

First evidence for the joint dispersal of mycorrhizal fungi and plant diaspores by birds

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Summary

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• Seed dispersal allows plants to colonise new sites and escape from pathogens and intraspecific competition, maintaining plant genetic diversity and regulating plant distribution. Conversely, most plant species form mutualistic associations with arbuscular mycorrhizal (AM) fungi in a symbiosis established immediately after seed germination. Because AM fungi are obligate symbionts, using the same dispersal vector as their host should be highly advantageous for their survival, but the co-dispersal of seeds and AM fungal spores has never been confirmed.

• We aim to clarify the potential role of European birds, essential dispersers for many plant species, as co-dispersers of seeds and AM fungal spores.

• In total, 63 bird droppings with intact seeds were placed in sterilised soil and maintained for 4 months in a protected environment to avoid contamination. Additionally, 173 bird droppings and 729 gauze swabs used to clean birds' feet were inspected for AM fungal spores.

• Although no spores were detected by direct observation of these samples, seven *Rubus ulmifolius* seedlings obtained from four independent droppings of *Erithacus rubecula* and *Sylvia melanocephala* were colonised by AM fungi. Our results show that birds can effectively co-disperse viable seeds and AM fungal spores, potentially over long distances, providing a pivotal mechanism to understand the cosmopolitan distribution of AM fungi.

Introduction

Approximately 80% of all terrestrial plant species form mutualistic associations with arbuscular mycorrhizal (AM) fungi, an interaction that enhances the uptake of mineral nutrients by plants, which can improve plant growth and fitness (Smith & Read, 2008). Mycorrhizal fungi also play an important role in the structure of terrestrial ecosystems, influencing plant community composition (Klironomos *et al.*, 2011; van der Heijden *et al.*, 2015) and ecological succession (Allen & Allen, 1990; Francis & Read, 1994). Most AM fungi are cosmopolitan and the same species can be found on multiple continents and remote oceanic islands (Davison *et al.*, 2015, 2018; Savary *et al.*, 2018). Such a broad distribution pattern suggests a highly effective long-distance dispersal strategy, but the dispersal mechanisms of AM fungi remain very poorly understood (Smith & Read, 2008; Davison *et al.*, 2015). In fact, long-distance dispersal of AM fungi might be difficult because they have hypogeous, relatively large spores (0.01–1 mm) with limited dispersal ability compared to other fungal groups (García de León *et al.*, 2016). Also, AM fungi are obligate symbionts and, therefore, successful colonisation is contingent on the coordinated arrival of fungal spores and suitable

plant hosts to new locations. Microbial dispersal is traditionally assumed to be largely stochastic (Allen *et al.*, 1989; Peay & Bruns, 2014; Evans *et al.*, 2017), although AM fungal spores, hyphae or colonised root fragments can be transported by several vectors (Smith & Read, 2008). There is empirical evidence of dispersal of AM fungal propagules by wind (Warner *et al.*, 1987; Egan *et al.*, 2014), ingested by or attached to invertebrates (McIlveen & Cole, 1976; Warner *et al.*, 1987; Gange, 1993; Harinikumar & Bagyaraj, 1994), and ingested by rodents (Janos *et al.*, 1995; Mangan & Adler, 2002; Vernes & Dunn, 2009) and large mammals (Lekberg *et al.*, 2011). Yet, we remain largely ignorant regarding the mechanisms by which AM fungi travel over long distances. Interestingly, having specialised structures for seed dispersal is significantly more frequent in obligate mycorrhizal plants than in nonmycorrhizal plants (Correia *et al.*, 2018). Thus, the joint dispersal (i.e. co-dispersal) of plants and symbiotic fungi might be potentially advantageous for both groups. Discussion of the possibility of mechanisms for co-dispersal has been present in the literature for a long time (Nicolson, 1959; Koske & Gemma, 1990). Pirozynski (1983) suggested that the concomitant dispersal of mycorrhizal fungus and host may be necessary to explain the wide distribution of the ectomycorrhizal

Pisonia sp. shrub in coral cays of the Indian and Pacific oceans. Nonetheless, actual reports of long-distance co-dispersal of mycorrhizal fungal spores are incredibly scarce. It has been observed in two coastal strand plants from Hawaii (*Sporobolus virginicus* and *Jacquemontia sandwicensis*) with rhizomes containing AM fungi that break off and disperse through ocean currents (Koske & Gemma, 1990), and more recently, for spores of ectomycorrhizal fungi attached to the surface of fruits of *Coccoloba uvifera* drying on the beach and later dispersed by seawater (Séne *et al.*, 2018).

By virtue of their worldwide distribution, abundance and high mobility, birds are important frugivores and well-known dispersers of seeds from a large variety of fleshy- and dry-fruited plants (Whelan *et al.*, 1998; Heleno *et al.*, 2011). Moreover, birds are important dispersers of a vast array of bacteria (Comstedt *et al.*, 2006; Elfving *et al.*, 2010), viruses (Reed *et al.*, 2003) and fungi (Cafarchia *et al.*, 2006; Belisle *et al.*, 2012; da Silva *et al.*, 2016; Suthers, 2016). Thus, birds could also be important dispersal vectors for AM fungi due to their exceptional long-distance dispersal potential (McIlveen & Cole, 1976). Many forest birds are voracious frugivores across the world (Kissling *et al.*, 2009; Garcia *et al.*, 2010; Heleno *et al.*, 2013; Jordano, 2014), and might disperse AM fungal diaspores both internally after ingestion, or externally if diaspores attach to their feathers and feet while foraging on the ground. Here we report on a systematic survey set out to clarify the potential role of European forest birds as co-dispersers of the seeds of fleshy-fruited plants and AM fungal diaspores, both internally and externally.

Materials and Methods

Study site and sampling

The study was carried out in Larçã, central Portugal (40°19'N, 8°24'W), in a maritime pine *Pinus pinaster* L. plantation where old decaying trees have been mostly replaced by Mediterranean scrubland dominated by strawberry tree *Arbutus unedo* L., Portuguese oak *Quercus faginea* Lam, and an understorey rich in fleshy-fruited native species (Supporting Information Table S1; da Silva *et al.*, 2016).

Birds were captured from October 2015 to December 2016 with 168 m mist-nets operated twice per month (minimal interval of 8 d) during the entire period of the study, and on every day of favourable weather ($n=19$ d) in September 2016, which includes the peak of avian migration and the fruiting period for most fleshy-fruited plants. In each session, mist-nets were operated for 5 h after dawn and visited every 30 min (Heleno *et al.*, 2013).

We collected samples in two different ways to assess the dispersal of AM fungal spores both internally, in droppings, and externally, attached to birds' feet. Captured birds were kept inside clean individual holding bags and released after producing a dropping (Heleno *et al.*, 2013). Droppings with undamaged seeds were collected and stored in 2 ml sterile Eppendorf tubes to be screened under a magnifying microscope and used in the

manipulative experiments. In addition, sterile gauze swabs were used to clean the feet of all birds captured and stored individually in sterile envelopes for later observation in the laboratory.

Establishment of mycorrhizas between co-dispersed seeds and fungi

To evaluate the co-dispersal of seeds and AM fungal diaspores in the same dropping, the content of 63 bird droppings obtained in the peak fruiting season (September–October 2016) was buried 2 cm deep individually in 0.25-l pots filled with sterile sand. Sand was autoclaved twice for 1 h at 121°C, left to cool inside the autoclave and transferred to bleach-cleaned pots on a clean lab bench. After being filled with sand and droppings, pots were placed individually in Sunbags (Sigma) to avoid contamination from external AM fungal spores (Walker & Vestberg, 1994). Watering was done once per week using autoclaved distilled water. Pots were kept under controlled conditions in a glasshouse at the Botanical Garden of the University of Coimbra (Portugal).

Simultaneously, seeds of the same species found on the droppings were directly collected from ripe fruits standing on plants at the same site to be used in the control treatments. The seeds were manually extracted from the fruits, washed with distilled water and dried, and the undamaged seeds were stored for the trials. Two control treatments were conducted, a positive control to check natural mycorrhiza formation using field soil, and an experimental control using sterile sand to ensure that there was no contamination during the experiment. The number of seeds placed in each control pot depended on their availability and size, in an effort to maximise the probability of having germinated seeds. For the positive control, 4–6 seeds of each plant species were sown in 0.25-l pots filled with soil from Larçã. Four to six replicates were used in this treatment per plant species. For the experimental control, 4–6 seeds of each plant species were sown in each 0.25-l pot filled with sterile sand. In this treatment, seed surfaces were disinfected in 96% ethanol for 30 s and 4% bleach for 2 min and washed with sterile distilled water. Six to eight replicates were used in this treatment per plant species. All pots were maintained in Sunbags (Sigma), under the same conditions.

Seed emergence was checked every 2–4 d from the beginning of germination. Plants were watered with sterile distilled water every 4 d and grown in the glasshouse under a natural photoperiod for 14 wk. If plants had enough space inside the Sunbags they were left to grow for an additional 2 wk (i.e. 16 wk). Plants were then harvested to evaluate the formation of mycorrhizal associations between the germinated seeds and eventual AM fungal diaspores contained in the sample. Roots were carefully washed and stained following a modification of the method of Vierheilig *et al.* (1998), where roots were cut into 1 cm fragments, cleared in 10% KOH and stained with Parker Quink (Walker, 2005). The assessment of root colonisation by arbuscular mycorrhizal fungi was done by counting arbuscules, vesicles, hyphae and spores using the grid-intersection method (McGonigle *et al.*, 1990). Briefly, stained roots were placed on microscope slides and observed under a compound microscope at 200× (Leica DM 5000-D, Wetzlar, Germany). The presence of

mycorrhizal structures was counted in 200 intersections between root fragments and the hairline for each plant and used to calculate the percentage of root length colonised by AM fungi.

Presence of spores in bird droppings and on feet

The presence of AM fungal spores was checked in all remaining droppings that contained at least one undamaged seed ($n=173$) and in the gauze swabs ($n=729$) obtained from cleaning birds' feet. Each sample was individually placed in sterilised Petri dishes and carefully screened under a stereomicroscope (Leica EZ4 HD). Observation of the droppings took 75 h and the feet-cleaning gauze swabs required 125 h. All structures that broadly resembled fungi spores were carefully examined and compared with images from the international collection of arbuscular and vesicular-arbuscular mycorrhizal fungi (INVAM, <http://invam.caf.wvu.edu/>).

Results

Establishment of mycorrhizas between co-dispersed seeds and fungi

Overall, nine plant species were detected in 63 droppings produced by eight bird species (Table 1). *Rubus ulmifolius* Schott and *Rhamnus alaternus* L. were the most frequent and abundant plant species found in the droppings, while blackcap (*Sylvia atricapilla* L.), Sardinian warbler (*Sylvia melanocephala* Gmelin) and European robin (*Erithacus rubecula* L.) were the most common dispersers (Fig. 1; Table S2).

A total of 54 seedlings from six species emerged from 34 bird droppings (Fig. 1; Table S2). Root colonisation by AM fungi was detected in seven *R. ulmifolius* seedlings that germinated from two droppings of European robin and two droppings of Sardinian warbler, representing 13% of all harvested plants (Fig. 1; Table S2). The mean value of root colonisation by AM fungi in *R. ulmifolius* germinated from droppings was *c.* 3% (mean = 2.5%, SE = 1.02, $n=7$). All *R. ulmifolius* plants growing in field-collected soil (positive controls) were mycorrhizal with mean values of root colonisation by AM fungi of 12.5% (SE = 1.35, $n=12$). Spores, vesicles and hyphae were more frequent and abundant than arbuscules in the colonised roots (Fig. 2). Root colonisation was not detected in any of the experimental control plants ($n=25$ for *R. ulmifolius*, $n=64$ for all plant species) grown in sterilised soil.

Presence of spores in bird droppings and feet

In total, 173 droppings produced by 14 bird species and 729 feet-cleaning gauze swabs from 31 bird species were checked under a compound microscope for the presence of AM fungal diaspores (Table 1). These droppings carried a total of 520 entire seeds from 14 plant species (Table S3). The most frequent and abundant plant species were *Pistacia lentiscus* L., *Ficus carica* L., *R. ulmifolius*, *R. alaternus* and *Vitis vinifera* L. (Table S3). Despite careful inspection (*c.* 200 h), the presence of AM fungal spores was not detected in bird droppings or feet-cleaning gauze swabs.

Table 1 Number and type of samples analysed from each bird species for the visual detection of arbuscular mycorrhizal (AM) fungal spores.

| Bird species | Droppings | Feet-cleaning gauze swabs |
|--------------------------------|------------|---------------------------|
| <i>Accipiter nisus</i> | | 1 |
| <i>Acrocephalus scirpaceus</i> | | 2 |
| <i>Aegithalos caudatus</i> | | 23 |
| <i>Caprimulgus europaeus</i> | | 1 |
| <i>Caprimulgus ruficollis</i> | | 1 |
| <i>Chloris chloris</i> | 2 | 14 |
| <i>Carduelis spinus</i> | | 4 |
| <i>Cyanistes caeruleus</i> | | 11 |
| <i>Dendrocopos major</i> | | 1 |
| <i>Erithacus rubecula</i> | 20 | 168 |
| <i>Ficedula hypoleuca</i> | 2 | 17 |
| <i>Fringilla coelebs</i> | | 10 |
| <i>Hippolais polyglotta</i> | | 4 |
| <i>Muscicapa striata</i> | 2 | 1 |
| <i>Parus major</i> | | 18 |
| <i>Periparus ater</i> | | 12 |
| <i>Phoenicurus ochruros</i> | | 1 |
| <i>Phylloscopus collybita</i> | | 12 |
| <i>Phylloscopus ibericus</i> | | 2 |
| <i>Phylloscopus trochilus</i> | | 21 |
| <i>Prunella modularis</i> | | 1 |
| <i>Pyrrhula pyrrhula</i> | | 1 |
| <i>Regulus ignicapillus</i> | | 45 |
| <i>Serinus serinus</i> | | 2 |
| <i>Sylvia atricapilla</i> | 62 | 219 |
| <i>Sylvia borin</i> | 54 | 9 |
| <i>Sylvia communis</i> | 5 | 1 |
| <i>Sylvia melanocephala</i> | 19 | 18 |
| <i>Sylvia undata</i> | 1 | |
| <i>Turdus iliacus</i> | | 7 |
| <i>Turdus merula</i> | 7 | 64 |
| <i>Turdus philomelos</i> | | 38 |
| Total | 173 | 729 |

Discussion

Our results provide the first evidence that forest birds can co-disperse viable seeds and viable AM fungal diaspores, postulating a mechanism for the establishment of mycorrhizal associations after seed dispersal and for the colonisation of new sites by AM fungi over long distances.

Several below-ground animals feed on AM fungi and can influence their abundance and distribution either reducing abundance through consumption or increasing dispersal through ingestion and ejection of spores (Allen, 1991; Gange & Brown, 2003). Above-ground mammals can also ingest AM fungal propagules and disperse them through their faeces, and several studies have shown that AM fungal spores can remain viable following passage through the digestive tract of mammals (Gehring *et al.*, 2002, and references therein) further improving the germination of ectomycorrhizal fungi spores (e.g. Cork & Kenagy, 1989; Claridge *et al.*, 1992). However, dispersal by terrestrial mammals and underground invertebrates is limited to short and medium distances and cannot explain the colonisation of discontinuous territories (McIlveen & Cole, 1976; Mangan *et al.*, 2004; Davison *et al.*, 2018).

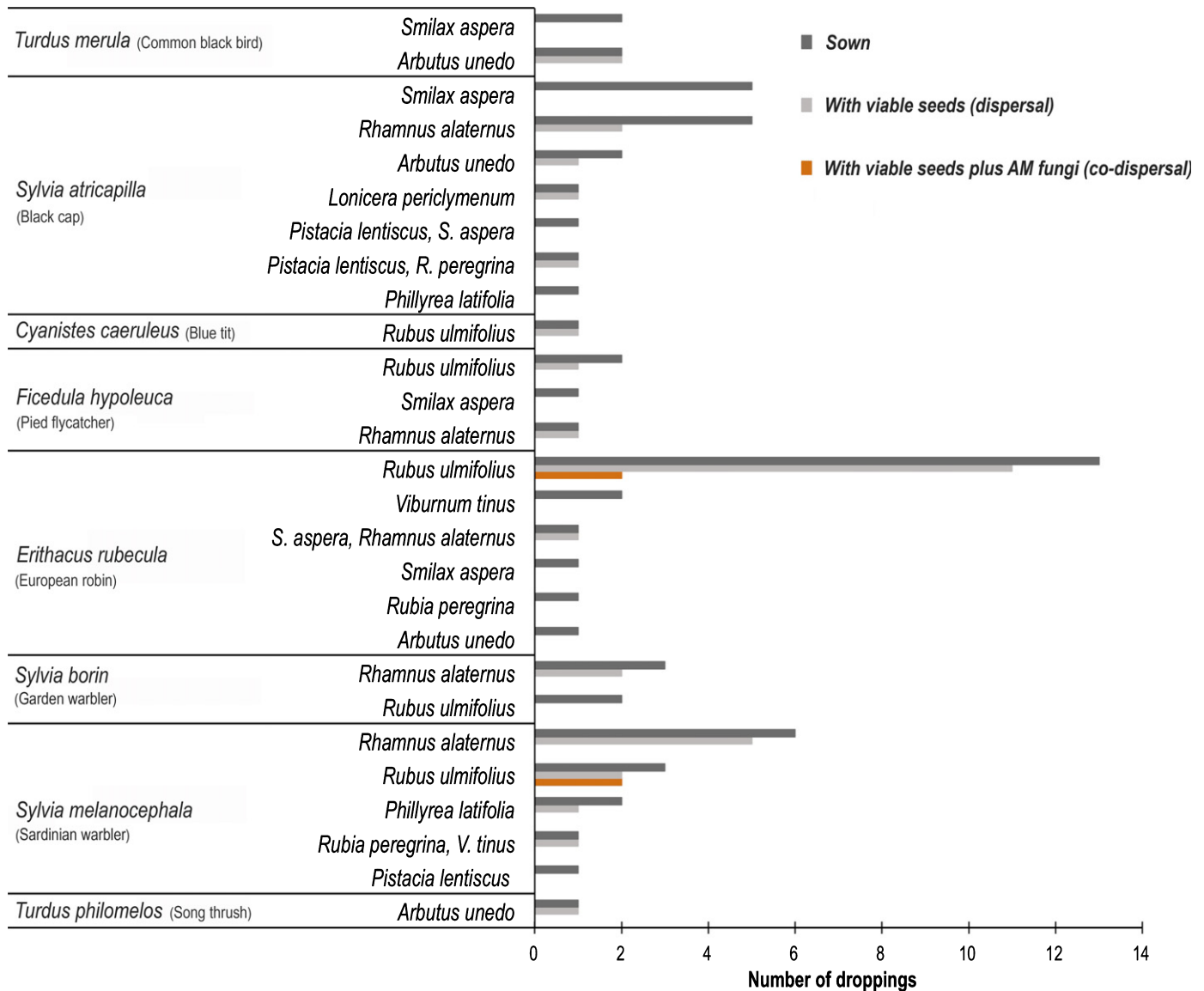


Fig. 1 Details of the glasshouse experiment: identity of seed dispersers and plant species, number of droppings with seeds collected and sown (dark grey bars), number of droppings with viable seeds (light grey bars), and number of droppings containing viable seeds with arbuscular mycorrhizal (AM) fungi (orange bars).

Birds can move spores of different fungi in their beaks, mouth parts, attached to body feathers or internally (da Silva *et al.*, 2016, and references therein). However, spores of AM fungi have only been found anecdotally in droppings of cassowaries (*Casuaris casuaris* L.), Australian brush turkeys (*Alectura lathami* Gray) (Reddel *et al.*, 1997) and unspecified species of goose (Nielsen *et al.*, 2016). All those studies used droppings collected from the soil, and thus it is not clear whether the spores were actually dispersed internally or if they were acquired from the soil after dropping deposition. In this study, AM fungi were detected in the roots of *R. ulmifolius* grown in sterilised soil from droppings of forest bird species collected from holding bags. We can exclude any contamination from air-borne AM fungal spores because plants were maintained inside Sunbags and none of the plants used as experimental controls ($n = 64$) were colonised. Thus, this

is the first report showing that at least two bird species, the European robin and the Sardinian warbler, may be viable vectors for the co-dispersal of plant seeds and spores of AM fungi. Previous studies that found AM fungal spores in animal droppings did not report on the viability of the spores, nor the presence of seeds. Thus, our study goes a step further, demonstrating that biotic co-dispersal of viable AM fungi and plant seeds is possible.

We could not detect the presence of AM fungal spores by direct observation of droppings or feet-cleaning swabs. This protocol was especially directed towards medium-sized birds that forage on the forest ground, such as *Turdus* spp. L., because they were likely candidates to interact with soil-borne fungal spores. More than 100 samples from *Turdus* spp. feet were analysed but we did not detect any AM fungal propagules. AM fungal spores can have ornamented walls but lack spines or similar structures

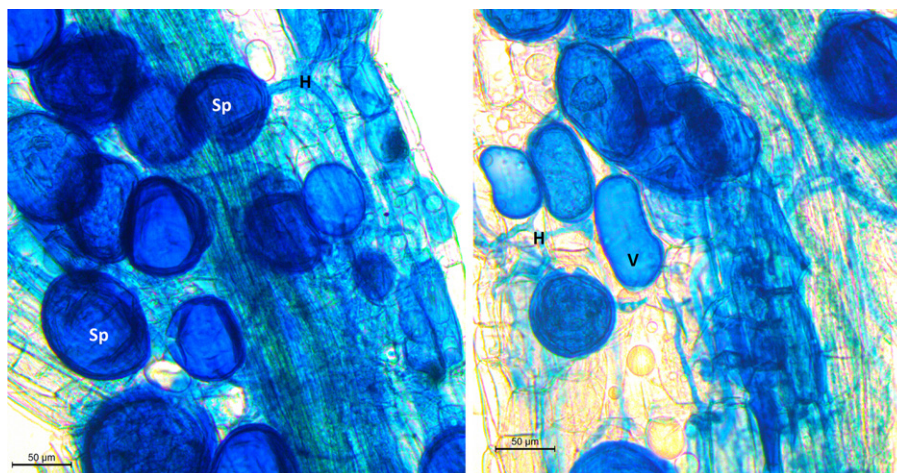


Fig. 2 Details of mycorrhizal roots from *Rubus ulmifolius* seedlings germinated from bird droppings. H, hyphae; Sp, intraradical spore; V, vesicle.

that would facilitate adhesion to animal feathers or feet and, thus, external transport might be difficult. However, we cannot exclude the possibility of false negatives, as spores could be present but not detected through this method. Indeed, direct detection of spores in droppings could be difficult because the number of AM fungal spores ingested by birds is probably low. First, AM fungal spores rarely appear in large groups, in many cases being heterogeneously distributed in the soil (Smith & Read, 2008). Birds probably ingest AM fungal spores during foraging activities on the ground, either by direct accidental ingestion or because spores are attached to fruits and seeds deposited on the ground. Another possibility is that spores are ingested through the consumption of soil invertebrates that feed on AM fungi such as earthworms, ants or millipedes (Harinikumar & Bagyaraj, 1994), although this process is probably not very frequent or efficient. A low number of spores may also explain the low level of root colonisation because colonisation depends firstly on the amount of viable fungal inoculum. Molecular tools could be useful to elucidate both the abundance and the diversity of AM fungi deposited in bird droppings and colonising seedlings, improving our understanding of this co-dispersal process.

Here we provide the first account of co-dispersal of AM fungal diaspores and seeds of suitable host plants in the same dropping, providing a potential mechanism for long-distance dispersal of AM fungi. Although the proportion of co-dispersal events and plants colonised by AM fungi detected in this study was relatively low, 6% of the droppings and 13% of the seedlings, the extremely high bird mobility, the diversity and abundance of frugivorous birds coupled with the frequency and diversity of consumed fruits implies that even relatively rare phenomena can have important consequences, particularly in the colonisation of new territories (Jeltsch *et al.*, 1997; Nathan, 2006; Nogales *et al.*, 2012). The ecological consequences of the process described here are particularly clear in the case of migratory frugivorous birds. At large scale, the high abundance of frugivorous birds making seasonal migrations each year may transport millions of seeds, generating a constant propagule pressure that is capable of promoting and accelerating the colonisation of different biogeographical

regions (Nielsen *et al.*, 2016; Viana *et al.*, 2016). Such large numbers make the co-dispersal of seeds and AM fungal spores a relevant ecological process because they can be transported together between distinct bioregions in a few hours (Alfonzo *et al.*, 2013; Somveille *et al.*, 2013) and moved between continents and isolated islands (Lewis *et al.*, 2014; Viana *et al.*, 2016). One of the confirmed co-dispersers of seeds and AM fungal diaspores, the European robin, is partially migratory with migrations across the entire Western Palearctic region (Adriaensen & Dhondt, 1990; Pérez-Tris *et al.*, 2000; Ambrosini *et al.*, 2016). Recently, it was found that AM fungal communities on islands are as diverse as mainland communities, suggesting that the island biogeography of AM fungi is characterised by an efficient dispersal that outweighs the potential effects of speciation and extinction (Davison *et al.*, 2018). Thus, birds might be important vectors for the long-distance dispersal of AM fungi with the additional advantage of transporting seeds of their symbiotic partners into particularly suitable germination sites (Wenny, 2001; Traveset *et al.*, 2014).

It has recently been shown that mycorrhizal plant species are more likely to invest in long-distance seed dispersal than nonmycorrhizal plants (Correia *et al.*, 2018). We argue that the likely interplay between these two critical ecosystem functions (i.e. seed dispersal and mycorrhizal associations) deserves further attention, especially the role of migratory birds, in what seems to be a pivotal mechanism to understand the broad distribution of most mycorrhizal fungi.

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Author contributions

MC, RH and SR-E planned and designed the research. MC, LPdS and JMC collected the data. MC performed the glasshouse experiment. MC and SR-E analysed the data. MC wrote the first draft of the manuscript and all authors contributed substantially to the revisions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Characterization of the mycorrhizal type and seed dispersal syndromes of the plant species found in the bird droppings used in the experimental trials.

Table S2 Details of the glasshouse experiment, with the description of the bird droppings that were sown.

Table S3 Detailed information on the bird droppings and the plant species dispersed.

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