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FUNGAL MICROBIOLOGY

Birds Mediate a Fungus-Mite Mutualism

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Abstract Mutualisms between ophiostomatoid fungi and arthropods have been well documented. These fungi commonly aid arthropod nutrition and, in turn, are transported to new niches by these arthropods. The inflorescences of *Protea* trees provide a niche for a unique assemblage of ophiostomatoid fungi. Here, mites feed on *Sporothrix* fungi and vector the spores to new niches. *Protea*-pollinating beetles transport the spore-carrying mites between *Protea* trees. However, many *Protea* species are primarily pollinated by birds that potentially play a central role in the *Protea-Sporothrix*-mite system. To investigate the role of birds in the movement of mites and/or fungal spores, mites were collected from *Protea* inflorescences and cape sugarbirds, screened for *Sporothrix* fungal spores and tested for their ability to feed and reproduce on the fungal associates. Two mite species where abundant in both *Protea* inflorescences and on cape sugarbirds and regularly carried *Sporothrix* fungal spores. One of these mite species readily fed and reproduced on its transported fungal partner. For dispersal, this mite (a *Glycyphagus* sp.) attached to a larger mite species (*Proctolaelaps vandenbergi*) which, in turn, were carried by the birds to new inflorescences. The results of this study provide compelling evidence for a new mite-fungus mutualism, new mite-mite commensalisms and the first evidence of birds transporting mites with *Sporothrix* fungal spores to colonise new *Protea* trees.

Keywords Acari · Mutualism · Phoresy · Promerops · Protea · Sporothrix

Introduction

Animal-fungal mutualisms are associations between fungi and faunal hosts where both parties benefit from their interaction (e.g. attine ants, fungus-growing termites and ambrosia beetles) [1]. Many fungi that are not freely mobile via water and air currents or that associate with highly disjunct and ephemeral niches rely on their associated faunal hosts for transport to new localities, and in turn, often offer nutritional benefits to their phoretic faunal partners [2–7]. Disruptions in these mutualisms, such as reduction in abundance (or extinctions) of one of the interacting partners, or changes in resource quality and/or quantity, can cause additional species extinctions (coextinctions) or reduction of ecological fitness of interacting partners [8, 9]. Understanding the role of all interacting partners in multipartite symbioses in the maintenance of biodiversity and ecological function is of major importance for assessing ecological threats for conservation management [10–12].

The ophiostomatoid fungi [13] include well-known tree pathogens in genera such as *Ceratocystis*, *Ophiostoma* and *Sporothrix* [14, 15]. The group represents a polyphyletic assemblage of fungi that share morphologically convergent traits, such as the production of sticky spores, for dispersal via arthropods [2–4]. Best-known vectors include bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae and Platypodinae) that often obtain additional nutrition from their mutualistic fungal

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partners when feeding on inoculated vascular tissues [16–19]. Mites, phoretic on the beetles, commonly also transport ophiostomatoid fungi [17, 18, 20–23] with some having evolved specialised spore-carrying structures known as sporothecae [24]. These associations are often mutualistic because the mites obtain complete nutrition from their fungal partners [25–27].

Members of two ophiostomatoid fungal genera, *Sporothrix* and *Knoxdaviesia*, live in a very unusual niche. Here, they are the dominant saprobic fungi within the inflorescences and infructescences of *Protea* trees in Africa [28]. *Protea*-associated mites such as *Proctolaelaps vandenbergi*, *Tarsonemus* sp. A and a *Trichouropoda* sp. act as primary vectors of fungal species including *Sporothrix phasma*, *Sporothrix splendens* and *Knoxdaviesia proteae* [29–31]. The association between the *Trichouropoda* mite and the *Sporothrix* fungi from *Protea* trees is mutualistic because the mites can use the fungi as only nutritional source to complete an entire life cycle [29].

Mites disperse the fungi by crawling between infructescences and inflorescences on individual *Protea* trees [30]. For longer distance dispersal, the mites are vectored by *Protea*-associated Cetoniidae beetles (e.g. *Genuchus hottentottus* and *Trichostetha facicularis*) [29, 30]. It was recently demonstrated that *Knoxdaviesia* fungal populations distantly separated from each other are in near-genetic panmixia, suggesting a prevalence of long distance dispersal in the *Protea* system [32–35]. However, the ubiquitous distribution of *Sporothrix* and *Knoxdaviesia* fungi within the inflorescences and infructescences of host *Protea* species [29, 36] and the lack of population genetic differentiation of populations separated by more than 200 km, is difficult to explain based purely on dispersal via beetles [34]. This is because the mountainous nature of the region where these *Protea* trees are found would impede free movement of insects over very long distances and these beetles are encountered within structures in low frequencies [37–39]. To explain the observed lack of population differentiation of the fungi [34], hypothesised that birds could possibly be involved in the long-distance dispersal of these unusual *Protea*-infecting mite-associated fungi.

Insects such as *Genuchus* and *Trichostetha* beetles involved in carrying mites, that in turn vector ophiostomatoid fungi, are important pollinators of many *Protea* species [37]. It is thus interesting that most *Protea* hosts of ophiostomatoid fungi are primarily pollinated by nectarivorous birds [37, 40–42]. Dominant avian *Protea* visitors in the biologically diverse Cape Floristic Region of South Africa are the endemic orange-breasted sunbird (*Nectarinia violacea*) and cape sugarbird (*Promerops cafér*) with the latter species being the primary pollinator [43, 44]. These birds are capable of flying vast distances (more than 160 km have been recorded for *Promerops cafér*) in search of suitable habitats [45, 46], where they predominantly feed on *Protea* nectar [47, 48]. Any phoretic organisms present on these birds would consequently spread over these same distances.

While no previous study has considered the role of birds as vectors of *Protea*-associated mites, numerous observations of *P. vandenbergi* mites on especially the cape sugarbird have been made (T. Rebelo, pers. com., www.ispotnature.org, www.proteaatlas.org.za). *Proctolaelaps vandenbergi* is known to attain very high numbers (over 60,000 individuals) within the inflorescences of bird-pollinated *Protea* species where they likely feed on pollen and nectar [49, 50]. This mite species has been implicated in the transport of the ophiostomatoid fungus *S. phasma* [30], and it is possible that it utilises the fungus as an additional food source. If this mite (or any other *Protea*-associated mite) can regularly spread *Sporothrix* fungal species via birds, the ubiquitous distribution of *Sporothrix* in *Protea* and the near panmictic population structure of ecologically similar mite-associated fungi from this niche could be explained.

In this study, we consider whether birds play a role in the complex and intriguing fungus-mite symbiotic interactions found in the *Protea* system. We hypothesise that *Protea*-pollinating birds carry *Protea*-associated mites, that in turn, carry spores of the same fungal species (*Sporothrix*) that are present in *Protea* inflorescences. We further hypothesise that mites that vector *Sporothrix* fungal species can utilise these fungi as a food source indicating a possible mutualistic association. Results of this study may shed light on the possible cascading effects of ecosystem disruptions on multipartite mutualisms on the maintenance of normal ecosystem functioning.

Methods

Mites Associated with Protea Neriifolia Inflorescences

Mites associated with the inflorescences of *Protea neriifolia*, one of the most wide-spread bird-pollinated *Protea* species in the Western Cape Province (Fig. 1a, b) were surveyed. This *Protea* provides the niche for two ophiostomatoid fungi, *Knoxdaviesia capensis* and *S. phasma* [31] and three mites (*Trichouropoda* sp., *Tarsonemus* sp. A and *P. vandenbergi*) that are known vectors of ophiostomatoid fungi [29, 30]. Twenty inflorescences at early to mid-flowering stage (where 30–50% of the individual flowers within the inflorescences were open and when birds actively visit for nectar) were sampled during October 2014 in Jonkershoek Nature Reserve (33° 59′ 24.5″ S, 18° 57′ 25.2″ E), Stellenbosch, stems submerged in a water-filled bucket to keep them fresh and transported to the laboratory. Inflorescences were placed in separate water-filled glass containers to maintain freshness for extended periods. After 2 days, mites that accumulated at the tops of flowers in anticipation of arriving flower visitors (Fig. 1c) were collected from each inflorescence by patting a 5-cm-long by 1-cm-wide strip of adhesive tape (Sellotape, Henkel Limited, UK) across the top of the inflorescence for 40 s. This

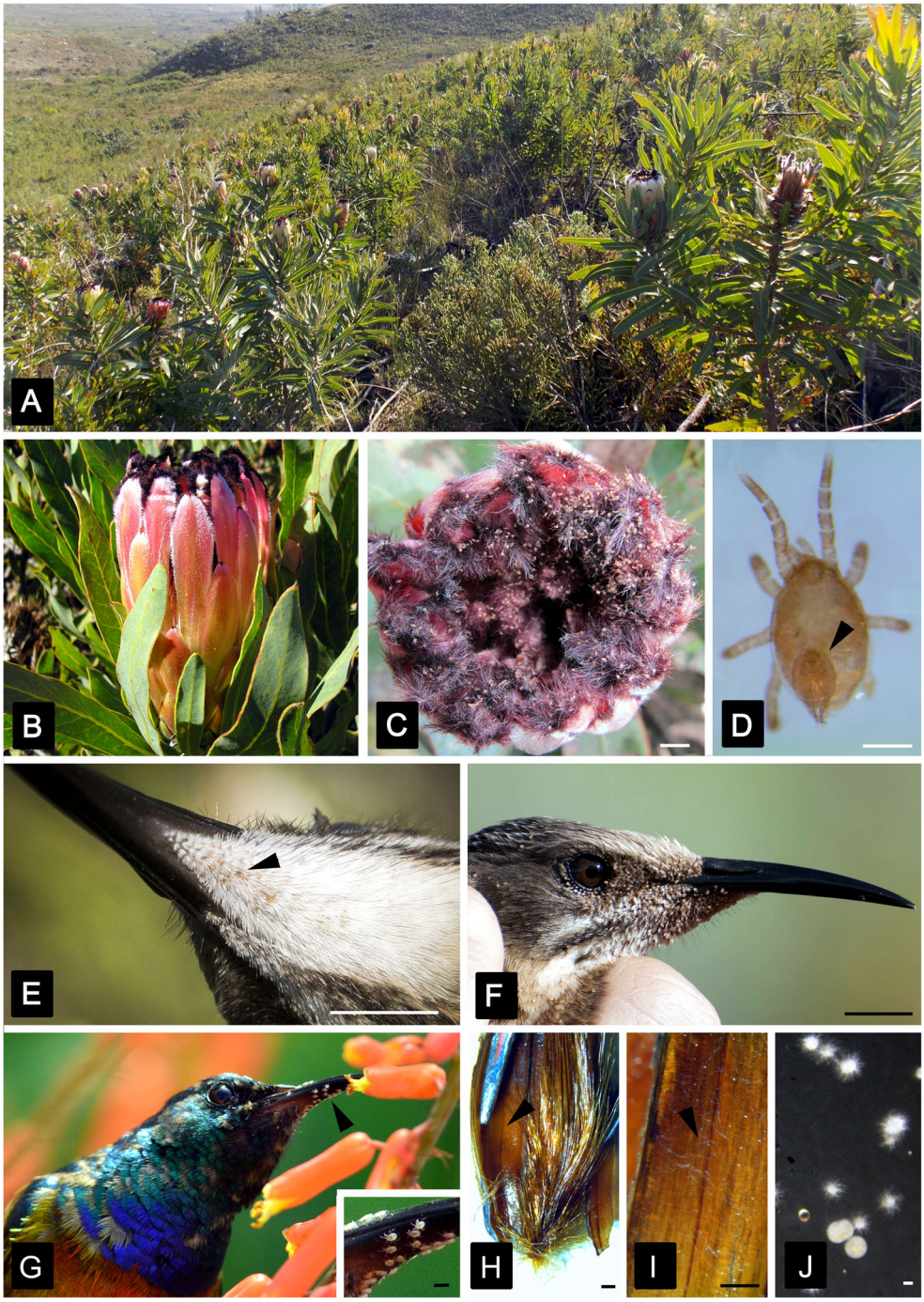

Fig. 1 **a** *Protea neriifolia* population (foreground) in the Jonkershoek Nature Reserve, Western Cape Province, South Africa. **b** *Protea neriifolia* inflorescence. **c** Mites accumulating at the top of an inflorescence in anticipation of flower visitors. **d** Hypopus of a *Glycyphagus* mite (arrow) attached to *Proctolaelaps vandenbergi* mite from a *P. neriifolia* inflorescences. **e** *Proctolaelaps vandenbergi* mites visible under the beak of a cape sugarbird (photo by Carina Wessels). **f** Cape sugarbird covered with *Proctolaelaps vandenbergi* mites (photo by Alan Lee). **g** Orange-breasted sunbird with *Proctolaelaps* mites on its beak (Insert to (**g**)). Same, with beak area enlarged (photo by David Parker). **h** *Protea neriifolia* fruit surrounded by perianth forming a nectar well (arrow). **i** Close-up of same perianth in region of nectar well showing fine whitish fungal hyphae (arrow), later identified as *Sporothrix phasma*. **j** *Sporothrix phasma* fungal colonies (white, fluffy) and two colonies of an unidentified yeast (lower left) originating from mites allowed to crawl on the surface of petri-dishes after 7 days

method did not collect all mites present but gave some indication of relative abundance of each species per inflorescence. The adhesive strips were mounted on clear transparent cellophane sheets to trap mites between the adhesive tape and the sheet and kept at 4 °C. All mites collected from inflorescences were sorted into morpho-species and identified to the lowest taxonomic rank possible. Phoretic associations between mites were also documented. The numbers of each mite species collected per inflorescence were counted and median abundance compared using a Kruskal-Wallis ANOVA in Statistica 13 (StafSoft Inc., Tulsa, OK, USA) for the non-parametrically distributed data (as determined by a Shapiro-Wilk test in Statistica). Significant differences are reported at $p \leq 0.05$.

Mites Phoretic on Cape Sugarbirds

Sites for bird captures were selected based primarily on the presence of substantial populations of *P. neriifolia* that were frequented by bird visitors. The main *Protea*-visiting species *Promerops cafér* (cape sugarbird) was selected because they

occur in fairly high numbers in *Protea* populations, they have a relatively large body size making handling easier and they are highly active [51]. Mist nests (ECOTONE, 15 mm × 15 mm netting) with a total span of 21 m × 2 m were set up in three areas of natural CFR vegetation (Franschoek Pass (33° 55′ 10.2″ S, 19° 09′ 42.0″ E), Jonkershoek Nature reserve and Du Toits Kloof Pass (33° 41′ 45.2″ S, 19° 05′ 14.2″ E) in the Western Cape Province, South Africa from April to June 2014. Mist nets were set up early in the morning (08:00 am–11:00 am) because this is a time of peak activity for this bird species [52]. Birds were removed from nets as soon as possible after capture. Non-target bird species were very rarely caught and were immediately released. Collected sugarbirds were placed into small cotton bags, weighed and measured in accordance with guidelines of South African Bird Ringing Authority (SAFRING) by ringer no. 1600 (A. Heystek) and thereafter scanned for the presence of mites. Because the beak and breast areas of these birds make most contact with *Protea* flowers when probing inflorescences during feeding [53, 54], these areas were targeted for the removal of mites. Mites were collected from the birds using adhesive tape strips, 10 cm long and 1 cm wide, that were repeatedly dabbed over the target areas of the bird (one strip per bird) and then adhered to a clean transparent sheet as described for mite collection from inflorescences. The sheets were placed within a cooler box and transported to the laboratory where it was stored at 4 °C. Importantly, this method did not capture all mites present on birds even in the targeted areas, because mites are agile and were able to escape between the feathers. In order to minimise stress on the birds, handling time was also kept to a minimum, which further hampered exhaustive mite collection. In addition to our own collections, a few random collections of mites (using the adhesive tape method) received from SAFRING ringers that were active in other areas of the CFR, were also added.

All samples were stored at 4 °C until further analyses could be conducted in the laboratory within 12 h of collection. All mites collected from birds were sorted into morpho-species under sterile conditions (and using tools that were flame-sterilised between handling of individual mites); all individuals were placed in separate sterile eppindorf tubes and were then identified to the lowest taxonomic rank. The abundance of the different mite species sampled from birds was compared using a Mann-Whitney *U* test in Statistica for the non-normally distributed data.

Fungal Isolation from Mites and Young Inflorescences

Twenty individuals of each mite species encountered within each of five randomly collected *P. neriifolia* inflorescences (at the mid flowering stage) from Du Toits Kloof Pass during June 2014 were used to determine the presence of *Protea*-associated *Sporothrix* fungi. For each inflorescence, mites were collected by shaking the inflorescence over a Petri dish under sterile conditions, after which 20 mite individuals of each mite species were taken from the Petri dish and placed individually into micro-tubes filled with 100 μl sterile distilled water using a sterile needle and with gloved hands. The needle was sterilised between each individual mite using a flame. Tubes were vortexed (VX-200 Lab Vortexer, Labnet International, Inc., Edison, NJ, USA) for 1 min to loosen and displace fungal spores.

A sub-set of mites collected from birds using the adhesive tape method were also screened for the presence of *Sporothrix* fungi. Seven sugarbirds were caught at Du Toits Kloof Pass during a single day in August 2015 using methods described above. For the collection of the mites from these birds, care was taken to minimise possible contamination with *Sporothrix* fungi from external sources such as soil and plant material adhering to hands. Precautionary measures included reducing collecting time to 30 s, wearing sterile gloves and sticking the adhesive tape strips onto sterilised clear plastic sheets (wiped clean using 70% ethanol). In the laboratory, ten mite individuals per species per bird (where possible) were individually removed using fine tweezers (sterilised between handling of each individual mite) and placed in separate micro-tubes filled with 100 μl distilled water that were again vortexed for 1 min.

The content of all tubes containing individual mites from inflorescences and birds were individually plated onto selective medium for *Sporothrix* fungi prepared from Malt Extract Agar (MEA, Merck, Wadeville, South Africa) containing 0.1 g/l cycloheximide and 0.05 g/l streptomycin [29]. Plates were monitored daily for 2 weeks, and all fungal colonies that resembled *Sporothrix* fungi were counted. Up to five colonies per plate were selected at random and purified as representatives of the *Sporothrix* species present on mite individuals. The percentage of mites that carried spores of *Sporothrix* fungi and the number of colony forming units of *Sporothrix* fungi isolated per mite individual from birds were compared using a Mann-Whitney *U* test in Statistica. The percentage of mites that carried spores of *Sporothrix* fungi and the number of colony forming units of *Sporothrix* fungi isolated per mite individual from each mite species collected from inflorescences were compared using generalised linear mixed models (GLMM) using R software (R Development Core Team 2013) and the *lme4* package [55]. Data on counts of colony forming units was fitted to a Poisson curve and percentage data was fitted to a binomial curve (with Laplace approximations). For analyses of fungi from mites from infructescences, the structure from which the mites were collected was included as random variable. These models followed the formulas: glmer (cbind (number of mites carrying spores, number of mites not carrying spores) ~ mite species + (1|infructescence), family = "binomial") for data on the percentage of mites that carried fungal spores and glmer (number of colony forming units ~ mite species + (1|infructescence), family = "Poisson")

for counts data. These models were tested against models that only contained the random variable and in both cases models including mite species identity were significantly better as judged by the Akaike Information Criterion using the *anova* function (for percentage data: AIC = 87.3 vs. AIC = 174.998, X^2 (2) = 91. 616; $p < 0.001$; for counts data: AIC = 3511.4 vs. AIC = 5205.6, X^2 (2) = 1698.2; $p < 0.001$). In addition, Tukey *post-hoc* tests in the R package *multcomp* were used to determine the pairwise differences in colony forming units and percentages of mites associated with *Sporohrix* fungi between the different mite species [55].

To determine whether mites could transfer *Sporothrix* fungal spores to uninhabited material, ten living mites per species collected from inflorescences and birds were placed on Petri dishes containing *Sporothrix* selective media. This was replicated 10 times for each mite species. These plates were monitored for the presence of fungal colonies that were subsequently purified.

Sexual fruiting structures (ascomata) of *Sporothrix* fungi are not usually encountered in inflorescences, as these form only after flower fertilisation and initiation of infructescence formation [36]. We consequently determined the site of first growth of these fungi in their asexual conidial-producing state in young inflorescences (only ca. 50% of individual florets open). Inflorescences were dissected and individual flowers were scanned for hyphal growth using a dissection microscope. We assumed that the area in the inflorescence in which we encountered *Sporothrix* fungi early in its development would represent the site of inoculation. Observed hyphae were collected by lifting individual mycelial strands with a sterile needle and plating these onto selective media as described above. All fungal cultures obtained from all mite individuals and inflorescences were grouped according to morpho-type based on colony growth form, texture and colour. Three to five individuals of each morpho-type were selected for further identification using DNA sequence comparisons.

Fungal Identification

Fungal DNA was extracted using a modified CTAB procedure following the methods of [32]. The internally transcribed spacer regions I and II (including 5.8S) of the rDNA of selected strains where amplified using primers ITS1F and ITS4 [56, 57]. Amplification reaction mixtures comprised 1 μl DNA template, 9 μl distilled water, 2.5 μl $MgCl_2$ (2.5 mM), 0.25 μl (10 mM) of each primer and 12 μl KAPA Taq ReadyMix (Kapa Biosystems, Inc. Boston, USA). Negative controls were included. PCR products were amplified using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed for an initial denaturation step for 3 min at 95 °C, followed by 40 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 50 s and a final elongation step at 72 °C for 7 min. Amplified PCR products were purified and sequenced at the Stellenbosch University Central Analytical Facility, Stellenbosch, South Africa. Species identities were established by performing Basic Local Alignment Search Tool (BLAST) searches on the GenBank data base (http://www.ncbi.nlm.nih.gov) using BIOEDIT, version7.2.5.0 and manually corrected ITS sequence data [58].

Fungi as a Food Source for Mites

To study the interaction between collected mite species and *Sporothrix* fungi, feeding and reproduction of mites that had been confirmed to carry *Sporothrix* fungal spores were tested on the various fungi following the methods described by [29]. Mites were collected in *P. neriifolia* inflorescences from Du Toits Kloof Pass in November 2015 and tested on a diet of *S. phasma* and *S. splendens*. Ten individuals of each mite species were placed on MEA plates (without antibiotic supplementation) that contained three-week-old cultures of either *S. splendens* or *S. phasma*, respectively. Mites on plates containing only MEA served as controls. Mites were prevented from escaping the plates by applying a thick layer of petroleum jelly on the inside of the lid, which formed a seal between the base and lid of the Petri dish, by sealing plates with Para film (Parafilm M®, Bemis Company, Inc.), and by floating plates in large trays containing water with a few drops of added detergent. The experiment was replicated five times with plates kept in the dark at 25 °C for 40 days. Thereafter the numbers of living mites (including adults and immatures) on each plate were counted. Differences in mite numbers between the different treatments per mite species were statistically compared using a *t* test [59] in Statistica 13 for the normally distributed data.

Results

Mites Associated with Protea neriifolia Inflorescences

Three mite species, *Proctolaelaps vandenbergi*, *Tarsonemus* sp. A and a heteromorphic deutonymphs (hypopodes) of a *Glycyphagus* sp., were associated with the top surface of *P. neriifolia* inflorescences at the stage when these structures are pollinated. *Proctolaelaps vandenbergi* and the *Tarsonemus* mites were the same species implicated in the dispersal of ophiostomatoid fungi from *Protea* infructescences by [29, 36]. The *Glycyphagus* mite was previously recorded from the infructescences of various *Protea* species [60]. Mites differed in their abundance on these inflorescences (H(2) = 38.048; $p < 0.0001$), with *Proctolaelaps vandenbergi* significantly more abundant than either the *Tarsonemus* or *Glycyphagus* ($Z = 5993$, $p < 0.0001$ and $Z = 4246$, $p < 0.0001$, respectively) (Table 1). More than a thousand individuals of *P. vandenbergi* mites were commonly collected from a single inflorescence.

Table 1 Number of mites collected from the top of *Protea neriifolia* inflorescences

Mite species	n^a	Min (25%)	Median (75%)	Max	n^b	% with phoretic mite partner
Proctolaelaps vandenbergi	19,808	17 (417)	706.5 (1142.5)	3697	50	0.25[c]
Glycyphagus	582	1 (4.5)	13 (25.5)	245	42	7.22[d]
Tarsonemus	224	0 (1.5)	2.5 (9.5)	99	13	5.8[d]

[a] Total number of individuals collected from 20 inflorescences

[b] Total number of individuals with a phoretic partner

[c] Percentage of individuals associated with *Glycyphagus* and/or *Tarsonemus*

[d] Percentage of individuals associated with *Proctolaelaps vandenbergi*

The other two mite species were collected in very similar numbers ($Z = 1.747$; $p = 0.242$). Interestingly, a phoretic association was commonly observed between the *Proctolaelaps vandenbergi* and the smaller *Tarsonemus* and *Glycyphagus* mites (Table 1; Fig. 1d). In some cases, both the *Tarsonemus* and the *Glycyphagus* mites were found carried on a single *Proctolaelaps vandenbergi* individual.

Mites Phoretic on Cape Sugarbirds

A total of 54 cape sugarbirds were captured from which 549 *Protea*-associated mites were removed. Only the *Protea*-associated *Proctolaelaps vandenbergi* (431 individuals) and hypopodes of the *Glycyphagus* sp. (55 individuals) were collected on these birds (Table 2). Overall, *P. vandenbergi* was significantly more abundant on the birds than the *Glycyphagus* sp. ($U = 636.500$, $Z = 5.044$, $p < 0.001$). All *Glycyphagus* mite individuals collected from birds were phoretic on *P. vandenbergi* mites with no individuals collected separately.

Mites were collected from both the beak and breast areas of the birds with the mites most commonly encountered on the undersides of the beaks (Fig. 1e). Photographic evidence suggested that when infestation levels increase, individual birds can carry more than 1000 mites (Fig. 1f), which can cover the entire head and body of a bird. In addition, photographic evidence suggested that the orange-breasted sunbird (*Anthobaphes violacea*) can also vector these mites as demonstrated by a photograph taken at Kirstenbosch National Botanic Garden, Cape Town, South Africa during the main flowering season of the numerous *Protea* spp. in the vicinity (Fig. 1g).

Fungal Isolation from Mites and Young Inflorescences

Eighty-three per cent of all the *Proctolaelaps vandenbergi* mite individuals collected from inflorescences were associated with fungi that morphologically resembled *Sporothrix* spp. This is significantly more than *Glycyphagus* ($Z = 10.479$; $p < 0.001$) and *Tarsonemus* ($Z = 12.601$; $p < 0.001$) (Fig. 2; Table 3). Isolations from *Proctolaelaps vandenbergi* mites resulted in significantly greater numbers of colony forming units of *Sporothrix* fungi compared with the *Glycyphagus* ($Z = 23.78$; $p < 0.001$) and *Tarsonemus* ($Z = 26.24$; $p < 0.001$) mites (Fig. 2; Table 3). *Glycyphagus* mites carried significantly larger numbers of *Sporothrix* spores than *Tarsonemus* mites ($Z = 12.60$; $p < 0.001$; Fig. 2). DNA sequence-based identification confirmed that all isolates belonged to the genus *Sporothrix* (Table 4). *Sporothrix phasma* was the dominant fungal species present and was collected from all three mite species (Table 4). However, *S. splendens*, a species not thought to be associated with this host [61], was also regularly isolated from the collected mites (Table 4). Hyphae of both *S. splendens* and *S. phasma* were commonly observed in the nectar well formed between the ovaries and the surrounding perianths in open florets, i.e. florets where the petals no longer covered the pollen presenter (Fig. 1h, i). These fungi were never observed in any other area of the individual florets or on florets that were still closed.

Table 2 Cape sugarbird sampling areas with total number of birds, *Proctolaelaps vandenbergi* and *Glycyphagus* mites collected

Locality	GPS co-ordinates	Number of birds	Total number of mites collected from birds
Vermont	34° 24′ 38.5″ S, 19° 09′ 19.1″ E	11	7[a]
Helderberg	34° 03′ 55.3″ S, 18° 52′ 26.3″ E	4	3[a]
Port Elizabeth	33° 35′ 23.9″ S, 23° 24′ 15.9″ E	19	155[a], 2[b]
Franschoek	33° 55′ 10.2″ S, 19° 09′ 42.0″ E	4	15[a], 4[b]
Jonkershoek	33° 59′ 24.5″ S, 18° 57′ 25.2″ E	6	43[a], 13[b]
Du Toits Kloof	33° 41′ 45.2″ S, 19° 05′ 14.2″ E	10	208[a], 32[b]

[a] *Proctolaelaps vandenbergi*

[b] *Glycyphagus*

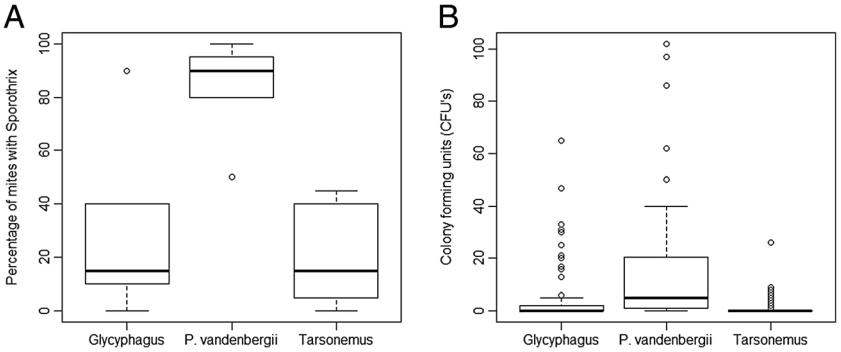

Fig. 2 **a** Median percentage of mites (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers) collected from *P. neriifolia* inflorescences from which *Sporothrix* fungi could be isolated. **b** Median number of colony forming units (CFUs) of *Sporothrix* fungi originating from mites collected from inflorescences (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers)

These same areas often contained the exuviae of *Glycyphagus* mite hypopodes and in many cases also adult *P. vandenbergi* mite individuals as well as the larvae, nymphs and adults of *Glycyphagus* mites. Only a few *Tarsonemus* mites were observed during this period in this part of the floret. The only other arthropods observed on florets during this young stage of the inflorescence development were a few individuals of Thysanoptera, Psocoptera and the bright orange larvae of a small Diptera species.

Twenty-one per cent of *P. vandenbergi* mite individuals and 20% of *Glycyphagus* mite individuals collected from birds were associated with *Sporothrix* fungi ($U = 0$; $Z = 0$; $p = 1.000$). However, isolations from *P. vandenbergi* mites resulted in greater numbers of colony forming units of *Sporothrix* fungi in total, compared with *Glycyphagus* mites, although this difference was not significant ($U = 343.00$; $Z = 0.132$; $p = 0.925$). Both *S. phasma* and *S. splendens* were isolated from the mites collected from birds.

When mites were placed on *Sporothrix*-selective media and allowed to crawl over the surfaces, all plates contained colonies of *Sporothrix* fungi (Fig. 1j). The numbers of colony forming units per plate could not be reliably counted because mites initially transferred many spores and they also transferred spores between developing colonies as they moved

Table 3 Results of GLMM models, including summary statistics of effects included in the final models, testing for the effects of mite species on number of individuals that were associated with *Sporothrix* fungi (model 1) and number of colony forming units of *Sporothrix* fungi isolated per mite individual, (model 2) for mites collected from the infructescences of *Protea neriifolia*

	Model 1				Model 2			
	Estimate	Standard error	*z* value	*p* value	Estimate	Standard error	*z* value	*p* value
Fixed parts								
Intercept	− 1.8925	0.4263	− 4.439	*< 0.001*	0.90204	0.90204	1.68	0.093
Proctolaelaps vandenbergi	3.2687	0.4315	7.575	*< 0.001*	1.33566	0.05616	23.78	*< 0.001*
Tarsonemus spp.	0.4373	0.3835	1.140	0.254	− 1.43759	0.11406	− 12.60	*< 0.001*
Random parts								
N (group)	5				5			
Variance	0.4629				1.423			
Standard deviation	0.6804				1.193			
Observations	14				300			
Summary								
AIC	87.3				3511.4			
BIC	89.9				3526.2			
Loglink	− 39.7				− 1751.7			
Deviance	79.3				3503.4			
Degrees of freedom for residuals	10				296			

Significance levels lower than 0.05 are highlighted in italics

Table 4 Fungal species isolated from mites that were collected from young *Protea neriifolia* inflorescences and cape sugarbirds

Fungal species	Vector mite	Frequency of association (%)	Representative culture and GenBank accession number	Accession of closest match on GenBank	Similarity (gaps)
Sporothrix phasma	*Proctolaelaps vandenbergi*	72	P8 (MF490797)	DQ316216	100% (0)
	Glycyphagus	66			
	Tarsonemus	73			
Sporothrix splendens	*P. vandenbergi*	28	P7 (MF490798)	DQ316205	100% (0)
	Glycyphagus	34			
	Tarsonemus	28			

The frequency (as percentage) of mites from which the *Sporothrix* fungi could be isolated are also provided

around on the plates. All plates were dominated by *S. phasma* with some also containing *S. splendens*.

Sporothrix as Food Source for Mites

All *P. vandenbergi* and *Tarsonemus* mites that were allowed to feed on *S. phasma* or *S. splendens* had died after 40 days and they were never observed to feed on these colonies. All three mite species placed on the control plates were also dead after 40 days, and these plates often contained contaminant fungi transferred by the mites. *Glycyphagus* mites placed on colonies of *S. phasma* or *S. splendens* were observed to feed on these fungi and their numbers increased substantially over 40 days. Populations of *Glycyphagus* mites increased from 10 individuals to an average of 372.2 (± 38) individuals on colonies of *S. phasma* over this time period. Colonies on *S. splendens* had significantly larger population sizes of *Glycyphagus* mites than when these mites fed on *S. phasma* after the same time period ($t = -10.5019$; $p < 0.0001$) with an average of 3527.2 (± 298) individuals counted per plate.

Discussion

Results of this study show for the first time that various *Protea*-associated mites are phoretic on birds. But more importantly, in terms of complex symbiotic patterns, these mites, vectored by birds were shown to carry fungi that live in a specific association with *Protea* inflorescences that are pollinated by these birds. The mites, in turn, transfer the fungi to the lower parts of the developing inflorescences, where the fungi grow and provide a food source for the mites. While it has previously been shown that mites vector and are engaged in 'agriculture' with *Sporothrix* fungi in *Protea* fruiting structures, this is the first evidence of a mite-fungus-bird symbiosis.

Proctolaelaps vandenbergi and the *Tarsonemus* mites collected from inflorescences and birds are well-known associates of *Protea* trees [30, 61] and transmit *Sporothrix* fungi from fruiting structures via beetles [29, 31]. Here, we show for the first time that *Glycyphagus* mites are also involved in these mite-fungi symbioses. Strong evidence is provided that, other than for the aforementioned species that have a commensal relationship with the fungi, *Glycyphagus* mites have a mutualistic association with *Sporothrix* fungi [62]. This is the second mutualism between mites and *Sporothrix* fungi discovered in *Protea*, the other involving *Trichouropoda* mites from fruiting structures dispersed by *Genuchus* beetles [30]. Fungus-mite-insect interactions are well-known for ophiostomatoid fungi associated with conifer-infesting bark beetles [27, 63], but they are less known in other environments such as the one studied here. Sporotrichosis disease caused by *Sporothrix schenckii* [64] can infect numerous distantly related animals such as armadillos, cats, dogs, dolphins, fish, horses, insects, parrots and rodents and be transmitted to humans [65]. *Sporothrix*-mite symbioses could be a common phenomenon and may well be relevant to the control and the spread of socially and economic important species such as the human pathogens *S. schenckii* and *S. brasiliensis* [66].

Glycyphagus mites are not known to be phoretic on *Protea*-associated beetles [29, 30]. Rather than direct transport by birds, the *Glycyphagus* mites were transported secondarily by the larger *P. vandenbergi* mites. Mite-mite hyperphoresy is a rare phenomenon [27, 67, 68] and mostly observed between the Uropodidae and Macrochelidae. In the present study, we document what is to the best of our knowledge, the first case of members of the Glycyphagidae as hyperphoretic on members of the Ascidae. It is also the first record of mite-mite hyperphoresy involving the Chordata and birds in particular. To the best of our knowledge, the only threat these mites, more specifically *Procotlaelaps vandenbergi*, potentially pose to the birds is to directly compete with birds for resources such as nectar [59].

Other than the beetle-mediated mite-fungus mutualism between *Trichouropoda* mites and *Sporothrix* fungi that commences only after the formation of *Protea* fruiting structures [29, 30], the bird-mediated mite-fungus mutualism between *Glycyphagus* mites and *Sporothrix* fungi starts long before the formation of *Protea* fruiting structures and is continuous throughout the *Protea* flowering season. *Sporothrix* occupies nectar wells as soon as the first florets of very young *Protea* inflorescences open. The presence of exuviae of *Glycyphagus*

mite hypopodes (specialised inert deutonymph stages) where their sole role is survival during phoresy [6, 69] in nectar wells indicates that these are among the earliest visitors to *Protea* florets. When hypopodes reach a new habitat (e.g. after reaching a *Protea* inflorescence) and find a suitable location (e.g. a nectar well) they moult, transfer *Sporothrix* fungal spores and begin to feed. *Proctolaelaps vandenbergi* mites are also expected to visit these sites early in the development of inflorescences, as they likely feed on pollen and nectar [7, 70]. Mites will continuously feed on cultivated *Sporothrix* fungi and/or nectar and pollen, and reproduce rapidly within developing inflorescences until maturity. Thereafter, spore-laden mites congregate in very large numbers at the apices of mature inflorescences in anticipation of arriving vectors in the form of *Protea*-pollinating birds such as cape sugarbirds and sunbirds. This fungus-mite-bird symbiosis will result in a very rapid colonisation and spread of *Sporothrix* fungi throughout the *Protea* flowering season.

Mites disperse over short distances using branches, dispersing *Sporothrix* fungal spores from infructescences to developing inflorescences on the same plant [30]. However, *P. vandenbergi*, the *Tarsonemus* and the *Trichouropoda* mites utilise *Genuchus* beetles for transport over longer distances from old *Protea* infructescences to young inflorescences [29, 30]. *Proctolaelaps vandenbergi* and the *Tarsonemus* mites also use *Protea*-pollinating *Trichostetha* beetles for dispersal between inflorescences over longer distances [29, 30]. Therefore, *Protea*-associated *Sporothrix* fungi engage in multiple symbiotic interactions to ensure dispersal and dominance within this fire-ephemeral niche during all phenological stages of the trees [63]. For example, the fungi have mutualistic associations with *Glycyphagus* mites during the flowering stage and *Trichouropoda* mites during the non-flowering stage of *Protea* trees, and commensal associations with *P. vandenbergi* and *Tarsonemus* sp. A mites during both stages of plant development. All of these mites are transported over long distances either directly, or indirectly via hyperphoresy on *P. vandenbergi* mites, on *Protea*-associated beetles and/or birds. Unlike *Protea*-associated beetles, cape sugarbirds disperse over hundreds of kilometres in search of flowering *Protea* populations for food [51, 71] and this likely explains the lack of genetic structure between distant populations of ecologically similar fungi from this niche as recently described by [32, 34]. If we consider that these birds can carry hundreds of mites between distant *Protea* populations, and that the vast majority of these mites carry fungal spores, then a single long-distance dispersal event by the bird could lead to the dispersal of thousands of fungal spores. Therefore, sporadic dispersal of only a few bird individuals between various *Protea* populations will lead to continuous genetic intermixing of fungal populations (panmixia) over the entire distribution range of the bird species.

Although a considerable proportion of the dispersal ecology of two *Protea*-associated *Sporothrix* fungal species has been clarified in this study, many questions remain. For example, in addition to the dominant *S. phasma*, we provide the first confirmed report of *S. splendens* on *P. neriifolia* trees since the formal description of the fungus more than 20 years ago [72]. *Sporothrix splendens* is dominant within *P. repens* inflorescences, a species that often occurs sympatrically with *P. neriifolia*, but does not host *S. phasma* [73]. Cape sugarbirds and sunbirds are known to visit both of these hosts [74] and could easily transfer spore-laden mites, also known from both hosts [61, 75], between them. However, the low numbers of *S. splendens* fungal isolates found on *P. neriifolia* trees indicates that it is not the preferred host. The growth of *S. splendens* on media prepared from *P. neriifolia* is also significantly more rapid than when it is grown on material prepared from its preferred *P. repens* host [61]. Differential competitive abilities between different fungal species due to differences in host chemistry may therefore be an additional complicating factor in determining host range and dispersal ecology of *Protea*-associated *Sporothrix* fungi and should be explored in future studies.

Symbiotic interactions may lead to the coevolution of the interacting partners and multiple dependencies on other mutualisms [76] as in the case of the attine ants, their cultivated fungi and their bacteria [77, 78]. The mutualistic interactions between the ants, which act as protectors and transporters of the fungal cultivar they feed on, and the bacterium which protects the fungal cultivar against pathogens, are all depended on the successful cultivation of the fungus [77]. Resent work also suggests a role for bacteria in the release of nutrients from plant material collected by the ants which may prove to enhance the growth of the fungi [79]. Therefore, the mutualism between the fungus and the ant may be dependent on the mutualism between the bacteria and the fungus. A similar symbiotic relationship has been found within the beetle-fungus mutualism. The southern pine beetle and its fungal cultivar are threatened by an antagonistic fungal species that can outcompete the fungal cultivar and interfere with beetle development [80]. The success of this beetle-fungus mutualism is strengthened by a bacterium that produces antibiotics against the antagonistic fungal species, assisting the successful cultivation of the fungal cultivar [80]. The mutualism between the fungus and the beetle may therefore also depend on a mutualism between the fungus and the bacterium. In these examples, mutualisms between all organisms are strongly interdependent and the entire system would collapse if one of the interacting partners is removed. This could have large consequences for forest ecosystems that are dependent on the ecological functions performed by these multipartite symbioses. This contrasts with the fungus-mite-bird symbioses described here as the mutualistic association between the birds and the plants do not dependent on the interaction between the mites and the fungi. Also, the larger *Proctolaelaps* mites that transport the fungus-carrying *Glycyphagus* mites do

not seem to benefit from these associations. However, species that rely heavily on interactions with other organisms for reproduction or survival (such as the fungi and/or mites in the *Protea* system), often have higher partner diversity (revised by [12]). This would decrease the chances of coextinction with the removal of a single interacting partner, as also suggested by simulated network models (e.g. [12, 81]).

Networks of interacting species can behave unpredictably with anthropogenic interference, and the effect of changes in interaction networks on ecosystem function and evolutionary processes, remains unclear [10]. The loss of birds in the *Protea* system may, for example, lead to disruptions in the extremely long-distance dispersal processes that are characteristic for the fungi in this niche and disrupt normal evolutionary processes [33–35]. Importantly, loss of interacting partners in networks and subsequently ecosystem function do not only depend on species extinctions (e.g. loss of pollinators, fungi or mites in the *Protea* system), but could also be realised by ecological mismatches driven by environmental change [10]. For example, changes in flowering and/or fungal growth and sporulation times due to climate change or other factors, could lead to mismatches between the timing of sporulation and the availability of fungal vectors. Alternatively, environmental change could change the nature of the interactions between interacting partners from mutualistic or commensialistic (e.g. fungi-plant or fungi-mite interaction), to antagonistic due to changing cost: benefit ratios [9]. The conservation of networks of interacting species should therefore be a focus for biodiversity conservation management [11].

Conclusions

This study has shown that *Protea*-associated birds such as the cape sugarbird carry *Protea*-associated mites such as *Proctolaelaps vandenbergi* and a *Glycyphagus* sp. In addition, these birds act as tertiary vectors for ophiostomatoid fungi such as *Sporothrix phasma* and *S. splendens*. A new mutualistic interaction between *Glycyphagus* mites and these *Sporothrix* fungi was recorded and the hyperphoretic behaviour of *Glycyphagus* mites on *Proctolaelaps* mites was revealed. The exact nature of the mutualism between the fungi and the mites needs further exploration. For example, it is possible that the fungi may, in addition to being a food source for the mites, also protect mites from other antagonistic organisms such as contaminating fungi. Inter-fungal competition studies and the influence on mite survival should be conducted to clarify these potential interactions. This study has also provided clear evidence for the very early colonisation of *Protea* inflorescences with *Sporothrix* fungi via mites. The impact of the fungi on *Protea* ecology is, however, not currently known. It is possible that this early occupation of this niche by the fungi and their mutualistic mites may well influence seed viability and/or the behaviour of potential pollinators which could impact *Protea* populations.

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