P. neriifolia* inflorescences, there would be a continuous increase in available pollen mass from stage 1 to stage 3 (assuming no pollen removal) with an estimated 40 µg pollen at stage 1, 80 µg pollen at stage 2 and 133 µg of pollen available when all flowers have opened at stage 3. Flowers within inflorescences opened at a rate of 8.43 ± 3.66 flowers/day/inflorescence. The total mass of pollen that became exposed per day per inflorescence was therefore estimated at 8.43 flowers × 0.431 µg pollen per flower = ~3.63 µg pollen per day.

Unlike pollen, nectar production would be continuous per flower and could therefore not be estimated. In addition, due to a lack of inflorescences that were void of pollen and nectar consumers, the nectar availability reported here is likely underestimated. Nectar availability (as measured from field-collected inflorescences) differed between the different stages (F = 3.50, df = 2, 57, p = 0.037), with the highest volume of nectar available during stage 2 when ~60% of flowers were open (Figure 3A). Nectar availability was statistically similar at stage 1 and stage 3 (t = 0.934, p = 0.621), and stages 1 and 2 (t = 1.68, p = 0.223). Nectar availability decreased significantly from stage 2 to stage 3 (t = 2.610, p = 0.031).

Numbers of *P. vandenbergi* mites present

The model investigating *P. vandenbergi* abundance between different flowering stages had an AIC value of 4271.5 (residual deviance = 3898.6 at df = 57). Abundance was significantly different between all stages with a peak during flowering stage 2 (Figure 3B). Statistical differences as compared to stage 1 were: stage 2, Z = 29.30, p < 0.001; stage 3, Z = 7.28, p < 0.001 and between stage 2 and stage 3, Z = 23.061, p < 0.001.

Pollen and nectar as a food source for P. vandenbergi *mites*

All *P. vandenbergi* mites that fed on the control diet consisting only of water died after 4 days even when ingesting water (Table 1). Mites in other treatments were observed to regularly ingest pollen and nectar and many of these survived for at least 6 days (Table 1). The survival of mites that fed only on nectar

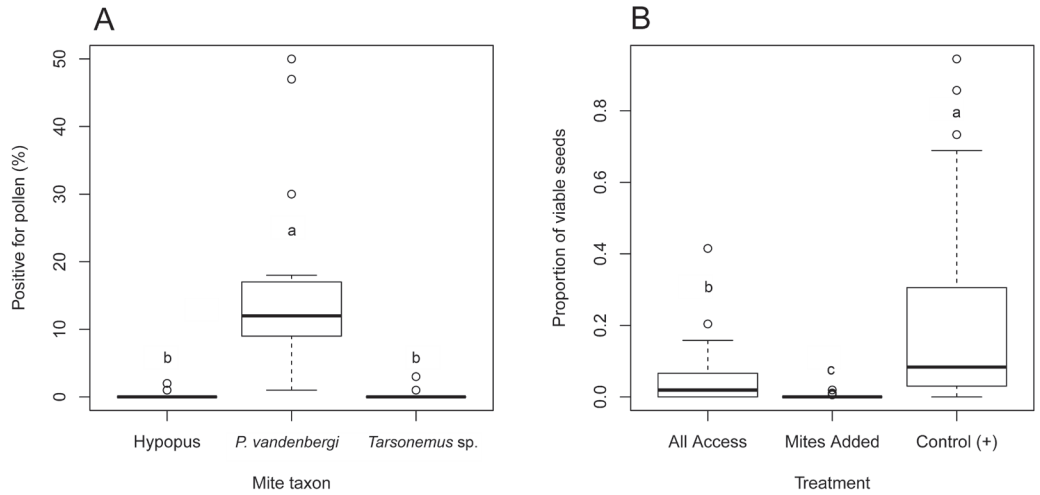

Figure 2. (A) The percentage of three different mite taxa (n = 100 individuals per taxon per inflorescence) collected from *Protea neriifolia* inflorescences (n = 20) that carried *Protea* pollen; (B) *Protea neriifolia* seed set between inflorescences to which mites and other pollinators had access (all access), to which only mites were added (mites added) and positive control to which all flower visitors had access throughout the entire flowering period. Box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range and dots represent outliers.

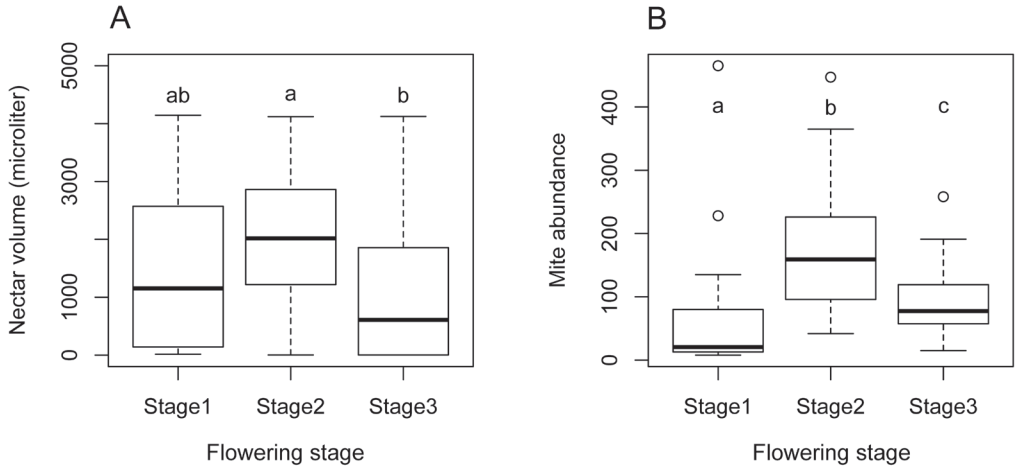

Figure 3. (A) Total volume of nectar (µl) available inside field-collected *Protea neriifolia* inflorescences during three flowering stages (stage 1 = 30% flowers open, stage 2 = 60% flowers open and stage 3 = 100% flowers open); (B) Abundance of *Proctolaelaps vandenbergi* mites collected from *Protea neriifolia* inflorescences at three flowering stages (stages as mentioned for A). Box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range and dots represent outliers.

Table 1. *Proctolaelaps vandenbergi* survival (%) and egg, larvae, and adult numbers on day 6 (D6) after feeding experiments on *Protea neriifolia* pollen and/or nectar. Growth rate (%), pollen consumption (µg) and fluid consumption (µl) were calculated on day 10 (D10). Except for pollen and fluids consumed, all data are presented as (1st Quartile)Median(3rd Quartile) and were analysed using. Data on pollen (µg) and fluids (µl) consumed are presented as mean (standard deviation). Initial pollen in chambers = 0.431 µg, Initial fluid in chambers = 5 µL. Superscript letters indicate significant differences ($p < 0.05$) between treatments. *p*-values in bold indicate significance ($p < 0.05$).

	Water	Nectar	Pollen	Nectar and Pollen	X^2	*p*-value
Survival$^{(D6)}$	0	(0)20(45)^{a}	(20)50(65)^{b}	(0)0(0)^{c}	171.56	**<0.001**
Eggs$^{(D6)}$	0	n.a	(0)0(1)^{a}	(0)0(2.1)^{b}	60.00	**0.044**
Larvae$^{(D6)}$	0	n.a	(1)2.5(4)^{a}	(0)1(1)^{b}	157.75	**0.007**
Adults$^{(D6)}$	0	(0)1(2.3)^{ab}	(1)2.5(3.3)^{a}	(0)0(0)^{b}	142.1	**<0.001**
Growth Rate$^{(D10)}$	0	n.a	(20)60(100)	(0)40(60)	89720	0.242
Pollen consumed$^{(D10)}$	n.a	n.a	0.3 (±0.1)^{a}	0.1 (±0.1)^{b}	F= 21.85	**<0.001**
Fluid consumed$^{(D10)}$	2.78 (±1.1)	2.35 (±1.2)	n.a	n.a	F=1.407	0.240

was like those feeding on a combination of pollen and nectar. However, mites that only fed on pollen had significantly higher survival compared to those feeding on nectar and a combination of nectar and pollen. Eggs and larvae were observed from day 4 onwards, but only in treatments that contained pollen. Significantly more larvae were found within the treatment that only contained pollen as a food source compared to the treatment that contained both pollen and nectar (Table 1). The mass of pollen consumed by mites was significantly higher for treatments where mites fed only on pollen than those that were provided with both pollen and nectar (Table 1).

It was not possible to precisely determine the amount of pollen and nectar consumed per mite individual over the experimental period of 10 days as numerous individuals died (presumably of old age and/or malnutrition) and in some cases, larvae were produced that also consumed resources. However, for mites that were fed only nectar, and where no larvae were produced, all available nectar was consumed within 10 days in some replicates. This indicated that 5 mature mites are capable of consuming 5 µl of nectar within 10 days (= 0.1 µl nectar consumed per mite per day). For nectar consumption at stage 1 (30% of open flowers = 1385.75 µl available), 71 mites may consume up to 7.1 µl nectar per day. At stage 2 (60% open flowers = 2060.75 µl available) there was an average of 178 mites per inflorescence that could consume ~17.8 µl nectar per day. The study of Theron-de Bruin et al. (2018) reported the collection of a median of 706.5 adult *P. vandenbergi* mites per inflorescence at the mid-flowering stage (30–50% of open flowers) from the Jonkershoek Nature Reserve in spring. This is expected to only represent a small portion of the total number of mites in these inflorescences, as not all mites that gathered at the top of inflorescences could be collected, and all immature individuals within inflorescences were discounted. This number of adult mites would be able to consume at least 70.65 µl nectar per day.

For mites that were fed both pollen and nectar and where no larvae were produced, mites could consume up to ~ 0.10 µg of pollen over the 10 days (= 0.002 µg on average of pollen consumed per mite per day). When mites were fed pollen only, most tubes contained larvae after 10 days. For those that did not, maximum pollen consumption was ~ 0.14 µg after 10 days (= 0.0028 µg of pollen consumed per mite on average). These values represent minimum values, as all mites in these tubes were dead by day 6. By using these values, it was possible to calculate predicted consumption rates for pollen and nectar by mites in *P. neriifolia* inflorescences. For pollen consumption at stage 1 (30% of open flowers), when there was a mean number of 71 mites in inflorescences, these can consume ~ 0.142–0.199 µg of pollen per day (= 3.91–5.48% of daily available pollen). At stage 2 (60% open flowers) there was an average of 178 mites per inflorescence. These may be capable of consuming 0.356–0.498 µg of pollen per day (= 9.81–13.72% of daily available pollen). Using data from the study of Theron-de Bruin et al. (2018), the adults collected in that study may be capable of consuming 1.43–1.98 µg of pollen per day (= 39.39–54.55% of daily available pollen).

DISCUSSION

We showed that *P. vandenbergi* flower mites do not significantly contribute to the pollination of *P. neriifolia*. In contrast, they readily fed and reproduced on a diet consisting only of *P. neriifolia* nectar and pollen. Consumption of nectar likely has little effect on *Protea* pollination, as we have shown that *P. neriifolia* produces vast volumes of nectar for its avian pollinators. However, pollen consumption by mites can be quite substantial. The reduction in pollen availability for pollinators may lead to a decrease in male fitness and ultimately influence *Protea* seed set and population dynamics.

No viable seeds formed within inflorescences that acted as negative controls, indicating that *P. neriifolia* is non-autogamous. When mites were added, very few viable seeds formed, demonstrating that pollen transfer by mites is possible but very limited. It was not possible to determine whether successful pollination in these cases resulted from cross-pollination (i.e. from pollen carried by mites in the initial transfer between inflorescences) or from self-pollination (via the transfer of pollen from anthers and receptive stigmas within the inflorescence) when mites moved between flowers while feeding on pollen and nectar. If the seed set resulted from the latter, the reduction in out-crossing could lead to inbreeding depression that is known to cause decreased fitness and future reproductive success in the Proteaceae (Eckert 2000; Johnson and Nilsson 1999; Robertson et al. 2011). This very low successful seed set excludes *P. vandenbergi* as pollinators of *P. neriifolia*, unlike in some hummingbird-pollinated systems (Dobkin 1984; Kaufmane and Rumpunen 2002; Lara and Orneals 2001).

Protea generally has a low seed set (2–30%) (Rebelo and Rourke 1986). Seed set for *P. neriifolia* in previous studies varied between 1.5–6.4%, which represents a mere 5 to 18 seeds per infructescence (*e.g.* Collins and Rebelo 1987; Maze and Bond 1996). In the present study, the natural seed set of *P. neriifolia* varied between 5–25%, depending on the study site. Low seed set therefore seems to be the norm for *P. neriifolia* as in other *Protea* species, but the reasons for this are generally unclear. Various proposed reasons include a shortage in viable pollen (inadequate pollen transfer, vector shortage or unsuitable pollen), resource limitations, predation, a lack of space within the inflorescence, or genetic polymorphism (Ayre and Whelan 1989; Collins and Rebelo 1987; Rebelo and Rourke 1986; Wiens 1984). In the present study, we suggest that pollen consumption by mites may be a contributing factor to the low seed set in *P. neriifolia*. At the mid-flowering stage, the mites can consume up to 2% of available pollen. At some sites, and perhaps during warmer periods, mite numbers can be very high (e.g. Theron-de Bruin et al. 2018) and may consume more than an estimated 50% of available pollen. This is high in comparison to pollen robbing by some

hummingbird-associated flower mites. For example, Paciorek et al. (1995) found that *Proctolaelaps kirmsei* can consume on average 5.4% and 16% of *Hamelia patens* pollen (which is believed to be an overestimation). Velázquez and Ornelas (2010) found a decrease of 69% in available pollen in *Moussonia deppeana*, 36% in *Lobelia laxiflora* and 63% in *L. cardinalis* flowers after 24 hours of consumption by the hummingbird flower mites *Tropicoseius* sp. nov. and *T. chiriquensis*. This reduction in pollen availability negatively affects male fitness, as *H. patens* is self-incompatible and mites did not assist in pollination (Paciorek et al. 1995). Similarly, it is expected that *P. vandenbergi* mites negatively influence male fitness in *P. neriifolia* by reducing the amount of available pollen for transfer by birds and insects (Hargreaves et al. 2009).

Proctolaelaps vandenbergi mites regularly consumed nectar in our study. However, even when mite numbers were very high (Theron-de Bruin et al. 2018), daily nectar consumption by mites remained less than 6.5% of the total available nectar. In addition, nectar production is expected to be continuous throughout the flowering season, diminishing the impact of nectar robbing by these mites. This contrasts with results from studies on nectar consumption by flower mites associated with hummingbirds. For example, Colwell (1995) showed that *Proctolaelaps kirmsei* mites consumed on average 40% of available nectar within *H. patens*. Lara and Orneals (2001) found that flower mites removed 50% of nectar from *Moussonia deppeana* flowers. Da Cruz et al. (2007) found that flower mites from *Heliconia laneana* and *H. spathocircinata* reduced nectar between 33% and 49% and consequently led to the decrease of nectar sugars within nectar due to continuous nectar production to compensate for nectar robbery. A *Proctolaelaps* sp. was also found to decrease nectar availability by 22% for pollinators of *Neoregelia johannis* flowers (Guerra et al. 2010).

From the feeding experiments, it was evident that *P. vandenbergi* mites could survive and reproduce on a diet consisting of *P. neriifolia* pollen and nectar. Members of this genus have diverse ecologies and can feed on various arrays of substances including fungi, pollen and other mites (Krantz and Walter 2009). A previous study indicated that this mite does not appear to feed on *P. neriifolia* flower-associated fungi (Theron-de Bruin et al. 2018). It is unknown whether *P. vandenbergi* is also predaceous on other arthropods, but as *Protea* flowers are not consistently available throughout the flowering season, they may switch to a more predaceous lifestyle when they live within *Protea* infructescences during the non-flowering stages (Roets et al. 2007, 2009, 2011, 2013; Theron 2011; Theron et al. 2012; Theron-de Bruin et al. 2018). However, as far as we know, predatory behaviour has not been documented for other flower associated *Proctolaelaps* species.

Both adults and immature *P. vandenbergi* individuals fed on pollen and nectar in experimental units. Interestingly, mites reproduced only when *Protea* pollen was available within experimental units, even though they could survive for prolonged periods when feeding on *Protea* nectar only (compared to when offered water only). Mites therefore seem to be able to differentiate between suitable breeding sites (those containing *Protea* pollen) and non-suitable breeding sites (areas without pollen), even when some resources are available (*Protea* nectar). Pollen provides high quantities of nutrients such as amino acids that are scarce in nectar (Stanley and Linskens 1974). Amino acids are particularly important for egg development in female mites and growing juveniles (Chmielewski 1999; Gilbert 1972; Royce and Krantz 1989). The ability to survive only on nectar may be an adaptation to use this nearly continuous source of carbohydrates at the end of the flowering stage of inflorescences, when all available pollen is depleted, and mites await the last few visits by pollinators to transport them to uncolonized inflorescences (Roets et al. 2009; Theron-de Bruin et al. 2018).

Previous feeding studies that used flower-associated mites (including *P. kirmsei*) in preference experiments indicated that these mites could distinguish between and show preference towards their host plants (Cutraro et al. 1998; Heyneman et al. 1991). These flower mites are therefore very host-specific (monophagous) as only ~ 1 in 200 individuals were found on another host (Heyneman et al. 1991). We expect that this monophagous habit persists in species that are associated with flowering plants that flower throughout the year. As *P. neriifolia* does not flower throughout the year, *P. vandenbergi* mites need additional host species to survive, except if they switch diet to other sources as mentioned above. However, *P. vandenbergi* mites are associated with numerous *Protea* species (Theron 2011) and may therefore be sequential specialists in that they specialise on the genus *Protea*, but switch host *Protea* species according to the availability of flowering inflorescences (Colwell 1973). More feeding and survey studies are needed to corroborate this.

Nectar thieves are generally considered to have negative impacts on their hosts. However, a study of the effect of *Tropicoseius* flower mites on *M. deppeana* (Lara and Ornelas 2001, 2002a) showed the opposite. The authors found that the nectar and pollen-robbing flower mites aided outcrossing in this species by influencing the behaviour of hummingbird visitors. It was found that when mites were absent, hummingbird visitation was less frequent, but lasted longer. In the presence of mites, hummingbird visitations were more frequent, but with shorter durations. This had positive consequences for seed production. A similar situation may exist in the *Protea* system. When birds perch on *Protea* inflorescences or probe them for nectar, *P. vandenbergi* mites swarm to the top to climb on birds for transport (pers. obsv.). When the mites are particularly numerous, they may irritate the bird to such an extent that it remains on the inflorescences for shorter periods. This would therefore decrease visitation times, but increase the frequency of visits, which could ultimately improve prospects for outcrossing and increased fitness (Lara and Ornelas 2001).

To conclude, *P. vandenbergi* mites feed and reproduce on *Protea* pollen and are pollen and nectar thieves. They have the potential to drastically decrease pollen availability within inflorescences and therefore may pose a risk to *Protea* reproduction, at least at certain sites and/or during certain times of the year and if *Protea* is pollen-limited. Conversely, they may promote outcrossing by interfering with usual pollinators. The reasons for these large differences in mite numbers are unclear but may be important considerations under future predicted climate change scenarios and accompanying shifts in flowering phenology. These mites offer very little in terms of pollination of *Protea* plants and may even reduce fitness if successful pollination is due to selfing. The impact of mites on avian visitation duration and frequency should be investigated further in future studies to determine possible trade-offs between pollen robbing and outcrossing success.

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