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The tripartite relationship between a bioturbator, mycorrhizal fungi, and a key Mediterranean forest tree

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Abstract Mycorrhizal fungi serve important functions in Australian ecosystems by forming mutualistic symbioses with plants that facilitate water and nutrient uptake. Scat analyses have shown that southern brown bandicoots (*Isoodon obesulus fusciventer*; 'Quenda') regularly feed on fungi, including ectomycorrhizal (ECM) species. Many of these ECM species are hypogeous and rely on mycophagous animals such as quenda to disperse their spores. We explored the tripartite relationship between a keystone Mediterranean tree, its associated mycorrhizal fungi, and a mammalian disperser. Wild-collected quenda scats were used as a mycorrhizal inoculum to grow *Eucalyptus gomphocephala* from seeds under glasshouse conditions. Autoclaved scats were used as a negative-control and sporocarp tissue from *Pisolithus* and *Scleroderma* ectomycorrhizal species were mixed together as a positive-control inoculum. Seedlings were harvested at 10 weeks to assess seedling growth and early mycorrhizal colonization of roots by high-throughput DNA sequencing. Quenda scat successfully introduced fungi to seedlings, shown by a 56% overlap of fungal operational taxonomic units (OTUs) detected in the scats and roots grown in fresh scat inoculum. Scat-inoculated seedlings had richer root mycorrhiza fungal assemblages and a higher proportion of mycorrhizal taxa compared to negative- and positive-controls. However, no difference in shoot or root mass in these young seedlings could be attributed to root fungi assemblages at this early growth stage, possibly reflecting that the role of mycorrhizae in these early seedlings was parasitic, rather than facilitatory. Our study has shown that spores of mycorrhizal fungi from the quenda scat inoculum can successfully germinate and colonize seedling roots after passage through the quenda gut.

Key words: *Eucalyptus gomphocephala*, Mycorrhizae, Peramelidae, plant health, rhizosphere.

INTRODUCTION

Healthy ecosystem functioning involves complex interactions between flora, fauna, and the physical properties of the ecosystem. Fauna may increase the resilience of ecosystems and plants to environmental stressors by bioturbation. Bioturbation is the manipulation of soil while foraging or creating shelter, thus improving soil turnover, capturing organic matter and increasing water infiltration (Fleming *et al.* 2014). While foraging, bioturbators not only change physical properties of soil, but also contribute to biological processes like seed and fungal spore dispersal (e.g. James *et al.* 2009; Valentine *et al.* 2017).

Fungal spore dispersal by bioturbators, especially of hypogeous mycorrhizal species, is expected to play an important role within Australian ecosystems where landscapes are characterized by infertile and weathered soils (Orians & Milewski 2007). These nutrient-poor soils (especially deficient in phosphorous and nitrogen) can greatly benefit from mycorrhizal interactions, and this is evident through the vast majority of native Australian plants (e.g. *Eucalyptus* spp.) having evolved symbiotic relationships with mycorrhizal fungi (Brundrett 2009). Mycorrhizal fungi allow plants greater access to the limited soil nutrients and water (Harley 1989; Landeweert *et al.* 2001), thus playing an important role in healthy plant functioning. In return, the fungi are provided with photosynthates from the plant.

Mycophagy is widespread among Australian mammals and likely plays an important role in the maintenance of both fungi and plant community dynamics (Claridge & May 1994). The presence of mycophagous animals can alter or maintain rates of mycorrhizal colonization by the dispersal of spores in their scats (e.g. Cázares & Trappe 1994; Terwilliger & Pastor 1999). Mammals are capable of travelling larger distances than other dispersal methods (e.g. air turbulence, root-to-root contact) (Johnson 1996), and are able to move spores between distinct habitat patches. The role of mammals as fungal dispersal agents has been widely investigated (Blaschke & Bäumler 1989; Cázares & Trappe 1994; Johnson

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1994; Frank *et al.* 2006; Vernes & Dunn 2009), and spores have been demonstrated to remain viable after passage through the mammalian gut (Trappe & Maser 1976; Lamont *et al.* 1985; Claridge *et al.* 1992; Reddell *et al.* 1997; Colgan & Claridge 2002). Indeed, for some mycorrhizal fungi, greater spore viability is achieved for spores that have passed through the gut of a mammal (Colgan & Claridge 2002).

In Australia, mycophagous animals include most terrestrial herbivorous and omnivorous mammals under 3 kg (Johnson 1996). While many native mycophagous species have greatly declined from their range, the southern brown bandicoot (*Isoodon obesulus fusciventer*) still persists in urban and peri-urban environments. Known locally as 'quenda', these medium-sized marsupials occur throughout the Swan Coastal Plain in southwestern Australia. Quenda turn over soil as they forage for subterranean food (Valentine *et al.* 2012), and their digging actions improve soil properties, increase water infiltration, and accelerate nutrient cycling (Valentine *et al.* 2017). *Isoodon* species are omnivores, known to feed opportunistically on fungi (Quin 1988; Claridge & May 1994; Mcilwee & Johnson 1998; Keiper & Johnson 2004). DNA analysis of scat content has demonstrated that they consume a wide variety of fungal species, many of them known to form mycorrhizal associations with *Eucalyptus* species (A. Hopkins, pers. comm.). Quenda home ranges vary between 0.28 and 8.1 ha (Broughton & Dickman 1991), theoretically giving them the ability to disperse fungal spores over several hundred metres. In suburban and peri-urban areas, such a distance could be sufficient to disperse fungus spores cross barriers such as roads and housing developments that surround and isolate patches of remnant woodland.

Eucalyptus gomphocephala ('Tuart') is a keystone tree species endemic to the southwestern Australian Floristic Region. A marked reduction in health and vitality of the *E. gomphocephala* population has managers and conservationists concerned about their persistence (Scott *et al.* 2013; Ishaq *et al.* 2013). A positive association has been documented between the presence of ectomycorrhizal (ECM) fungi and healthy *E. gomphocephala* trees (Scott *et al.* 2013; Ishaq *et al.* 2013), however, little is known about the relationship between ECMs, plant health and mammal bioturbators as spore vectors. We therefore investigated the effect of a scat inoculum from quenda, on the initial establishment, growth and rhizosphere fungi of *E. gomphocephala* seedlings grown in sterile river sand under glasshouse conditions. We predicted that the presence of fungal ECM spores from scats would increase the species richness of mycorrhizal rhizophere fungi, and therefore improve seedling establishment (shoot or root growth). Identifying the potential role bioturbators play in sustaining mycorrhizal community diversity will help inform management actions regarding bioturbator populations and *E. gomphocephala* woodland remnants.

METHODS

Scat and fungi collection

A total of 106 reserves within the City of Mandurah, Western Australia, were surveyed for quenda activity in 2014 (Bryant *et al.* 2017) and reserves with the highest levels of activity were subsequently surveyed for quenda scats over winter (May-June 2015). Six of these sites were determined to have sustained and reliable quenda activity during the 2015 scat collection and were chosen for this study. Habitat variables of these sites are outlined in Table 1. Scats were collected via area searches within each study site, with a variable number of scats found at each site (range: 5–11). Scats were identified by size and shape (Triggs 2003), and only collected if found in close proximity (<1 m) to fresh quenda digs to ensure the correct species identification. Quenda diggings were identified based on their size and shape: they are generally conical with an average (±1SD) diameter of 100.9 ± 3.9 mm and depth of 69.6 ± 3.9 mm (depth range 35–135 mm for $n = 47$ measured) (Valentine *et al.* 2012). Scats were stored individually at −18°C; however, at the time of the glasshouse trial, to have sufficient volume for analyses, all scats samples were pooled by site. Scats were macerated using a sterilized mortar and pestle, a portion set aside for DNA sequencing, and the rest mixed with deionized water to form a homogenized slurry for the glasshouse trial ('base inoculum').

Sporocarps from *Scleroderma* and *Pisolithus* spp. were collected from the same study sites in early July 2015. Both genera of mycorrhizal fungi have been associated with *E. gomphocephala* (Ishaq *et al.* 2013) and have easily recognizable sporocarps. To ensure no cross-contamination with the scat samples, fungi were collected on separate days from scats. Fungal specimens were air-dried and stored at room temperature. At the time of the glasshouse trial, spore-bearing tissue from both *Scleroderma* and *Pisolithus* spp. sporocarps were sampled and combined with deionized water to make a spore mixture for planting.

Experimental design

To investigate early establishment of mycorrhizal associations in *E. gomphocephala* seedlings, we used a factorial experiment consisting of four inoculum treatments across the six sites, with three replicates per site (total $n = 72$ pots). Treatment 1 (F Scat) used fresh scat from the base inoculum. Treatment 2 (A/C Scat) was a negative scat control with the base inoculum autoclaved before application (121°C for 20 min). A positive control, treatment 3 (F Spore), used fresh spore mixture and autoclaved base inoculum. Treatment 4 (A/C Spore) was a negative spore

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Table 1. Site habitat variables of the six urban reserves within the City of Mandurah that were the source of inoculum used in this study. Habitat variables were collected as part of a larger survey of 106 sites and detailed in Bryant *et al.* 2017. Distance to urban area and native vegetation extent (%) were determined by GIS analyses. Other variables were recorded from on the ground surveys from 10 × 10 m quadrats (vegetation type, condition and canopy cover) and at 5 × 5 m quadrats nested within the larger quadrat (leaf litter depth, woody debris and soil type)

Site	Latitude	Longitude	Distance to urban area (km)	% Native vegetation (200 m buffer)	Vegetation type	Vegetation condition	Canopy cover %	Leaf litter depth (cm)	% Woody debris	Soil type
FERN	32°38′47.3″S	115°38′45.0″E	0.25	69.43	Woodland	Excellent	10	2	5	Loamy sand
GUMN	32°38′18.6″S	115°38′27.9″E	0.15	79.75	Woodland	Excellent	25	6	5	Loamy sand
ISLA	32°45′18.8″S	115°41′34.4″E	9.57	78.72	Woodland	Good	50	4	10	Sand
MARL	32°29′16.9″S	115°46′19.7″E	2.15	90.14	Woodland	Excellent	70	6	5	Sand
SOUT	32°46′37.9″S	115°41′48.2″E	11.12	82.58	Estuary	Good	15	1	2	Loamy sand
TOUC	32°38′31.5″S	115°38′16.1″E	0	34.08	Woodland	Excellent	30	3	20	Loamy sand

control using both autoclaved spore mixture and autoclaved base inoculum. Treatments (0.5 g dry weight of each mixture) were applied approximately 0.5 cm below the soil surface in each pot.

Eucalyptus gomphocephala seeds were collected from the Lake Clifton region, Yalgorup National Park, southwestern Australia, where previous research on quenda, mycorrhizal fungi and *E. gomphocephala* have been carried out (So *et al.* 2011; Scott *et al.* 2013; Valentine *et al.* 2012, 2017; Ishaq *et al.* 2013). Seeds were grown in steam-sterilized river sand in sterilized forestry tubes (50 × 50 × 120 mm). Forestry tubes were lined with plastic bags so they would not be free-draining to minimize the washing away of nutrients and the inoculum treatments. Individual pots were watered daily using deionized water to field capacity to avoid waterlogging. Seeds were germinated directly in the inoculated soil.

Pots were arranged in a randomized block design and were placed in a heated growth cabinet (25°C, 12 h light/dark cycle) for germination. At two weeks old, seedlings were thinned to two per pot and then moved into a glasshouse where they were exposed to natural photoperiods and daily temperatures between 8–27°C. Seedlings were further thinned to one per pot at six weeks old by removing whole seedlings (including roots) where possible. Seedlings were harvested 10 weeks after planting to investigate the early mycorrhizal colonization in roots and their effect on seedling growth.

Seedling growth

When harvested, seedlings were washed free of sand and any remaining treatment slurry. Shoots were cut at soil level and their wet weight recorded after being patted dry with paper towels. One seedling from treatment 2 (A/C Scat) had ceased root growth early and was removed from the analysis.

Whole roots were suspended in deionized water and inspected under a dissecting microscope (10 × magnification) to confirm mycorrhiza colonization of roots had occurred. Roots were not stained to allow for DNA sequencing to be conducted later and no attempt was made to quantify the incidence of mycorrhizal association due to the early development stage of the mycorrhiza. Roots were then patted dry and their wet weight recorded. Roots were carefully stripped and the fine roots frozen for DNA sequencing.

Differences in shoot and root wet weights were analysed using a mixed-model ANOVA (site as random factor, inoculum-type 'Scat versus Spore mixture' and 'fresh or autoclaved' as fixed factors) on BoxCox-transformed data which were not significantly different from a normal distribution (Shapiro Wilk test for shoots: $P = 0.986$; roots: $P = 0.972$) and showed homogeneity of variances (Levene's test for shoots: $P = 0.078$; roots: $P = 0.063$). Where significant interactions between inoculum-type and 'fresh or autoclaved' factors were evident, we carried out post-hoc pairwise analyses for each seedling treatment using Bonferroni corrections.

DNA extraction and amplification

Fine root material was thoroughly washed free of excess soil and DNA extracted from fine root samples (0.05 g each) using the PowerPlant® Pro DNA Isolation Kit following the manufacturer's protocols (MO BIO Laboratories 2014). To determine the fungal taxa present in the base inoculum, DNA was extracted from four sub-samples of each base inoculum mixture per site (0.25 g each) using the MoBio PowerSoil® DNA Isolation Kit (MO BIO Laboratories 2014). All extractions were performed with extraction controls.

The ITS2 region of the ribosome encoding genes was amplified using the fungal-specific primer fITS7 (GAACGCAGCRAAIIGCGATA, specific for higher fungi, Ihrmark *et al.* 2012) and the general primer ITS4 (White *et al.* 1990) with adapters attached. Amplification was performed following the protocols outlined in Ihrmark *et al.* (2012) using HotStarTaq (Qiagen, Valencia, CA, USA), 30 amplification cycles and an annealing temperature of 57°C. The

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ITS4 primer contained a 10-base pair tag specific to each sample. Two replicate PCRs were performed per sample and these were combined following amplification. Negative controls were undertaken for each primer pair. The products were visualized on a 1% agarose gel and then mixed in similar concentrations before being cleaned using an Agencourt AMPure® XP PCR purification kit (Beckman Coulter Inc., MA, USA).

High-throughput sequencing (HTS) DNA analysis

The final sequence library was subjected to 454 sequencing (454 Life Sciences Corp. 2011) on a Roche GS Junior at the Western Australian State Agricultural Biotechnology Centre, Murdoch University. Extraction controls and PCR controls were also tagged separately and sequenced. Deconvolution (sequence cleanup and clustering) was carried out in the Sequence Clustering and Analysis of Tagged Amplicons (SCATA) pipeline (scata.mykopat.slu.se) with standard settings according to Ihrmark *et al.* (2012) and a clustering threshold of 98.5% similarity. Prior to clustering, all sequences were trimmed to 300 bp length. Raw molecular data are stored at the Sequence Read Archive (SRA) curated by NCBI under the accession number SRP 132166.

Representative cluster sequences (molecular operational taxonomic units or OTUs) were identified by searching against internally curated SCATA databases. The OTUs were then blasted against NCBI's sequence database GenBank (Altschul *et al.* 1990) through Geneious. Potentially similar OTUs were aligned in Geneious to confirm identification. Where identifications could be made with sufficient accuracy, OTUs were designated to a putative ecological functional group (e.g. ectomycorrhizal (ECM), arbuscular (AM), pathogen, saprotroph) based on one of two criteria. The life history of the majority of identified OTUs was determined based on literature searches. For a small number of OTUs, searches on NCBI revealed a close alignment with sequences from a known source (e.g. fruitbody, mycorrhizal root tip etc.) and this information was used as an additional layer to guide putative functional group classification. For example, fungi were considered to be putatively mycorrhizal if they fell within orders, families or genera with predominantly mycorrhizal members or had sequences that closely aligned with known mycorrhizal isolates (ECM or AM).

To compare the fungal community composition in the base inoculum and root samples, we performed non-metric Multidimensional Scaling using Bray-Curtis similarity index (PAST v3) (Hammer *et al.* 2001). Differences in the root fungi community between seedling treatments was assessed using a two-way PERMANOVA (factors: 'inoculum-type' and 'fresh or autoclaved'), followed by a post-hoc pairwise analysis using Bonferroni corrections. SIMPER analyses were then carried out to identify which OTUs contributed to the treatment differences. Singleton and doubleton sequences/OTUs were removed from the dataset prior to this analysis. Correlation between seedling growth parameters (shoot and root mass) and the relative abundance of each of the top ten fungi OTUs from the SIMPER analysis was determined using Pearson's correlation coefficient (R). Values are presented as averages ± 1SD throughout.

RESULTS

Seedling growth

Taking into account site as a random factor in our analysis, there was a significant difference in shoot mass between scat/spore treatments ($F_{1,47} = 45.71$, $P = 0.001$) but no difference between fresh/autoclaved treatments ($F_{1,47} = 0.27$, $P = 0.625$). Scat-only treatments (T1 'F Scat' and T2 'A/C Scat') had smaller shoots than the spore treatments (T3 'F Spore' and T4 'A/C Spore') (Fig. 1a). There were no main effects of scat/spore ($F_{1,47} = 6.53$, $P = 0.051$) and fresh/autoclaved ($F_{1,47} = 0.76$, $P = 0.425$) treatments, but a significant interaction term for root mass ($F_{1,47} = 19.54$, $P = 0.008$). Post-hoc pairwise comparisons using Bonferroni corrections revealed a significant difference between T2 'A/C Scat' and T4 'A/C Spore' only ($P = 0.011$). Overall, T2 'A/C Spore' had the smallest root mass (Fig. 1b).

Fungi associated with the base scat inoculum

High-throughput sequencing of the base inoculum (fresh scat material not baited using seedlings) yielded 433 OTUs (singletons and doubletons excluded) from inoculum collected across the six sites, with an average of 129 ± 77 OTUs per site (range 76–278). The most common fungal OTU detected in the scat inoculum was a putatively sequestrate ECM *Russulaceae* species (12.6% of all sequence reads), followed by the ECM truffle *Descomyces angustisporus* (7.6%). Operational taxonomic units representing *Boletales* (16.7%) and *Agaricales* (15.9%) orders were also commonly represented in the scat inoculum.

Ninety OTUs (81.1% of all DNA reads) were identified and categorized to a putative ecological form (Table 2). The 27 ECM and five saprotroph OTUs were identified as putatively forming macrofruiting bodies (e.g. mushrooms, truffles, stinkhorns, cup fungi) that could potentially represent diet items for quenda. Only one AM fungal OTU was detected: a *Glomeraceae* species that made up only 0.04% of reads. Lichenised fungi and chytrids were also detected, but in very low numbers.

Root fungi

Sequencing of the seedling roots detected 288 distinct fungal OTUs (singletons and doubletons excluded). Of these, 185 OTUs (86.4% of all DNA reads from the seedling roots) were identified to their putative ecology. In contrast to the base inoculum,

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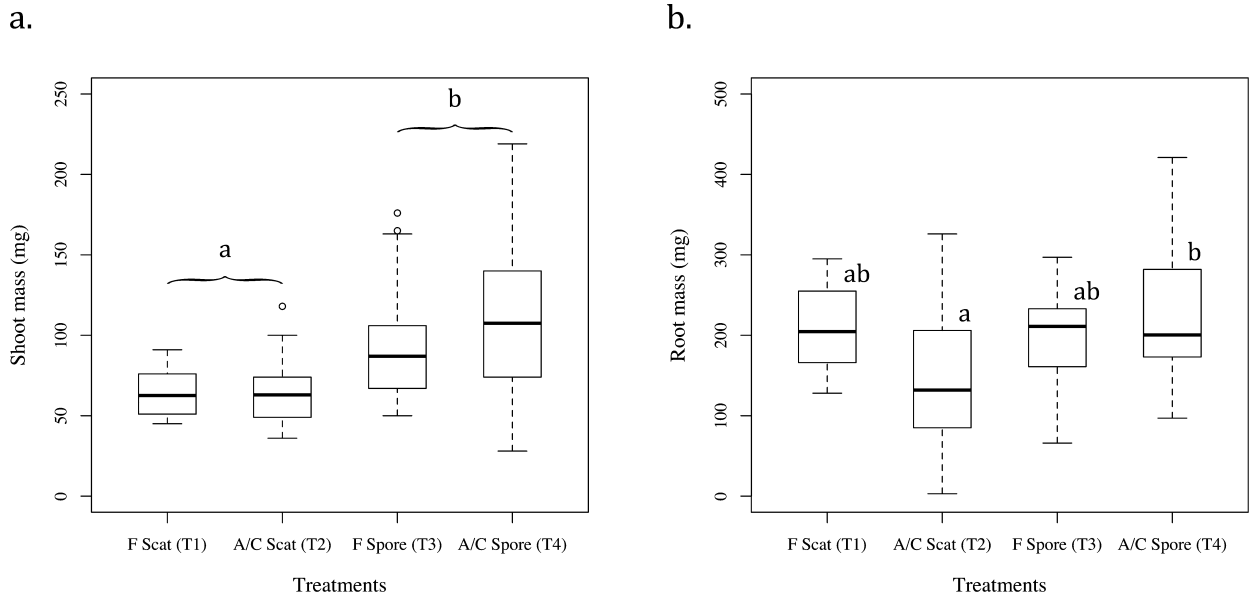

Fig. 1. (a) Shoot and (b) root mass (mg) of 10-week-old *Eucalyptus gomphocephala* seedlings by treatment. Raw values are represented for clarity, although statistical analyses were carried out on BoxCox-transformed values. Mixed-model ANOVAS using inoculum-type ('scat or spore') and 'fresh or autoclaved' as fixed factors were tested. Significant differences in shoot mass were driven by the main effect of inoculum type, while differences in root mass were driven by the interaction of the two fixed factors and thus evaluated using post-hoc pairwise analyses with Bonferroni corrections. Significance levels are denoted by letters above each box plot and outliers represented by hollow circles. F Scat (T1): fresh scat inoculum; A/C Scat (T2): autoclaved scat inoculum; F Spore (T3): fresh spore inoculum; A/C Spore (T4): autoclaved spore inoculum.

Table 2. Overall results of the total number of fungal operational taxonomic units (OTUs) sequenced as part of this study and their putative ecological functions. The overall percentage of sequence reads from each ecological functional group are shown for OTUs detected in (a) the base inoculum and (b) seedling roots

	Total No. OTUs	a. Base inoculum		b. Seedling roots		Overlap No. OTUs
		No. OTUs	% reads	No. OTUs	% reads	
Total sequenced	629	433	100.00	288	100.00	92
Identified ecology	199	90	81.09	185	86.39	76
ECM	49	27	56.14	43	11.27	21
AM	6	1	0.04	6	1.04	1
Saprotroph	58	20	8.70	55	45.56	17
Pathogen	28	15	1.36	27	13.85	14
Dung fungi	23	5	2.51	23	11.16	5
Yeast	20	17	8.24	17	2.02	14
Others *(eg. chytrids, endophytes, lichen)*	15	5	4.10	14	1.50	4
Unknown ecology	430	343	18.91	103	13.61	16

the majority of the sequence reads were from saprotrophic taxa rather than ECMs (Table 2).

Differences in the community composition of fungal taxa were also apparent between treatment groups. Roots treated with T1 (F Scat) showed the greatest proportion of root colonization by mycorrhizal species, both ECM and AM (Fig. 2), and also the greatest species richness of mycorrhizal species (Fig. 3). The presence of some fungal OTUs on seedling roots across all treatments despite not being detected in the source base inoculum (Fig. 3) was likely due to fungal species present in the glasshouse. Nevertheless, there were marked differences between seedling treatment groups (non-metric multidimensional scaling followed by a two-way PERMANOVA; Fig. 4). There was a significant difference between main effects of scat/spore treatments ($F_{1,67} = 4.10$, $P < 0.001$) and fresh/autoclaved treatments ($F_{1,67} = 5.40$, $P < 0.001$), as well as their interaction term ($F_{1,67} = 2.79$, $P = 0.003$). Post-hoc tests using Bonferroni corrections revealed significant differences in root fungi community between all treatments, except the two autoclaved treatments (T1 'A/C Scat' and T4 'A/C Spore': $P > 0.05$).

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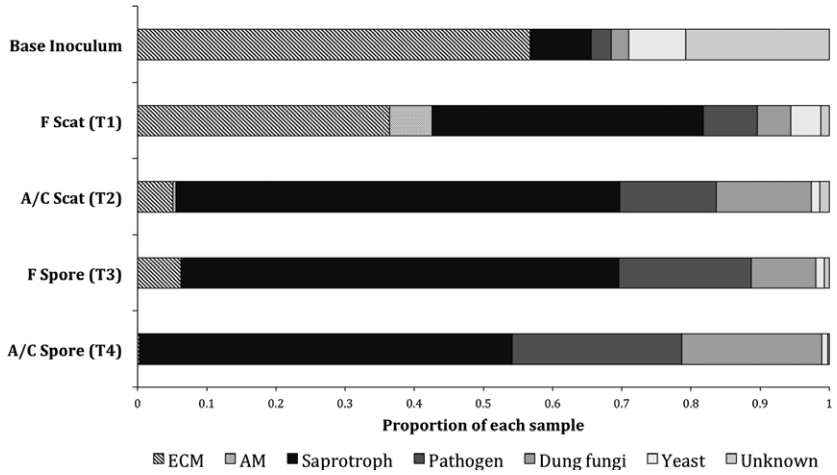

Fig. 2. The average proportion of each ecological functional group detected in base inoculum and *Eucalyptus gomphocephala* roots by treatments. Functional groups were classified as ectomycorrhizal (ECM), arbuscular mycorrhizal (AM), saprotrophic, pathogenic, dung fungi, yeast and unknown. Hashed regions represent mycorrhizal groups. Chytrids and endophytes were omitted from this analysis due to their very low proportions. Base inoculum: quenda scat not baited with seedlings; F Scat (T1): fresh scat inoculum; A/C Scat (T2): autoclaved scat inoculum; F Spore (T3): fresh spore inoculum; A/C Spore (T4): autoclaved spore inoculum.

There was a 56% crossover of fungal OTU reads between the base inoculum and root samples inoculated with T1 (F Scat). With the exception of a *Tuberaceae* species (likely belonging to either *Redellomyces or Labyrinthomyces*) and *Descomyces angustisporus*, few ECM from the base inoculum were found in high quantities in T1 (F Scat) roots. Ten fungal OTUs contributed to 50% of dissimilarity between root fungi assemblages across the seedling treatments (Table 3). Mycorrhizal taxa contributed only 11.88% to dissimilarity between treatments but were detected, on average, in higher proportions in T1 'F Scat' (Fig. 5a). A saprotrophic species, *Chaetomium nigricolor*, was found to be the major OTU contributing to dissimilarity due to its high relative abundance (it was present in every seedling treatment; Fig. 5b).

Despite the marked difference in mycorrhizal root colonization between the inoculation seedling treatments, there was no correlation found between seedling growth parameters (shoot and root mass) and any of the ten fungi taxa identified in the SIMPER analysis (Table 4).

DISCUSSION

Our study demonstrates that spores of mycorrhizal fungi from quenda (*I. obesulus fusciventer*) scat inoculum can successfully germinate and colonize seedling roots of a key eucalypt after passage through the gut. Clearly, fungal spores remain viable after excretion and successfully colonize plant roots. Thus, small mammals such as quenda play an important role as ecosystem engineers (Fleming *et al.* 2014) by preferentially eating a wide range of fungal fruiting bodies and dispersing spores, thereby facilitating mycorrhizal fungal associations. Their role as a dispersal agent could be particularly vital in degraded and fragmented habitats, especially where there is nutrient depauperate soil, such as across the urban landscape.

We found a large overlap between fungal OTUs from the quenda scat base inoculum (that is, not baited with seedlings) and from seedling roots treated with fresh scat inoculum (T1 'F Scat'), which indicated quenda scats successfully provided a source of suitable fungi. Furthermore, despite the colonization of fungi from environmental sources outside the inoculum (detected in negative-control treatments), seedlings grown in T1 (F Scat) had a much greater species richness of rhizosphere fungi and a greater proportion of sequence reads from mycorrhizal taxa compared to other treatments. This indicates that scats made a marked contribution towards the development of mycorrhizal associations.

While mycorrhizal inoculation using mycophagous marsupial scat has previously been reported to enhance eucalypt seedling growth (Lamont *et al.* 1985; Claridge *et al.* 1992), we did not record a positive effect of the presence of live scat material on seedling growth. Shoot mass of seedlings grown with fresh scat did not differ significantly from their autoclaved scat control, and were actually smaller than seedlings inoculated with the spore controls. Therefore, while the scat inoculated treatment showed a much greater species richness of rhizosphere fungi, especially of ECM species, no benefit of these mycorrhizal associations were detected in seedling growth

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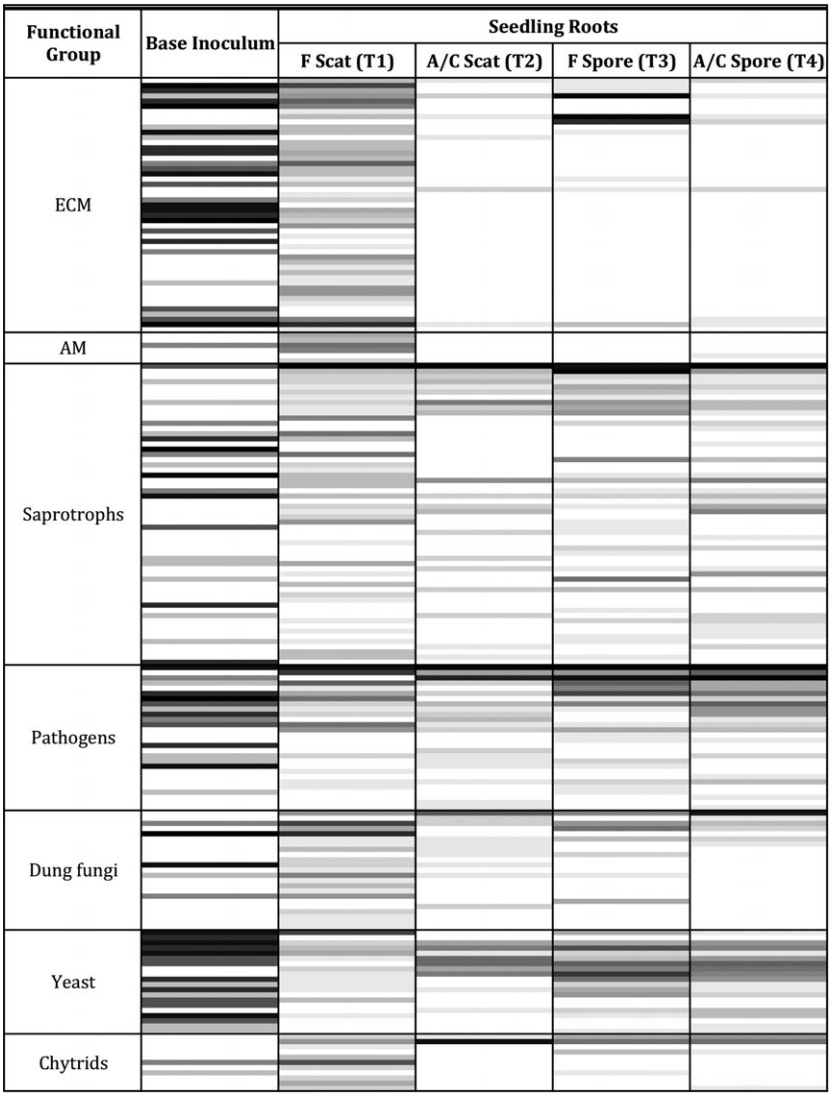

Fig. 3. Shadeplot showing the relative frequency of occurrence of each fungal operational taxonomic unit (OTU) detected by treatment. Operational taxonomic units are ordered top down according to the overall frequency of sequence reads within each ecological functional group, with the most common OTUs at the top. Functional groups were classified as ectomycorrhizal (ECM), arbuscular mycorrhizal (AM), saprotrophic, pathogenic, dung fungi, yeast and chytrids. Shade intensity represents the relative frequency of occurrence: black bars mean the OTU was detected in all samples of that treatment, white bars mean the OTU was not detected within that treatment. Base inoculum: quenda scat not baited with seedlings; F Scat (T1): fresh scat inoculum; A/C Scat (T2): autoclaved scat inoculum; F Spore (T3): fresh spore inoculum; A/C Spore (T4): autoclaved spore inoculum.

parameters at the 10 week harvest. This phenomenon is fairly common, with early growth depressions from mycorrhizal colonization reported in a number of experiments (e.g. Jones & Smith 2004). This may reflect that most energy is prioritized for root growth and establishment of mycorrhiza, rather than shoot growth in these early stages. The apparent 'parasitic effects' of mycorrhizal fungi have been reported in simplified one-off experiments, particularly in the developmental stage, but these fungal–plant interactions become beneficial for the plant when observed over a longer time scale (Johnson *et al.* 1997). Hence short-term losses may lead to long-term gains once the mycorrhiza have fully developed, and there is no longer a need to prioritize mycorrhizal formation on roots. The lower shoot mass for the scat-only treatments (T1 'F Scat' and T2 'A/C Scat') could also reflect the smaller quantity of nutrients available in the nutrient-deficient river sand substrate. Similar shoot and root masses in the positive sporocarp treatment (T3 'F Spore') and its autoclaved control (T4 'A/C Spore') suggests the increased access to nutrients from the addition of sporocarp tissue better enhanced seedling growth compared to the

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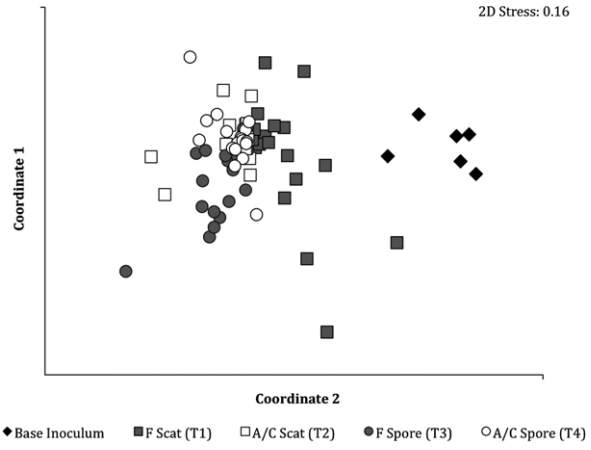

Fig. 4. Non-metric MDS ordination of the fungal assemblage found in the base inoculum and in *Eucalyptus gomphocephala* seedling roots. Black symbols represent base inoculum samples; grey symbols indicate seedling roots inoculated with live material; hollow symbols represent seedling roots grown in fully autoclaved material. Squares are scat-only treatments and circles are spore treatments. Base inoculum: quenda scat not baited with seedlings; F Scat (T1): fresh scat inoculum; A/C Scat (T2): autoclaved scat inoculum; F Spore (T3): fresh spore inoculum; A/C Spore (T4): autoclaved spore inoculum.

mycorrhizal root associations at this early stage. Fungal sporocarps are known to be a potentially rich source of nutrients (Wallis *et al.* 2012), and the presence of the sporocarp tissue, in addition to the autoclaved scat material, may have given seedlings in T3 and T4 a growth advantage.

Time successions of mycorrhizal colonization of roots have been shown in many species, with community composition of rhizosphere fungi changing with tree age (e.g. Dighton & Mason 1985). Patterns of succession are complex and are affected by factors such as spore germination time, host-specificity or competitiveness between fungal species (Dighton & Mason 1985; Twieg *et al.* 2007; Ishida *et al.* 2008). The low proportion of root colonization by ECM taxa for the fresh scat-inoculated seedlings (T1 'F Scat'), namely by *Russulaceae*, *Boletales* and *Agaricales* species, was surprising given their high occurrence in the base inoculum. It is possible that these mycorrhizal taxa do not play an important role in the initial establishment of seedlings and their early-stage development. *Descomyces angustisporus* was the only ECM species occurring at relatively high frequency in the base inoculum to be detected in *E. gomphocephala* roots. Instead, other ECM species detected in the base inoculum in lower frequencies such as the *Pyronemataceae* sp. and the *Tuberaceae* sp. were able to better colonize the developing roots of *E. gomphocephala* in this study. Competition or differential rates of colonization between fungal species could be at play here. The presence of *Trichoderma* in the seedling roots presents one such factor. *Trichoderma* is an endophytic plant symbiont, known to be an early and rigorous colonizer of roots due to their antagonistic interactions with other microbes in the rhizosphere (Verma *et al.* 2007). Such non-ECM associations may be quick to establish in the roots of newly established seedlings and compete with ECMs for colonization. The other fungi contributing to variation between treatments (Table 3) are ubiquitous species that are likely to be endophytic on the roots. Further investigation of interactions between these fungal taxa and their sequence of colonization of *E. gomphocephala* roots over time could give more insight to their potential fitness benefits for plant growth.

Quenda clearly have the potential to influence ecosystem processes through their foraging activities. The combination of soil bioturbation and mycorrhizal fungi dispersal have important flow-on effects for the health

Table 3. The top ten operational taxonomic units (OTUs) together contributing to a total 51.42% dissimilarity between root fungal assemblages from the four seedling treatments. Seedling treatments were as follows: F Scat (T1) 'fresh scat inoculum'; A/C Scat (T2) 'autoclaved scat inoculum'; F Spore (T3) 'fresh spore inoculum'; A/C Spore (T4) 'autoclaved spore inoculum'. Putative ecological function of each OTU identified as ectomycorrhizal (ECM), saprotrophs, pathogens and dung fungus

OTU Identity	Taxon	Habit	Ecology	Average Contribution %	Cumulative Contribution %
scata3214_0	*Chaetomium nigricolor*	Microscopic	Saprotroph	19.48 ± 5.36	19.48
scata3214_2	*Podospora* species	Microscopic	Dung fungus	6.50 ± 4.59	25.98
scata3214_3	*Fusarium oxysporum*	Microscopic	Pathogen	6.47 ± 2.96	32.45
scata3214_4	*Trichoderma* species	Microscopic	Saprotroph	5.31 ± 5.85	37.76
scata3214_97	*Russulaceae* species	Truffle	ECM	2.82 ± 3.61	40.59
scata3214_5	*Pyronemataceae* species	Macro fruiting body	ECM	2.61 ± 3.40	43.19
scata3214_8	*Descomyces angustisporus*	Truffle	ECM	2.55 ± 1.88	45.74
scata3214_6	*Tuberaceae* species	Truffle	ECM	2.15 ± 2.79	47.90
scata3214_11	*Lasiosphaeriaceae* species	Microscopic	Saprotroph	1.78 ± 1.14	49.68
scata3214_54	*Agaricomycotina* species	Macro fruiting body	ECM	1.74 ± 2.10	51.42

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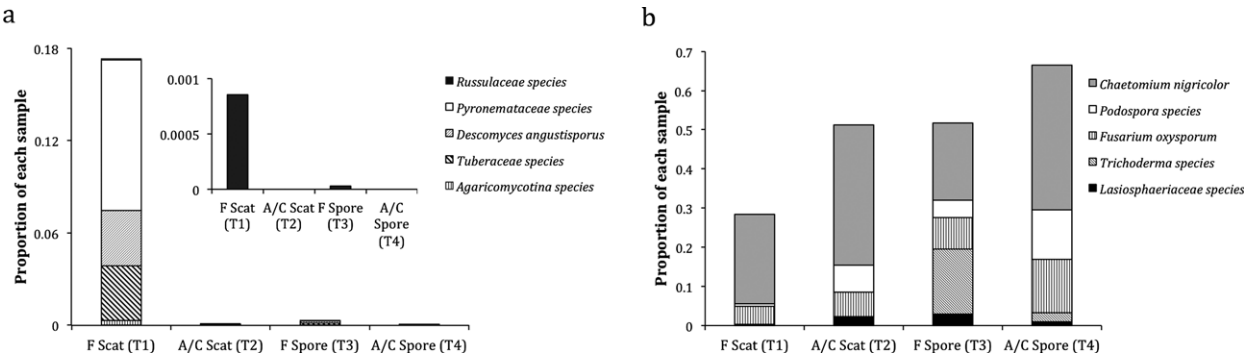

Fig. 5. Top ten contributors to dissimilarity between seedling treatments as shown in Table 3. Operational taxonomic units (OTUs) were separated into (a) mycorrhizal taxa (with an insert showing low proportions of a *Russulaceae* species), (b) non-mycorrhizal taxa. Values shown are the average proportion of each root sample contributed by each OTU. F Scat (T1): fresh scat inoculum; A/C Scat (T2): autoclaved scat inoculum; F Spore (T3): fresh spore inoculum; A/C Spore (T4): autoclaved spore inoculum.

Table 4. Correlations between seedling growth parameters and the relative abundance of each of the ten selected fungal operational taxonomic units (OTUs) detected in the corresponding seedling roots. Fungal OTUs tested were the top contributors to overall root fungal assemblage dissimilarity between seedling treatments from Table 3. Pearson's correlation coefficients (*R*) calculated using BoxCox-transformed shoot and root mass values

				Shoot mass		Root mass	
OTU Identity	Taxon	Habit	Ecology	*R*	*P*-value	*R*	*P*-value
scata3214_0	*Chaetomium nigricolor*	Microscopic	Saprotroph	0.04	0.72	−0.08	0.492
scata3214_2	*Podospora* species	Microscopic	Dung fungus	0.21	0.083	0.16	0.183
scata3214_3	*Fusarium oxysporum*	Microscopic	Pathogen	0.17	0.159	0.18	0.142
scata3214_4	*Trichoderma* species	Microscopic	Saprotroph	0.18	0.124	0.09	0.466
scata3214_97	*Russulaceae* species	Truffle	ECM	−0.16	0.177	0.01	0.922
scata3214_5	*Pyronemataceae* species	Macro fruiting body	ECM	−0.09	0.479	−0.08	0.510
scata3214_8	*Descomyces angustisporus*	Truffle	ECM	−0.04	0.720	0.14	0.250
scata3214_6	*Tuberaceae* species	Truffle	ECM	−0.10	0.385	0.11	0.364
scata3214_11	*Lasiosphaeriaceae* species	Microscopic	Saprotroph	−0.01	0.906	−0.11	0.343
scata3214_54	*Agaricomycotina* species	Macro fruiting body	ECM	−0.04	0.734	−0.03	0.825

and resilience of *E. gomphocephala* and other flora. Understanding the interactions between key players in maintaining ecosystem health is vital as any threat to any one component of this system can have subsequent consequences for the others. In this study, we explored the tripartite relationship between a keystone Mediterranean tree, its associated mycorrhizal fungi and their mammalian disperser. Our findings, that spores of mycorrhizal fungi from quenda scat inoculum can successfully germinate and colonize seedling roots after passage through the quenda gut, indicate a potential mechanism to improve seedling recruitment and growth under field conditions in the future.

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