UNIVERSIDADE DE
COIMBRA

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THE STRUCTURE AND DISRUPTION OF MUTUALISTIC NETWORKS

Tese de Doutoramento em Biociências, especialização em Ecologia, orientada pelo Doutor Ruben Hüttel Heleno e pelo Professor Doutor Jaime Albino Ramos e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Dezembro de 2018

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Doctoral thesis in Biosciences, specialization in Ecology, supervised by Doctor Rúben Hüttel Heleno and by Professor Doctor Jaime Albino Ramos, presented to the University of Coimbra.

Tese no âmbito do Doutoramento em Biociências, especialidade em Ecologia, orientada pelo Doutor Rúben Hüttel Heleno e pelo Professor Doutor Jaime Albino Ramos, e apresentada à Universidade de Coimbra.

December 2018

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This thesis was supported by:

FCT – Fundação para a Ciência e a Tecnologia
PhD grant SFRH/BD/96292/2013

CEF - Centre for Functional Ecology – Science for People & the Planet
Departamento de Ciências da Vida
Faculdade de Ciências e Tecnologia da Universidade de Coimbra (FCTUC)

MARE - Marine and Environmental Sciences Centre
Departamento de Ciências da Vida
Faculdade de Ciências e Tecnologia da Universidade de Coimbra (FCTUC)

Acknowledgements

I am very thankful to my supervisors, Dr. Rúben Heleno and Professor Dr. Jaime Ramos, for their support, teachings, and patience throughout the last five years. I thank them for the opportunity to get immersed in the world of ecological networks, which contributed to change my perception of the natural world.

I am grateful to António Rosa, Ana Cláudia Norte, Camilo Carneiro, Carlos Godinho, Cláudia Matos, Edna Correia, Luís Pascoal, Marcial Felgueiras, Paulo Cortez, Paulo Encarnação, Paulo Tenreiro, Pedro Andrade, Pedro Miguel Araújo, Pedro Pereira, Ricardo Ceia, Ricardo Lopes, and Sérgio Timóteo. This work was only possible due to the contribution of these ringers who collected bird droppings from the study sites throughout the country.

I also thank Luís Pascoal, Paco, and Sérgio Timóteo for the valuable suggestions and critiques in the manuscripts coming out from the work for this thesis, and for the friendship.

I am grateful to my mother, for all the support throughout these years (and for the help collecting the blackberries).

To Ricardo (Ruca), whose help in the first day of the field experiment was invaluable.

To all friends from CEF. A special mention to Andreia, Daniela, Inês, Lucie, Mariana, Marta, and Paco, for the conversations, support, and invitations to help them in the field work, which often helped to clear my mind. I also would like to thank sr. Arménio for his help in the identification of some seeds.

I also thank to Javier Pérez-Tris, Klaus Hövemeyer, Christopher Kaiser-Bunbury, and all the anonymous reviewers who provided valuable comments on the manuscripts. I also thank Jane Memmot for providing the Mathematica script to draw the networks.

I want to thank to "Fundação para a Ciência e Tecnologia (FCT)" for the financial support of this thesis through the grant SFRH/BD/96292/2013. I am also indebted to the two research institutions [Centre for Functional Ecology – Science for People & the Planet (CEF) and Marine and Environmental Sciences Centre (MARE)] for providing me the room to carrying out the laboratory work.

I also thank the Portuguese ringing authority [CEMPA/*Instituto de Conservação da Natureza e das Florestas* (ICNF)] for the permission to capture and ring wild birds. Without that, this work would have not been possible.

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Abstract

All species must interact with other species to survive and reproduce. However, the increasingly environmental degradation due to factors such as habitat loss, climate change, biodiversity loss, or biological invasions, pose a great threat to the stability of species interactions, namely mutualistic interactions, which may undermine important ecosystem processes. Among those ecological processes, seed dispersal constitutes a key mechanism shaping plant population dynamics and consequently the structure of terrestrial ecosystems. The main goal of this thesis is to assess the structure of avian-seed dispersal networks and their resilience to disturbances.

Due to their abundance and distribution, birds are one of the most important seed dispersers worldwide, either internally after ingestion (endozoochory) or externally by adhesion to their body (epizoochory). The external transport of seeds by adhesion is largely dismissed as a rare phenomenon in birds, especially in passerines; however, few studies have addressed this seed dispersal mechanism in birds. In **chapter I**, birds were captured during the autumn migration at seven sites across Portugal to compare the frequency of both seed dispersal mechanisms. Endozoochory was 85 times more frequent than epizoochory, but their occurrence in migratory birds suggests the great ecological relevance of both mechanism for long-distance seed dispersal. Interestingly, both pathways proved to be effective for the transport of seeds with and without specific adaptations for those particular mechanisms.

In the last decades, there has been an exponential growth of studies implementing a network approach to explore the relationship among the structure, functioning, and resilience of biological communities. These studies generally on several network descriptors to characterize these communities. However, these descriptors are affected by the sampling effort. In **chapter II**, I assessed the sampling effort needed to properly characterize the structure of endozoochorous seed dispersal networks by the identification of seeds in the droppings of mist netted birds. It was found that five days were enough to record most bird and plant species, but at least eight days are needed to derive robust network structure descriptors.

The studies of mutualistic network offer the opportunity to predict the consequences of primary extinctions on the ecosystem multiple trophic levels, namely on secondary extinctions. However, most *in silico* species extinction simulations do not allow the establishment of new interactions (rewiring), and the empirical validation of their output is almost inexistent. In **chapter III**, the structure of a seed dispersal network, before and after the experimental removal of the most commonly dispersed fruit species, is compared with two extinction simulation scenarios: without and with rewiring. It was observed that some bird species started to disperse new plant species, while others likely diverted their feeding preferences to animal prey. Although none of the simulation scenarios could accurately predict the observed species-level descriptors, accounting for rewiring considerably improved network structure predictions.

Long-term studies in ecology are crucial to identify potential trends in interaction patterns and species' roles. In **chapter IV**, the structure of a seed dispersal network was assessed at the peak of fruiting period across five consecutive years under an innovative combination of traditional monolayer and multilayer network approaches. In this chapter, a new species-level multilayer descriptor - species activity - is suggested, to reflect the number of layers (here, years) in a multilayer network framework where a given species occurs. The interannual network structure was generally constant, with four temporally consistent interaction modules spanning across all years of the study. The most important species to the seed dispersal service in all years were also those with highest species activity, independently of their abundance. This result suggests that the most regular species across time are essential to maintain the network temporal cohesion and the ecosystem functions that ensure the ecosystem functioning and resilience on the short- and long-term.

This thesis contributes to expand the existing knowledge on the structure and resilience of seed dispersal networks in several ways. Despite its low frequency when compared to endozoochory, the detection of epizoochory by migratory birds highlights the possibility of long-distance seed dispersal through this mechanism even of plants with no specific structures favouring external adhesion. Endozoochory is clearly the most frequent mechanism of seed dispersal by birds, and at least eight sampling-days are needed to correctly depict the emergent properties of these seed dispersal networks when using data from mist netted birds. While predicting the consequences of species extinctions is not trivial, including constrained rewiring opportunities into *in silico* extinction simulations seems to increase considerably the accuracy of the predictions. The structure of avian seed dispersal networks is relatively stable on the long-term due to the temporal reliability of the most important plants and dispersers on both short- and long-term, regardless of their abundance, which are also essential to the temporal network cohesion and ecosystem functioning.

Keywords: birds; endozoochory; epizoochory; multilayer networks; rewiring; sampling effort; seed dispersal; species activity

Resumo

Todas as espécies necessitam de interagir com outras espécies ao seu redor para sobreviver e para se reproduzirem. No entanto, os crescentes fatores de degradação ambiental, como a perda de habitats, alterações climáticas, perda de biodiversidade ou invasões biológicas constituem uma forte ameaça à estabilidade destas interações, nomeadamente as interações mutualistas, podendo comprometer processos estruturantes dos ecossistemas. Entre esses processos ecológicos, a dispersão de sementes constitui um mecanismo chave para a dinâmica das populações vegetais e consequentemente para a estrutura dos ecossistemas terrestres. O principal objetivo desta tese consiste na avaliação da estrutura de redes de dispersão de sementes por aves e na sua resiliência face a diferentes tipos de perturbações.

As aves são, em resultado da sua abundância e distribuição, um dos principais grupos de dispersores de sementes, podendo transportar sementes internamente após ingestão (endozoocoria), ou por aderência externa ao corpo dos animais (epizoocoria). O transporte externo de sementes é geralmente considerado raro em aves, principalmente em passeriformes; no entanto, este mecanismo encontra-se muito pouco estudado. No **capítulo I**, foram capturadas aves durante a migração outonal em sete locais em Portugal para comparar a frequência dos dois mecanismos de dispersão de sementes. A endozoocoria foi 85 vezes mais frequente do que a epizoocoria, no entanto a confirmação da dispersão interna e externa de sementes em aves migradoras sugere a elevada relevância ecológica de ambos mecanismos para a dispersão de sementes a longa distância. Tanto para a endozoocoria como a epizoocoria, verificou-se também a dispersão de algumas sementes sem adaptações específicas ao respetivo processo de dispersão observado.

Nas últimas décadas, o número de estudos a utilizar a teoria de redes para explorar as relações entre a estrutura e o funcionamento e resiliência das comunidades biológicas tem vindo a crescer exponencialmente. Estes estudos apoiam-se geralmente na caracterização das comunidades com base em múltiplos descritores de redes, no entanto estes são sensíveis a diferentes intensidades de amostragem. No **capítulo II**, foi avaliado o esforço de amostragem necessário para uma adequada caracterização da estrutura de redes de dispersão de sementes por endozoocoria, através da identificação de sementes em excrementos de aves capturadas com redes de anilhagem. Enquanto que cinco dias foram suficientes para detetar a maioria das espécies de aves e plantas nas redes, os resultados mostram que são necessários pelo menos oito dias para se obter uma caracterização fidedigna da estrutura da comunidade.

O estudo das redes mutualistas oferece um grande potencial para prever as consequências de extinções primárias nos vários níveis tróficos dos ecossistemas, nomeadamente ao nível de extinções secundárias. Contudo, a maioria das simulações de extinções de espécies *in silico* não permite o estabelecimento de novas interações (*rewiring*), e a validação dos resultados obtidos das simulações com estudos empíricos é praticamente inexistente. No **capítulo III**, a estrutura de uma rede de dispersão de sementes, antes e depois da remoção experimental da espécie de fruto mais dispersada, é comparada com dois cenários de extinção: com e sem *rewiring*. A remoção experimental levou à dispersão de novas espécies de plantas por algumas espécies de aves, mas algumas destas provavelmente direcionaram as preferências alimentares para presas animais. Embora nenhuma das simulações tenha previsto com exatidão os descritores de espécie observados, a inclusão de *rewiring* melhorou claramente a previsão da estrutura da rede.

Estudos de longo prazo são cruciais em ecologia, nomeadamente para identificar alterações nos padrões de interações e no papel funcional das espécies. No **capítulo IV**, a estrutura de uma rede de dispersão de sementes foi avaliada, no pico de frutificação, durante cinco anos consecutivos através de uma inovadora combinação das tradicionais redes mono-camada (*monolayer)* com redes multi-camada (*multilayer)*. Aqui, é sugerido um novo descritor ao nível da espécie – *species activity* -, representando o número de camadas (*layers*; neste caso, anos) numa estrutura de redes multi-camada, onde cada espécie ocorre. De um modo geral, a estrutura interanual das redes foi constante, com quatro módulos de interações temporalmente consistentes e abrangendo todos os anos do estudo. As espécies mais importantes para a dispersão de sementes em cada um dos anos foram também aquelas com maior *species activity* (i.e. mais regulares no tempo) independentemente da sua abundância. Estes resultados sugerem que as espécies temporalmente mais regulares são essenciais para manter a coesão temporal da rede de interações e os serviços ecossistémicos que asseguram o funcionalmente e resiliência dos ecossistemas no curto e no longo prazo.

Esta tese contribui para ampliar o conhecimento existente sobre a estrutura e a resiliência das redes de dispersão de sementes através de várias vertentes. Apesar da baixa frequência de epizoocoria quando comparada com a endozoocoria, a sua ocorrência em aves migradoras realça a possibilidade da dispersão de sementes a longas distâncias, mesmo quando estas não possuem estruturas anatómicas especializadas que favoreçam a sua aderência ao exterior do corpo das aves. A endozoocoria é claramente o mecanismo de dispersão de sementes mais frequente em aves, sendo necessários pelo menos oito dias para caracterizar corretamente a topologia de redes de dispersão de sementes com recurso a redes de anilhagem. Apesar de as previsões das consequências da extinção de espécies não serem fáceis de elaborar, a inclusão de possibilidades de *rewiring* em simulações de extinção de espécies *in silico* parece aumentar consideravelmente a exatidão dessas previsões. A estrutura das redes de dispersão de sementes redes é relativamente estável entre anos devido à regularidade temporal das plantas e dispersores mais importantes no curto e no longo prazo, independentemente da sua abundância, e que são essenciais para a coesão temporal e para o funcionamento do ecossistema.

Palavras-chave: aves; dispersão de sementes; endozoocoria; epizoocoria; esforço de amostragem; redes multi-camada; *rewiring*; *species activity*

General Introduction

General Introduction

The advent of a network approach

All organisms are embedded in a network of interactions with other organisms. Records of interactions between organisms date back at least as far as ancient Greece, including plant-animal interactions such as herbivory and seed dispersal (Thanos 1994), and in the eighth century, al-Jahiz provided the first known description of a food chain (Egerton 2002). Only a thousand years later the interest in the interactions between species emerged again thanks to several naturalists (reviewed in Ings & Hawes 2018), such as Charles Darwin who described the intricate interdependencies of species as an "entangled bank" (Darwin 1859). Later, Lorenzo Camerano suggested that food web communities are in a dynamic equilibrium where changes in the abundance of a certain species affect the abundance of other species (Camerano 1880). It was only in the twentieth century, that the study of species interactions increased in complexity, with the collection of more detailed data and the elaboration of food web models in an attempt to explain the observed trophic interaction patterns (Layman *et al.* 2015; Ings & Hawes 2018). Despite the remarkable insights provided by these studies, they were mostly focused on one or a few species and their respective interacting partners, which hinders the evaluation of the role of each species on community structure (Bascompte & Jordano 2007). One of the exceptions was the extensive sampling of plant-pollinator communities performed by Robertson (1929) from 1887 to 1916, which resulted in the identification of 15 265 pairwise interactions between 456 plant and 1430 flower-visitor animal species (Robertson 1929; Memmott & Waser 2002). However, the lack of appropriate tools at the time to assess the structure of these communities hindered such analysis for nearly a century.

Throughout the 20th century, the development of a network theory, focusing on the identification of patterns in graphs (i.e. networks) composed of nodes connected by links, inevitably attracted the attention of ecologists. Consequently, many ecological communities started to be studied under a network approach (where nodes usually correspond to species connected by links representing interactions), first in food webs and later in host-parasitoid and mutualistic networks (Ings *et al.* 2009). Multiple network descriptors characterizing different topological properties of ecological networks have been developed throughout the last few decades, which allied with better data and a higher computational power, increased substantially our knowledge on their structure and on the potential drivers contributing for such structure (Jordano 1987; Bascompte & Jordano 2007; Bascompte 2009; Vázquez *et al.* 2009a; Dormann *et al.* 2017).

Mutualistic network analysis and real-world communities

A worldwide pressure on ecosystems due to anthropogenic activities has been impacting negatively the structure and function of ecological communities (Vitousek *et al.* 1997; Barnosky *et al.* 2012; Sebastián-González *et al.* 2015). Under such scenario, there is a pressing need for conservation strategies to shift from an exclusive species-based to an interaction-based focus, as the loss of interactions may cease the functional roles of species in ecological communities before they actually go extinct (McConkey & Drake 2006; Valiente-Banuet *et al.* 2015; Harvey *et al.* 2016; Brodie *et al.* 2018). Network analysis arises naturally as an appropriate tool to such endeavour, allowing for example the assessment of the resilience of ecological communities to disturbances (Poisot *et al.* 2016). Accordingly, networks have been used to frame a wide spectrum of important conservation issues such as the impact of biological invasions (e.g. Traveset *et al.* 2013), ecological restoration (e.g. Heleno *et al.* 2010), defaunation (e.g. Kaiser-Bunbury *et al.* 2010), or biocontrol (e.g. López-Núñez *et al.* 2017). The value of a network approach is intrinsically related to data quality and how it accurately represents the structure of natural communities. Since sampling all species and interactions in a community is virtually impossible, a trade-off between sampling effort and data quality is crucial to the applicability of networks in conservation and predictive studies (Hegland *et al.* 2010; Jordano 2016). Therefore, understanding exactly how variable levels of sampling completeness affect network structure descriptors, and consequently the inferences we derive from them, is a critical topic in ecological networks (e.g. Nielsen & Bascompte 2007; Dormann *et al.* 2009; Rivera-Hutinel *et al.* 2012).

In order to interact, species must occur concurrently in space and time. However, interaction probability is also contingent on an array of other factors, such as species abundances, resource availability, trait matching, or phenological overlap, all of which vary across multiple spatial and temporal scales (Carnicer *et al.* 2009; Poisot *et al.* 2014). The interplay between all these factors means that interactions between species are highly dynamic. However, most studies on mutualistic network tend to ignore such dynamics, which may lead to an inaccurate characterization of ecological communities. Mutualistic network studies tend to be sampled over relative short time scales (often one year or less), and even when studies cover longer time spans, interactions are still invariably aggregated into well-defined temporal windows, precluding a true assessment of temporal dynamics (e.g. Kaiser-Bunbury *et al.* 2009; González-Castro *et al.* 2012a; Heleno *et al.* 2013a; Ramos-Robles *et al.* 2016). The recent development of a multilayer network approach, which explicitly connect different networks (i.e. layers) through interlayer links, constitutes a promising tool to the analysis of ecological networks, likely providing a more realistic characterization of ecological communities across space and time (Pilosof *et al.* 2017; Timóteo *et al.* 2018).

Network dynamics is largely ignored in many studies seeking to assess the consequences of *in silico* simulations of species extinctions in mutualistic networks (e.g. Memmott *et al.* 2004; Memmott *et al.* 2007; Bezerra *et al.* 2009; Rumeu *et al.* 2017). This approach may originate unrealistic results because it assumes that all possible interactions have been detected and therefore species cannot establish any new (or undetected) interaction (see Blüthgen 2010 for a critique on the subject). Species' extinctions can lead to a reorganization of network structure due to the interaction rewiring, i.e. the incorporation of new links to compensate for the lost ones (Brosi & Briggs 2013; Goldstein & Zych 2016; Timóteo *et al.* 2016). Recently, some studies started to incorporate interaction rewiring rules when predicting species extinction (Kaiser-Bunbury *et al.* 2010; Timóteo *et al.* 2016); nevertheless, these rewiring assumptions still require empirical validation from manipulative studies, particularly in the case of seed dispersal.

Long-term studies are fundamental to assess temporal trends in ecological communities, namely changes in species and interaction diversity, and its implications in the importance of a given species for the community (Petanidou *et al.* 2008; Lindenmayer *et al.* 2012; Herrera 2018). Despite their importance, this kind of studies is still scarce in mutualistic communities. Notable exceptions include the 18-year assessment of pollinator abundance trends in plant-pollinator communities by Herrera (2018), or a 12-year assessment of the interannual abundance of two frugivorus bird species, fleshy-fruit crops, and their respective bird-fruit interactions (Herrera 1998). To date, however, there are only a handful of studies assessing the interannual structure of mutualistic communities, namely plant-pollinator interactions, under a network approach. Overall, these studies show that the topology of pollination networks is relatively constant despite the occurrence of a variable species turnover between years, while species roles, expressed by species-level descriptors, tend to be variable between years (Alarcón *et al.* 2008; Petanidou *et al.* 2008; Olesen *et al.* 2011b; reviewed in Trøjelsgaard & Olesen 2016). However, there are no studies assessing the interannual variability in the structure of avian-seed dispersal networks. These communities are known to display an interannual variation in fruit production and ripening timing, which might affect the bird-fruit interaction patterns from year to year (Herrera 1998). Since seed dispersal networks are more generalist than pollination networks (Blüthgen *et al.* 2007), species roles in the former may be more constant across years despite any potential fluctuation in the availability of some interacting partners.

The study system: Seed dispersal by birds

Seed dispersal constitutes a critical step in the demography of plant populations, contributing to shape the composition and structure of ecosystems (Howe & Miriti 2004; Traveset *et al.* 2014). The dispersal of seeds away from the parent plant tends to increase the survival, growth, and establishment of new plants. Specifically, it decreases the density-dependent disproportional mortality of seeds and seedlings near conspecifics due to a higher incidence of predation, pathogens, and competition (Janzen-Connell hypothesis), allows the colonization of favourable sites unpredictable in space and time, and promotes the non-random dispersal toward sites with suitable conditions for survival (Janzen 1970; Connell 1971; Howe & Smallwood 1982; Packer & Clay 2000; Wenny 2001; Comita *et al.* 2014). At the population level, seed dispersal away from the parent plant entails important long-term beneficial effects such as the increment of genetic variability due to gene flow between plant populations, decreasing the probability of inbreeding depression, and species range shifts (Nathan & Muller-Landau 2000; Godoy & Jordano 2001).

Several vectors promote the dispersal of seeds, including water (hydrochory), wind (anemochory), and animals (zoochory). Diaspores (i.e. seeds with additional tissues to facilitate dispersal) often possess morphological traits that increase the probability of dispersal via a specific vector (i.e. seed dispersal syndromes): for example, wing and plumes promote the transport of seeds by wind, and nutritive fleshy tissues surrounding seeds attract frugivorous animals that consume the pulp and disperse viable seeds after ingestion (Van der Pijl 1982; Traveset *et al.* 2014). Zoochory is a particularly relevant seed dispersal mechanism, and it is estimated that up to 90% and 60% of plant species in tropical and temperate regions, respectively, have their seeds dispersed by animals (Howe & Smallwood 1982; Willson *et al.* 1990). Globally, the main groups of animal seed dispersers comprise birds (Wenny *et al.* 2016), mammals (Herrera 1989; Mello *et al.* 2011b), and to a lesser extent, reptiles (Olesen & Valido 2003). Nevertheless, a wider spectrum of other animal groups may also disperse seeds, such as fishes (Horn *et al.* 2011), beetles (de Vega *et al.* 2011), or ants (Handel & Beattie 1990).

In the Mediterranean area, birds are the most important seed dispersers of the vast majority of all fleshy-fruited plant species occurring in the area (Herrera 1995). Several bird species tend to be highly frugivorous when fruits are seasonally available, including migrating birds that feed intensively on fruits in order to build fat for their post-breeding migratory flights, often dispersing seeds inside their guts (endozoochory) (Fuentes 1994; Herrera 2004 and references therein). Further, fruit ingestion by birds tend to have a higher positive effect on seed germination than their ingestion by terrestrial mammals, probably due to a shorter seed retention period in the guts of birds (Traveset & Verdú 2002). Constrained by the non-mutually exclusive effects of bird's diet, morphological and behavioural traits, and species-specific seed traits (Traveset *et al.* 2001; Traveset *et al.* 2007; Pigot *et al.* 2016; González-Varo *et al.* 2017), benefits from seed dispersal via endozoochory may include the (1) dispersal toward suitable sites for germination and seedling survival, (2) removal of fruit pulp with potential germination inhibitors, (3) seed coat scarification, or (4) a fertilization effect provided by the faecal material (Wenny 2001; Robertson *et al.* 2006; reviewed in Traveset *et al.* 2007). Such high degree of frugivory, both in terms of abundance and species richness of consumed fruits, coupled with the high mobility and abundance of birds, makes seed dispersal performed by birds a critical process in plant population dynamics and consequently to the structure of ecological communities (Wenny *et al.* 2016).

Contrarily to endozoochory, epizoochory is a passive seed dispersal mechanism as seeds attach inadvertently to the animal's body, which is assumed to be more common on mammals (Roth 1986). The study of epizoochory in birds has been largely restricted to water birds, which live in muddy habitats rich in plants that produce small diaspores capable of getting attached to their feathers either directly or mixed with mud that stick to their body parts (Vivian-Smith & Stiles 1994; Figuerola & Green 2002; Raulings *et al.* 2011). Accordingly, the magnitude, extent, and ecological consequences of epizoochory by forest birds, such as passerines, is largely overlooked (Nogales *et al.* 2012). Given the ubiquity, high abundance, and global migratory patterns of many passerines, their potential role as epizoochorous seed dispersers clearly deserves further scrutiny.

Objectives

This thesis is set out to explore four important caveats currently precluding a deeper understanding of seed dispersal networks, namely (1) the nearly absent data on epizoochory by passerines, (2) determining the minimal adequate sampling effort for robust inferences of network structure, (3) improve current species extinction simulation models, and (4) understand the interannual temporal dynamic of seed dispersal networks.

Thesis structure

Chapter I

In this chapter, I compare the frequency of endozoochory and epizoochory performed by passerines at nine sites throughout Portugal during their post-breeding migratory period. This work was published on the Journal of Avian Biology.

Costa J.M., Ramos J.A., da Silva L.P., Timóteo S., Araújo P.M., Felgueiras M.S., Rosa A., Matos C., Encarnação P., Tenreiro P.Q. & Heleno R.H. (2014). Endozoochory largely outweighs epizoochory in migrating passerines. *Journal of Avian Biology*, 45: 59-64. https://doi.org/10.1111/j.1600-048X.2013.00271.x

Chapter II

Here, I evaluate how many sampling-days are necessary to detect most species and interactions in the seed dispersal community, and to calculate robust network descriptors. This work was published on Basic and Applied Ecology.

Costa J.M., da Silva L.P., Timóteo S., Ramos J.A. & Heleno R.H. (2016). Sampling completeness in seed dispersal networks: when enough is enough. *Basic and Applied Ecology*, 17: 155-164. https://doi.org/10.1016/j.baae.2015.09.008

Chapter III

In this chapter, I compare the consequences of an experimental removal of the most dispersed plant species with two *in silico* extinction scenarios (with and without rewiring) of that species. This work was published on Basic and Applied Ecology.

Costa J.M., Ramos J.A., da Silva L.P., Timóteo S., Andrade P. Araújo P.M., Carneiro C., Correia E., Cortez P., Felgueiras M., Godinho C., Lopes R.J., Matos C., Norte A.C., Pereira P.F, Rosa A. & Heleno R.H. (2018). Rewiring of experimentally disturbed seed dispersal networks might lead to unexpected network configurations. *Basic and Applied Ecology*, 30: 11-22. https://doi.org/10.1016/j.baae.2018.05.011

Chapter IV

In this chapter, I sample an avian seed dispersal network across five years to assess the interannual variability in network structure, the temporal persistence of species (birds and fruits) and interactions, and the variability of each species' role in the network.

Costa J.M., Ramos J.A., Timóteo S., da Silva L.P., Timóteo S., Ceia R.S. & Heleno R.H. Species activity promotes the stability of fruit-frugivore interactions across a five-year multilayer network. Published as a pre-print in bioRxiv. https://doi.org/10.1101/421941

Chapter I

Endozoochory largely outweighs epizoochory in migrating passerines

Costa J. M., Ramos J. A., da Silva L. P., Timóteo S., Araújo P. M., Felgueiras M. S., Rosa A., Matos C., Encarnação P., Tenreiro P. Q. & Heleno R. H. (2014). Endozoochory largely outweighs epizoochory in migrating passerines. *Journal of Avian Biology*, 45: 59-64. https://doi.org/10.1111/j.1600-048X.2013.00271.x

Abstract

Fruits and seeds are critical food sources for many European passerines during the autumn migration, which in turn contribute to disperse seeds either internally, i.e. after ingestion (endozoochory), or externally, when seeds adhere to the body surface (epizoochory). Despite the recognized importance of birds as seed dispersers, the vast majority of studies focused on endozoochory while the external transport of seeds is frequently invoked as being potentially important but remains largely unexplored. This is particularly important during the post-breeding migration of passerines, the most ubiquitous and diverse movement of potential seed carriers across Europe and into Africa, which coincides with the fruiting peak of many plant species (August-October). Our aim was to evaluate the role of migrating birds as potential long-distance seed dispersers and compare the prevalence of epizoochory and endozoochory during post-breeding migration. We sampled 926 wild birds at nine locations in Portugal and retrieved 1833 seeds of 19 plant species dispersed internally and only three seeds externally attached to three birds (*Serinus serinus*, *Locustella naevia*, and *Turdus merula*). Endozoochory prevalence was 85 times higher than that of epizoochory. Migrating and non-migrating passerines dispersed seeds in similar frequencies. While two of the three seeds transported externally had specific adaptations to epizoochory, namely spines (*Torilis arvensis*) and hooks (*Galium aparine*), the third is a large seed from a fleshy-fruited plant, *Frangula alnus* (i.e. with typical endozoochorous syndrome). These seeds were found on bird species with different diets, but similar behaviour (ground foragers), and in similar habitats (open agroecosystems). Our results highlight the strong role of migrating passerines as potentially long-distance seed dispersers and show that, at least in the autumn, the prevalence of epizoochory is several orders of magnitude lower than that of endozoochory.

Introduction

Seed dispersal is an important process in the life cycle of most plants, influencing community composition and ecosystem stability (Nathan & Muller-Landau 2000). The advantages of seed dispersal can be summarized into three broad categories: it (1) enables seeds and seedlings to escape from high mortality near conspecifics due to predation, pathogens, and competition, (2) allows the colonization of favourable sites unpredictable in space and time, and (3) promotes the non-random dispersal towards microsites with particularly suitable conditions for survival (Janzen 1971; Howe & Smallwood 1982; Wenny 2001). While plants can have short-distance dispersal mechanisms (autochory), they must rely on external vectors, such as water (hydrochory), wind (anemochory), and particularly animals (zoochory) for long-distance dispersal (Ridley 1930). Specifically, animals can disperse seeds either internally (endozoochory) or externally, when seeds get attached to the animals' fur or feathers (epizoochory) (Van der Pijl 1982). Diaspores, i.e. the plant structures that are actually transported, frequently have a series of morphological adaptations that promote their dispersal by a specific mechanism; these trait groups are known as dispersal syndromes. For example, light seeds with wings or plumes can easily be transported by wind, and fleshy nutritious tissues attract animals that may disperse their seeds after ingesting the fruits (Howe & Smallwood 1982).

Because some seeds can adhere to the body surface for large periods, these can potentially be dispersed over much longer distances by epizoochory than by endozoochory (Sorensen 1986; Whelan *et al.* 2008). However, while endozoochory in birds has been intensively studied all over the world (Forget *et al.* 2011), epizoochory has been largely neglected. Diaspores adapted to epizoochory usually have barbs, hooks, spines, or viscid mucilage, which enable the external adhesion to the body of animals (Fahn & Werker 1972; Sorensen 1986; Yang *et al.* 2012). The effectiveness of such adaptations will largely determine the retention time and the dispersal distance (Sorensen 1986; Guttal *et al.* 2011).

By virtue of their worldwide distribution, abundance, and high mobility, birds are important frugivores and disperse seeds from a large variety of fleshy- and dry-fruited plants (Whelan *et al.* 2008; Heleno *et al.* 2013b). Passerines (order Passeriformes) comprise more than half of the known extant bird species (Edwards & Harshman 2008), and are present in nearly all terrestrial habitats, being particularly well represented in agroforestry habitats, where fruiting plants are also common (Siriwardena *et al.* 1998; Robinson *et al.* 2002). During post-breeding migration, many passerine species that breed in temperate areas are highly frugivorous and potential long-distance dispersers of seeds either after ingestion (Herrera 1995) or by adhesion to their feathers (Choi *et al.* 2010), for example by transporting seeds between stopover sites.

Despite the undisputable attraction of birds for ecologists and the potential importance of passerines for epizoochory, there is a strong bias towards epizoochory studies in mammals when compared to birds (Heinken & Raudnitschka 2002; Couvreur *et al.* 2004; Manzano & Malo 2006; Picard & Baltzinger 2012) while epizoochory in passerines remains largely unexplored. When epizoochory was studied in birds, it was almost invariably centred in waterbirds (Vivian-Smith & Stiles 1994; Figuerola & Green 2002; Brochet *et al.* 2010; Raulings *et al.* 2011; Aoyama *et al.* 2012). Among these studies, only one compared the prevalence of endozoochory and epizoochory (Brochet *et al.* 2010). Similarly, to our knowledge, only one study evaluated the prevalence of epizoochory in several orders of birds, including passerines (Choi *et al.* 2010). This study revealed an overall low epizoochory prevalence (0.08%) and no seeds where found adhered to passerines. Our aim was to evaluate the role of migrating birds as potential long-distance seed dispersers by assessing their internal and external seed loads during post-breeding migration and compare to those of resident birds.

Material and methods

We carried out a countrywide sampling to compare the prevalence of endozoochory and epizoochory by passerines in the post-breeding migratory period. During five consecutive days (10-14 September 2012), we captured passerines (few individuals of other orders were also captured) in nine locations spread across 500 kilometres, from northern to southern Portugal (Fig. 1). We sampled in September because it corresponds to the peak of both avian migration and fruiting period of most fleshy-fruited plants. While the dry fruits of many annual plants, more likely dependent on epizoochory, are more abundant earlier in the year (early summer), these are still largely available in September. We sampled simultaneously at all locations for five consecutive days to reduce temporal variation in the data, thus capturing a snapshot of seed dispersal by non-migrating and migrating passerines at their stopovers. Our sampling locations were selected in a range of agroforestry mosaic systems, the most common habitat type in Portugal.

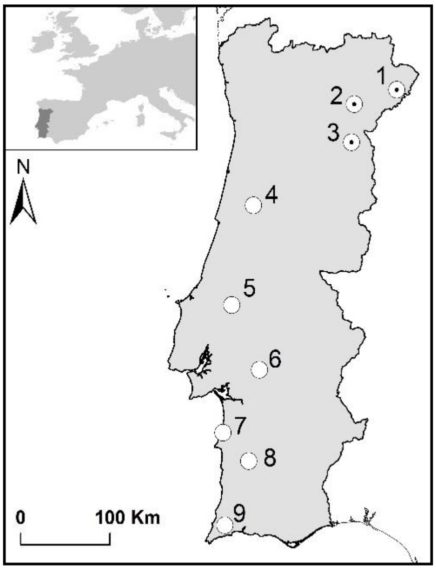

Figure 1 - Geographic location of the study sites: 1) Atenor – Miranda do Douro, 2) Nozelos – Torre de Moncorvo, 3) Barragem de Santa Maria de Aguiar – Figueira de Castelo Rodrigo, 4) Larçã – Souselas, 5) Casais da Estrada – Achete, 6) Herdade do Freixo do Meio – Foros de vale Figueira, 7) Lagoa de Santo André – Santiago do Cacém, 8) Água Branca – Odemira, and 9) Bensafrim – Lagos. Black dots within each circle represent the three sites where epizoochory was detected.

At each site, we operated mist nets during five hours after dawn (aprox. 06:30-11:30 a.m.), which were visited at a maximum of 30-minute intervals to extract mist-netted birds. During extraction, all birds were carefully inspected for external adhered seeds before being placed in ringing bags for up to 30 minutes to defecate. After this period, all produced droppings were collected, and birds were ringed, measured, and released. Birds' droppings were air-dried and stored until processing. All intact seeds retrieved from droppings and feathers were later identified with a dissecting microscope by comparison with a reference collection of seeds at the University of Coimbra. We considered each dropping containing at least one intact seed as one bird-plant interaction. The availability of seeds was estimated at each site by counting all ripe standing fruits along three linear transects of 25 metres x 2 metres running parallel to the mist nets.

Each passerine species was classified as a migrant or resident at the time and location of sampling (Supplementary Material: Table S1), following Cabral *et al.* (2005). We considered migrant species those that are known long-distance migrants, i.e. those that can be found in Portugal only during part of the year (either for breeding, wintering, or during migration towards lower or higher latitudes). Resident species are those of which most individuals remain in Portugal during the whole year. Finally, species with both migrant and resident populations were considered as partially-migratory. As we were only interested in seed dispersal by passerines, and non-passerines did not disperse any seed, we excluded these from further analysis

Differences on the frequency of occurrence of endozoochorus and epizoochorous seeds on captured birds were assessed with a chi-square test. Differences between the number of plant species dispersed by migrating (including partially-migrating) and resident birds were assessed with a Mann-Whitney test. The statistical tests were implemented in R 3.0.0. To visualize all seed dispersal interactions, a graph was build using specific code written in Mathematica 9.0 (Wolfram Research 2012).

Results

We captured 926 birds from 54 species, 29 families, and six orders (Passeriformes, 48 species; Coraciiformes, 2; Caprimulgiformes, Piciformes, Accipitriformes, Strigiformes, 1 each) (Supplementary Material: Table S1). Overall, 1833 seeds of 19 native plant species were retrieved from 254 droppings produced by 20 bird species (all native passerines); these interactions are represented in Fig. 2. All seeds were identified to species-level, except two species that were identified to family-level and morphotyped (Poaceae sp. 1 and Poaceae sp. 2). Endozoochory was significantly more common than epizoochory (98.8% and 1.2% of all retrieved seeds, respectively; $\chi^2 = 161.1$, d.f. = 1, $p < 0.001$).

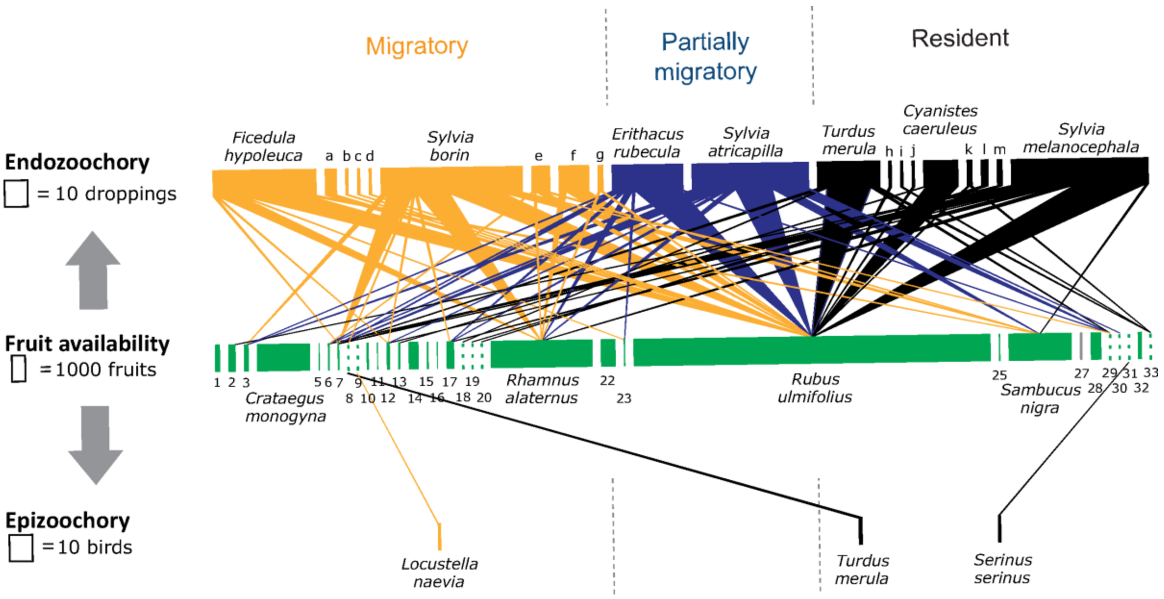

Figure 2 - Representation of the overall dispersal of seeds via endozoocory (top level) and epizoochory (bottom level). Species are represented by boxes. The width of the central boxes represents fruit availability (number of fruits counted along linear transects at each location). The width of the top and bottom boxes is proportional to the importance of each species as seed disperser. Migrant birds are shown in yellow, partially-migrant birds in blue, and resident birds in black. Dotted lines indicate dispersed plant species that were not detected in the fruit census. Birds: a – *Luscinia megarhynchos*, b – *Muscicapa striata*, c – *Phylloscopus boneli*, d – *Phylloscopus trochilus*, e – *Sylvia cantillans*, f – *Sylvia communis*, g – *Phoenicurus phoenicurus*, h – *Emberiza cia*, i – *Sturnus unicolor*, j – *Cettia cetti*, k – *Cyanopica cyanus*, l – *Fringilla coelebs*, m – *Passer domesticus*. Plants: 1 – *Arbutus unedo*, 2 – *Asparagus acutifolius*, 3 – *Bryonia dioica*, 5 – *Cydonia oblonga*, 6 – *Daphne gnidium*, 7 – *Ficus carica*, 8 – *Frangula alnus*, 9 – *Galium aparine*, 10 – *Juncus effusus*, 11 – *Lonicera periclymenum*, 12 – *Lonicera implexa*, 13 – *Myrtus communis*, 14 – *Olea europaea*, 15 – *Osyris alba*, 16 – *Phillyrea angustifolia*, 17 – *Pistacia lentiscus*, 18 – Poaceae sp. 1, 19 – Poaceae sp. 2, 20 – *Portulaca oleracea*, 22 – *Rosa canina*, 23 – *Rubia peregrina*, 25 – *Ruscus aculeatus*, 27 - *Scirpoides holoschoenus*, 28 – *Smilax aspera*, 29 – *Solanum nigrum*, 30 – *Tamus communis*, 31 – *Torilis arvensis*, 32 – *Viburnum tinus*, 33 – *Vitis vinifera*.

Overall, *Rubus ulmifolius* was by far the most dispersed plant (53.3% of all bird-seed interactions, dispersed by 17 bird species; Fig. 2). This plant species was also the most dispersed species in all but one site (Larçã), where *Rhamnus alaternus* was the most dispersed species. Most plant species were dispersed by blackcap *Sylvia atricapilla* (10 species), followed by garden warbler *S. borin* (nine species), and the Sardinian warbler *S. melanocephala* and robin *Erithacus rubecula* (both with eight species each). However, *S. borin* was the species that most frequently dispersed seeds (71.7%, 43 out of 60 individuals; excluding the western bonelli's warbler *Phylloscopus bonelli* with just one individual caught). Together, migratory and partially migratory birds seemed to disperse seeds more often than resident birds (Fig. 2), but this difference was not statistically significant (Mann-Whitney test: $Z = -1.758$, $n_{\text{(migratory)}} = 20$ species, $n_{\text{(residents)}} = 28$ species, $p = 0.079$).

Only three seeds were found externally adhered to bird feathers: one seed of *Torilis arvensis* on a serin *Serinus serinus* (granivorous, resident), one seed of *Frangula alnus* on a blackbird *Turdus merula* (frugivorous, resident), and one seed of *Galium aparine* on a grasshopper warbler *Locustella naevia* (insectivorous, migratory) (Figs. 2 and 3). Such findings result in an overall epizoochory prevalence of 0.33%, 85 times lower than that of endozoochory (3 and 254 interactions, respectively). These seeds were found on the side of the neck, nape, and belly of the hosts, respectively, and were recovered from the three northernmost locations (Table 1). These locations are essentially composed by a matrix of scrubland and low-intensity agricultural fields on river beds, with dispersed olive *Olea europaea*, almond *Prunus amygdalus*, and holm oak *Quercus rotundifolia* trees and abundant herbaceous vegetation.

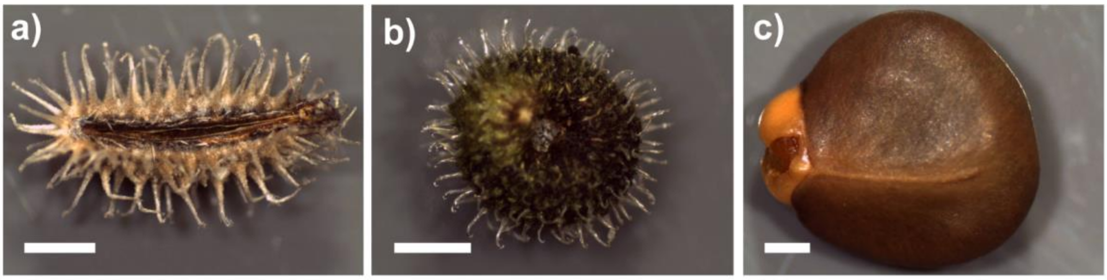

Figure 3 - Picture of the three seeds transported via epizoocory: (a) Torilis arvensis, (b) Galium aparine, and (c) Frangula alnus. Inset scale bars = 1 millimetre.

Table 1 - Captured birds and seeds dispersed per study site. "Richness" represents the total number of species, and "Abundance" the total number of passerines captured, or seeds dispersed.

	Passeriformes		Endozoochory		Epizoochory	
Site	Richness	Abundance	Richness	Abundance	Richness	Abundance
Bensafrim	17	40	4	95	0	0
Odemira	14	43	4	24	0	0
Santo André	13	51	1	15	0	0
Freixo do Meio	15	68	2	75	0	0
Achete	26	155	10	434	0	0
Larçã	13	82	6	138	0	0
Sta. Maria Aguiar	26	225	7	563	1	1
Nozelos	21	128	7	315	1	1
Atenor	27	122	5	174	1	1

Discussion

In this study, we found a much lower prevalence of epizoochory (0.33%) than endozoochory (27.8%) in passerines during the post-breeding migration. Despite our care in searching for seeds attached to birds, we cannot rule out the possibility that some adhered seeds have detached or that seeds were evacuated in faeces while the birds were on the net. Similarly, it is possible that some larger seeds are quickly regurgitated and thus not found in the droppings; however, the only plant species recorded in the transects that would be most likely regurgitated due to its size (> 5 millimetres) are the seeds of *O. europaea*, which are still unripe in September and therefore unlikely to be relevant for birds at the time of sampling. Furthermore, any seed regurgitated inside the bird-holding bags would have been collected and included in the analysis. Despite these eventual drawbacks, the total number of passerines captured, and the large spatial replication make us confident that our results are representative of seed dispersal during the migratory period in this group of birds.

We found no significative differences in the prevalence of endozoochory between migratory and resident passerines. Most resident species are mostly insectivorous or granivorous, seldom dispersing seeds; however, two resident species, the blackbird and the Sardinian warbler, are important seed dispersers (five and eight plant species in this study, respectively), which contributed to the lack of statistical difference. However, the biological importance of migratory passerines relatively to residents may be higher because the former group has more species dispersing seeds and from more plant species than the latter (Supplementary Material: Table S1). The fact that most seeds were dispersed by migratory species suggests a strong potential for long-distance seed dispersal.

Even non-typically frugivorous birds, such as the highly insectivorous blue tit *Cyanistes caeruleus* and pied flycatcher *Ficedula hypoleuca*, or granivorous such as the house sparrow *Passer domesticus* and chaffinch *Fringilla coelebs*, contributed to the dispersal of several plant species (Fig. 2). Insectivorous birds may feed on fruits as a nutritional complement (Fuentes 1994), while seed predators may disperse seeds when these are not destroyed during ingestion and digestion (Heleno *et al.* 2011). The finding of 19 intact seeds along with many remains of destroyed seeds of *Portulaca oleracea* in three chaffinch droppings renders further evidence of seed dispersal by typically seed predators. Thus, instead of a dichotomized view between seed dispersers and non-seed dispersers, our results support the view of a continuum of bird species importance for seed dispersal (Heleno *et al.* 2011).

The few studies that quantified epizoochory on birds were almost exclusively centred in waterbirds and found a variable prevalence of seeds adhered on birds' feathers and feet. In the USA, a study reported that 78% of the individuals of four waterbird species transported seeds from 12 salt marsh plant species, mostly attached to their feathers but also some on mud adhered to their feet (Vivian-Smith & Stiles 1994). In Spain, Figuerola and Green (2002) found that 35% - 100% of the individuals from six waterbird species carried propagules attached to their body; unfortunately, no distinction was made between seeds and invertebrates in their analysis. Regarding seeds alone, most were found adhered to the bird' feathers (Figuerola & Green 2002). More recently, Brochet *et al.* (2010) found diaspores (seeds and *Chara* sp. oogonia) attached on feathers and feet of 18% of inspected teals *Anas crecca* in France. These authors reported a similar prevalence of endozoochory (20%), but they did not considered only seeds, which hinders a comparison with our study. It was also estimated that 22% of the individuals of Pacific black duck *A. superciliosa* and chestnut teal *A. castanea* carried at least one viable seed via epizoochory (Raulings *et al.* 2011). On the Ogasawara Islands (Japan), epizoochory rates from 16% to 32% were found in four species of seabirds (Aoyama *et al.* 2012). To our knowledge, only one study evaluated the prevalence of epizoochory on a diverse assemblage of birds, including passerines, reporting a very low prevalence of epizoocory (0.08%) and no seeds found in passerine species (Choi *et al.* 2010).

The seeds of *T. arvensis* and *G. aparine* were attached to the birds' feathers by their specific adaptations to epizoochory: spines and hooks, respectively (Fig. 3). However, the epizoochory of the fleshy-fruited *F. alnus* is more puzzling as these seeds are considerably larger (*ca.* 5-6 milimetres), without ornamentation, and are generally ingested along with the fleshy pulp and dispersed via endozoocory (Hampe 2001). Naturally, the presence of a certain dispersal syndrome does not prevent the dispersal of seeds by other, non-standard mechanism (Higgins *et al.* 2003; Thomson *et al.* 2010). The transport of seeds without anchoring structures, via epizoochory, has been previously reported (Figuerola & Green 2002; Brochet *et al.* 2010; Raulings *et al.* 2011; Aoyama *et al.* 2012). However, in most cases those seeds either have structures that promote anemochory, which allow some anchoring to feathers, or they are very small seeds that can easily be arrested with mud on bird's feet (Carlquist 1966). Neither of these situations applies to the seeds of *F. alnus*. We hypothesize that residues of the fruit's pulp promoted the adhesion of the seed to the blackbird's nape, presumably when the bird was foraging. A similar situation may occur when seeds remain glued to feathers after defecation. Although the blackbird and serin are resident species in Portugal, the grasshopper warbler is a transient migrant which might be highly relevant given the disproportional importance of long-distance dispersal events for plant population dynamics (Nathan 2006). Epizoochorous seeds anchored to a migrating bird can potentially be dispersed over thousands of kilometres (until the bird dies or the seed gets detached). This long-distance dispersal allows plant species to eventually colonize new areas and can be especially troublesome in the case of potentially invasive species (Choi *et al.* 2010).

Our results offer support to the empirical suggestion by Nogales *et al.* (2012) that epizoochory in terrestrial birds must be "extremely rare". Despite the differences in sample size, we estimated a prevalence of epizoochory similar to that reported by Choi *et al.* (2010): 0.33% and 0.08%, respectively. However, we estimate a much lower frequency of epizoochory in passerines than that commonly found in waterbirds. Epizoochory results from the passive contact between the seeds and the animals (Burger 2005). Therefore, it seems likely that epizoochory might be more frequent in birds that forage or nest on the ground, particularly in habitats where epizoochorous plants (such as many annual species) or plants with small seeds are abundant. Furthermore, humid conditions, such as those in marshes, are likely to increase the frequency of epizoochory by promoting the adhesion of small seeds with mud on animals’ feet, regardless of the presence of adhesive structures on the seeds. In effect, the three passerines found here to carry seeds on their plumage are, despite having different main diets (blackbird –frugivorous, serin –granivorous, grasshopper warbler –insectivorous), species that usually live in habitats rich in herbaceous plants and may forage on the ground (Fernández-Juricic 2001; Cramp & Simmons 2004). Finally, all other things being equal, larger body-sized birds are more likely to disperse seeds externally due to their larger surface area. Thus, habitat, behaviour, and body size may explain the dissimilarity in epizoochory figures for waterbirds and those presented here for passerines. Accordingly, all birds reported in previous studies as carrying seeds externally have in common the fact that they live in habitats rich in plants that produce seeds prone to adhere to birds’ body and feet, and tend to nest on the ground (Vivian-Smith & Stiles 1994; Figuerola & Green 2002; Brochet *et al.* 2010; Choi *et al.* 2010; Raulings *et al.* 2011; Aoyama *et al.* 2012).

Chapter II

Sampling completeness in seed dispersal networks: When enough is enough

Costa J. M., da Silva L. P., Timóteo S., Ramos J. A. & Heleno R. H. (2016). Sampling completeness in seed dispersal networks: When enough is enough. *Basic and Applied Ecology*, 17: 155-164. https://doi.org/10.1016/j.baae.2015.09.008

Abstract

Ecological networks are an increasingly popular tool to explore community assembly rules and frame practical conservation issues. However, most described networks vary largely in sampling effort, hampering the distinction of true biological patterns from artefacts caused by poor sampling. Identifying entire seeds in the droppings of mist-netted birds is generally considered a preferred sampling method for building unbiased, quantitative seed dispersal networks. We retrieved seeds from the droppings of 936 mist-netted birds captured during five days in seven sites in Portugal and estimated sampling completeness as the diversity of seed species, disperser species, and links detected with respect to those predicted by the Chao 2 estimator. In one of those sites, sampling effort was extended to 25 days to evaluate the sensitivity of ten network structure descriptors to increasing sampling effort. After five sampling days we detected 93% of the seed species, 97% of the disperser species, and 79% of the links predicted by Chao 2, however sampling for 25 days resulted in the detection of more seeds, dispersers, and links than those estimated at day 5. Most network descriptors only began to stabilize around day 8, except for connectance and weighted connectance that stabilized earlier. Similarly, only after 8 days most networks descriptors significantly departed from the confidence interval estimated by null models exclusively constrained by species abundances, thus reflecting independent ecological patterns. Nestedness was the only exception, as it never departed from the null models. We suggest that Chao 2 may slightly underestimate the real diversity and that in our case at least eight sampling days were needed to build sound seed dispersal networks as 67% of the seeds, 88% of the dispersers, and 71% of the links were detected. Our results have important implications for the interpretation of seed dispersal networks because under-sampled networks may produce biased descriptors that do not suitably characterize the focal communities.

Keywords: Asymptotic estimators, Cumulative sampling, Endozoochory, Faecal analysis, Food-webs, Mist-nets, Rarefaction curves, Sampling effort

Introduction

The study of community-wide species interaction networks experienced a tremendous growth in recent years and proved a powerful tool to explore many processes in ecology, being particularly valuable in disentangling the relationships between the structure and function of nature's "entangled bank" (Darwin 1859; Olesen *et al.* 2012; Heleno *et al.* 2014). The use of ecological networks deepened our understanding of important conservation issues such as environmental degradation (e.g. Tylianakis *et al.* 2007; Traveset *et al.* 2013) or ecological restoration (Heleno *et al.* 2010).

Seed dispersal is one of the research areas where ecological networks attracted greatest interest (Carlo & Yang 2011; Donatti *et al.* 2011; Mello *et al.* 2011a; Heleno *et al.* 2013a). The dispersal of seeds away from the mother plant is a key process, allowing plants to avoid competition, find suitable conditions for germination, and expand their distribution range (Traveset *et al.* 2014). Due to their high mobility, frugivorous birds are the main seed dispersers in most ecosystems (Herrera 1984; Traveset *et al.* 2014). Avian seed dispersal data can be collected by: (1) recording fruit consumption by birds on focal plants (Olesen *et al.* 2011a), (2) identifying entire seeds in the droppings of mist-netted birds (Heleno *et al.* 2013a), and (3) identifying seeds in droppings collected in the field and identifying the disperser species with molecular techniques (González-Varo *et al.* 2014).

Most network structure descriptors are affected by sampling effort to some degree (Blüthgen *et al.* 2008; Blüthgen 2010; Rivera-Hutinel *et al.* 2012), with qualitative indices being more sensitive to sample size than quantitative analogues (Banasek-Richter *et al.* 2004). Specifically, poor sampling underestimates the real diversity of links, truncating estimated trophic breath and leading to a biased network structure (Blüthgen *et al.* 2008). On the other hand, as implementing such a holistic approach is inherently highly labour-intensive, it is important to know when further effort will not significantly increase the accuracy of the community description, thus avoiding unnecessary work load (Hegland *et al.* 2010). This effort is of utmost importance to allow meaningful cross-study comparisons (Heleno *et al.* 2014).

Richness estimators based on species and link accumulation curves are a powerful way to evaluate sampling completeness (Chacoff *et al.* 2012; Rivera-Hutinel *et al.* 2012; Olito & Fox 2015), where the number of missing species is estimated as those needed to reach a theoretical asymptote (Chacoff *et al.* 2012). Although some statistical methods have been suggested to ease the problem (e.g. Bartomeus 2013), there are no satisfactory solutions for poor sampling, and the sampling effort needed to build comprehensive seed dispersal networks from which theoretical and applied conclusions can be drawn has not yet been evaluated. In this study we aim to evaluate (1) the effect of sampling effort on the completeness of seed dispersal networks based on the analysis of droppings from mist-netted birds, and (2) the sampling effort needed to build high quality seed dispersal networks so that reliable network descriptors can be calculated.

Materials and methods

Study sites and data collection

On five consecutive days in the first half of September 2012, birds were captured in seven sites throughout Portugal ranging from agroforestry systems to secondary native forest (Fig. 4) (see Costa *et al.* 2014). At each site/day, 80-100 metres of mist-nets (according to the site-specific conditions) were operated during five hours after dawn. Nets were visited every 30 minutes and captured birds were individually placed in ringing bags to produce droppings (Heleno *et al.* 2013a). Intact seeds were later extracted from the droppings and identified under a dissecting microscope by comparison with a reference collection. Interaction frequency was quantified as the number of droppings from each bird species containing at least one intact seed of any of the plant species. We built quantitative seed dispersal networks for each site by considering the cumulative samples collected up to day *i*. We used the number of days as a measure of sampling effort because there were no significant differences in the number of droppings collected per day (average ± SD droppings collected per day = 124.8 ± 22.7, see Supplementary Materials: Table S2). The availability of fleshy fruits was independently assessed at each site by counting all ripe standing fruits along three 2 metre x 25 metre transects parallel to the mist-nets. In the site with highest fruit diversity (n=10; Fig. 4, Site A - Larçã), sampling continued for another consecutive 20 days.

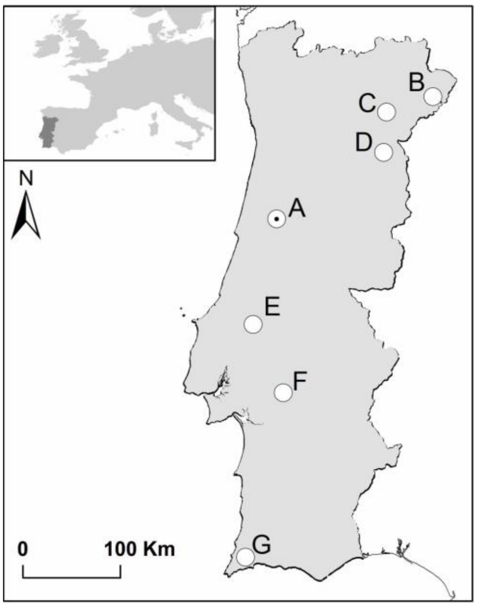

Figure 4 - Location of the study sites. A) Larçã, Souselas; B) Atenor, Miranda do Douro; C) Nozelos, Torre de Moncorvo; D) Santa Maria de Aguiar dam, Figueira de Castelo Rodrigo; E) Casais da Estrada, Achete; F) Herdade de Freixo do Meio, Foros de Vale Figueira; G) Bensafrim, Lagos. Site A was sampled for 25 consecutive days; all other sites were sampled for five days.

Network descriptors

The consistency of network structure descriptors to increasing sampling effort was evaluated with the data collected during five days in all sites (Table 1) and with the data collected during 25 consecutive days at the site with the highest fruit diversity. We explored the effect of increasing sampling effort in six qualitative and four quantitative network descriptors. Qualitative descriptors included: connectance, the proportion of realised links from all possible links (i.e. interactions) in the network (Jordano 1987); Nestedness (NODF) (Almeida-Neto *et al.* 2008) which reflects the degree of organization of interactions around a core of generalist species; Disperser richness; Seed richness; Link richness; and Qualitative Modularity, reporting the existence of clusters of tightly interacting species (Olesen *et al.* 2007). Qualitative Modularity was estimated with the algorithm QuaBiMo (Dormann & Strauss 2014) using both binary (qualitative) and weighted (quantitative) matrices. Although this algorithm has been specifically developed for weighted interaction matrices, we applied the same algorithm to binary versions of the original matrices in order to evaluate the effect of the input data (binary/weighted). The quantitative descriptors included: weighted connectance, the ratio between linkage density (mean number of links per species) and the number of species in the network (Bersier *et al.* 2002); weighted nestedness (WNODF), as NODF but takes into account interaction frequency (Almeida-Neto & Ulrich 2011); network specialisation index (H_2’), measuring the degree of the partner’s selectivity as the departure from a theoretical non-discrimination of interactions (Blüthgen *et al.* 2006); and quantitative modularity, as qualitative modularity but based on the original, weighted interaction matrices. The significance of each network descriptor was assessed by comparison with the 95% confidence interval of a set of 1000 matrix randomizations (100 for Modularity) using the Patefield´s algorithm (marginal totals equal to the observed matrix) (Patefield 1981). This allows us to distinguish if a certain parameter is driven by biological/ecological characteristics of the interacting species or results simply from random interaction patterns driven mostly by species abundances. Piecewise regressions were used to estimate the sampling effort needed to achieve asymptotic values (i.e. slope of partial regression line not being significantly different from zero). Network descriptors were calculated with packages bipartite 2.05 (Dormann *et al.* 2008) and vegan 2.2 (Oksanen *et al.* 2015), while piecewise regressions were performed with package segmented 0.5 (Muggeo 2008) in R 3.0.2 (R Core Team 2017).

Sampling completeness

The minimum expected asymptotic richness of seed species, disperser species, and links, was estimated with the Chao 2, a non-parametric estimator based on the proportion of uniques (here, species captured on a single day) relative to duplicates (here, species captured on two days) (Chao 1984; Colwell & Coddington 1994). The expected richness was computed using the program EstimateS 9.1.0 (Colwell 2013). The Chao 2 estimator was chosen for being more robust to reduced sample size (Colwell & Coddington 1994), performing considerably better than other richness estimators (Walther & Moore 2005).

Sampling completeness was considered as the percentage of observed richness relative to the estimated asymptotic richness. By extrapolation of the estimated rarefaction curves (Colwell *et al.* 2012), we estimated the additional number of sampling days needed to detect 80%, 90%, and 100% of the total estimated richness. To evaluate whether sampling completeness is affected by fruit diversity across sites, we used a generalized linear model with quasibinomial error distribution (due to data underdispersion).

Results

Network descriptors

During the five sampling days, different network descriptors showed different trends regardless of whether they were based on qualitative or quantitative links (Fig. 5). Both connectance and weighted connectance of all networks stabilized at day 3. NODF, weighted NODF, and H_2' showed a higher site-specific variability (Fig. 5).

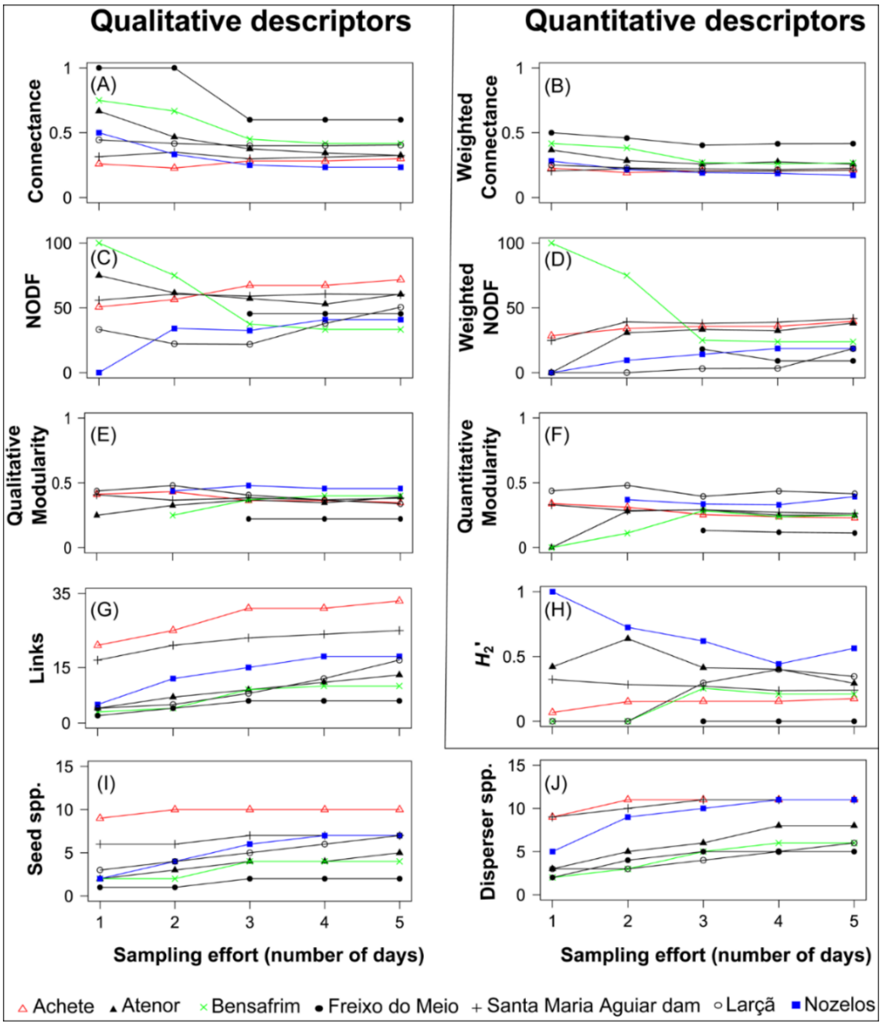

Figure 5 - Effect of sampling effort (measured as the number of days) on the connectance (A), weighted connectance (B), nestedness [NODF (C) and weighted NODF (D)], modularity [qualitative

(E) and quantitative (F)], specialization H_2’ (H), richness of seeds (I), dispersers (J), and links (G) detected in seven seed dispersal networks sampled simultaneously during five consecutive days throughout Portugal. Missing values correspond to days/sites where the network was too small to allow the calculation of the respective network descriptors.

All but two descriptors (seed species and link richness) stabilised before the end of the study at the site sampled for 25 days (Supplementary Materials: Table S3) but different metrics showed distinct patterns in respect to increasing sampling effort. Both connectance and weighted connectance remained relatively stable throughout the study period (connectance = 40% - 50% ($-0.02 < \beta_{95\%\ CL} < 0.0005$, Fig. 6A); weighted connectance = 21% - 27% ($-0.001 < \beta_{95\%\ CL} < 0.0002$, Fig. 6B)). NODF stabilized after day 8, (day 8-21: $-0.01 < \beta_{95\%\ CL} < 1.17$; day 21-25: $-5.09 < \beta_{95\%\ CL} < 2.01$; Fig. 6C) while weighted NODF tended to stabilize at day 8 but formally reached its asymptotic value only on day 16 ($-0.51 < \beta_{95\%\ CL} < 0.58$, Fig. 6D). The qualitative and quantitative modularity as well as network specialization H_2’ stabilized around day 8 (qualitative modularity: $-0.004 < \beta_{95\%\ CL} < 0.00002$, Fig. 6E; quantitative modularity: $-0.002 < \beta_{95\%\ CL} < 0.0008$, Fig. 6F; H_2’: $-0.004 < \beta_{95\%\ CL} < 0.001$, Fig. 6H). The number of detected seed species and links was still increasing as revealed by the positive slopes of the partial regressions (seeds: $0.19 < \beta_{95\%\ CL} < 0.42$, Fig. 6I; links: $0.62 < \beta_{95\%\ CL} < 0.80$, Fig. 6G). However, disperser’s diversity stabilised at day 8 with no further detected species ($-0.02 < \beta_{95\%\ CL} < 0.02$, Fig. 6J).

Sampling completeness

After five sampling days, we detected, on average, 93% (range: 71% - 100%) of the estimated seed species, 97% (92% - 100%) of the estimated disperser species, and 79% (57% - 100%) of the estimated links on the seven study sites (Table 2). Fruit diversity at a given site did not significantly affect sampling completeness (GLM: $\beta = -0.06$, $t = -0.464$, $p = 0.662$; Supplementary Materials: Fig. S1). After the first five sampling days in Larçã, we detected only 58% of the seed species, 75% of the disperser species, and 45% of the links detected within 25 days. The detection rate improved to 67% of the seeds, 88% of the dispersers, and 71% of the links by day 8. After 25 sampling days, we estimated to have recorded all seed species (n=12), all disperser species (n=8), and 73% (38 out of 52) of the estimated number of links at site A (Table 2). The observed richness in this site after 25 days was higher than that estimated using Chao 2 at day 5 for all levels considered (seeds, dispersers, and links; Table 2). A total of 50 days were considered necessary to detect 90% of the links and 72 days would be needed to detect all links (Table 3).

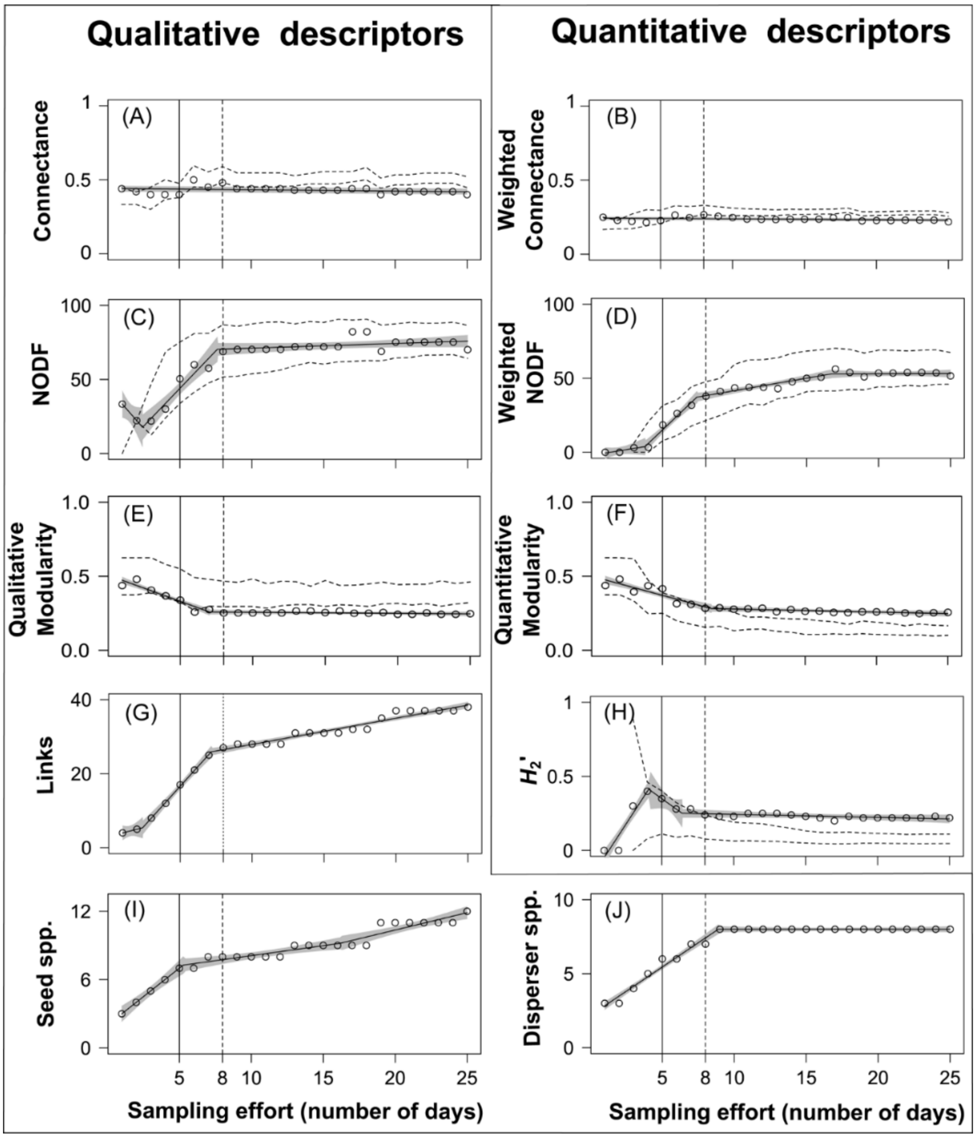

Figure 6 - Effect of the sampling effort (measured as the number of days) on the connectance (A), weighted connectance (B), nestedness [NODF (C) and weighted NODF (D)], modularity modularity [qualitative (E) and quantitative (F)], specialization H_2’ (H), richness of seeds (I), dispersers (J), and links (G) detected on the seed dispersal network at Larçã (Fig. 1). The dashed lines indicate the 95% confidence interval of 1000 runs (100 for modularity) of a Patefield null model. The fitted lines represent the theoretical values estimated by piecewise linear regressions, except for connectance, which was fitted with linear regression, with the grey area indicating the 95% confidence interval of the regression slope. The dashed vertical line at day 8 indicates the

minimum adequate sampling effort suggested for this site, while the full line at day 5 indicates the duration of the sampling period on the other sites.

Table 2 - Percentage of disperser species, seed species, and links detected during five consecutive sampling days across the seven sites. Estimated values of richness were computed using the Chao 2 estimator. Estimates marked with * were computed based on the classic formula of Chao 2 because the coefficient of variation for incidence distribution > 0.5; **O** – observed richness, **E (95% C.I.)** – estimated richness (95% confidence intervals), **%** - sampling completeness (=O/E*100).

Site	**# days**	**Seeds**			**Dispersers**			**Links**		
		O	**E (95% C.I.)**	**%**	**O**	**E (95% C.I.)**	**%**	**O**	**E (95% C.I.)**	**%**
A - Larçã	25	12	12 (12-16)	100	8	8* (8-21)	100	38	52 (42-93)	73
A - Larçã	5	7	7 (7-12)	100	6	6 (6-7)	100	17	30 (20-68)	57
B - Atenor	5	5	7* (5-24)	71	8	8 (8-15)	100	13	18 (14-40)	72
C - Nozelos	5	7	8 (7-19)	88	11	12 (11-21)	92	18	23 (19-44)	79
D - Santa Maria de Aguiar	5	7	7 (7-18)	100	11	12 (11-24)	92	25	29 (26-45)	86
E - Achete	5	10	11 (10-22)	91	11	11 (11-15)	100	33	41 (35-62)	81
F - Freixo do Meio	5	2	2* (2-3)	100	5	5 (5-6)	100	6	6 (6-14)	100
G - Bensafrim	5	4	4 (4-5)	100	6	6 (6-14)	100	10	13 (10-28)	77

Table 3 - Number of additional sampling days needed to detect 80%, 90%, and 100% of the diversity estimated by the Chao 2 estimator.

Network descriptor	**Sampling period**	**Target sampling completeness**		
		80%	**90%**	**100%**
Seed species	5 days (mean of 7 sites)	0	0	2
	25 days (1 site)	0	0	0
Disperser species	5 days (mean of 7 sites)	0	0	1
	25 days (1 site)	0	0	0
Link diversity	5 days (mean of 7 sites)	1	2	5
	25 days (1 site)	8	25	47

Discussion

In this study we show that while some network descriptors can be accurately estimated with lower sampling effort (number of disperser species, connectance, and weighted connectance), most network metrics only started to stabilize after eight mist-netting days (number of sinks and seed species, NODF, weighted NODF, modularity (both quantitative and qualitative), and H_2’).

Network emergent properties

While five sampling days suitably captured the biological diversity across the seven study sites, it was not sufficient to deliver a completely sound representation of the community structure measured by all but two network descriptors (connectance and weighted connectance). Contrary to our expectation, we could not detect a trend for quantitative indices performing better to lower sampling efforts. In fact, weighted NODF took slightly longer to stabilize than its qualitative counterpart.

Both connectance and weighted connectance remained relatively stable throughout the study period and therefore were accurately predicted even with low/moderate sampling effort. However, only after day 7/8 the network is significantly less connected than we would expect according to a random association among species (Fig. 6). Still, given the small difference between observed and predicted values, such significance may be explained by the intrinsic mathematical behaviour of the used null model, which tends to generate matrices with higher connectance than the observed matrix (Dormann *et al.* 2009). Additionally, these descriptors showed similar trends in all of our study sites, suggesting that such robustness to sample size is not network-specific but also that these descriptors are of limited informative value to compare seed dispersal networks. Previous studies on pollination networks found a tendency for an initial increase in connectance towards an asymptote that might be reached at relatively low (Nielsen & Bascompte 2007) or higher (Rivera-Hutinel *et al.* 2012) levels of sampling completeness. Our results suggest that, despite both connectance and weighted connectance of seed dispersal networks may be stable even with a low sampling effort, they are of limited use if we want to compare at least similar seed dispersal networks. Moreover, despite its wide use in ecological network studies and apparent robustness to sample size, connectance seems to have no relation between its value and the network conservation status (Heleno *et al.* 2012). Further work is required to confirm the extent of this generalization.

The two measures of nestedness (NODF and weighted NODF) were more sensitive to sampling effort than connectance. Asymptotic values of NODF were achieved earlier (day 8) than for weighted NODF (day 16), but overall our results coincide with those of Rivera-Hutinel *et al.* (2012) who found NODF to be relatively stable if at least 30% of the network had been sampled. However, the observed network was not significantly nested

(Fig.6) even after 25 sampling days. This pattern was not the result of sampling limitations, as both NODF and weighted NODF values were relatively stable, with as low as half of the full sampling effort and thus not to be expected to change with a higher sample size, but likely driven by species abundances. Taken altogether our results question the ecological relevance of nestedness indices (James *et al.* 2012). Modularity has been increasingly used in network studies as it is strongly related to nestedness (Fortuna *et al.* 2010). We found both qualitative and quantitative modularity to stabilize after 7 and 8 sampling days, respectively, and to encompass real ecological information as it cannot be explained by null models based solely on species abundances. However, it is important to note that wile quantitative modularity revealed a network more modular than expected by chance, the analyses based on binary matrices revealed the opposite pattern, reinforcing the value of weighted interaction networks in the capacity of this algorithm to detect modules (Dormann & Strauss 2014). Network specialization H_2' stabilized around day 6 to 8 and was higher (i.e. more specialized) than expected under random species associations. These results corroborate the stability of this metric to moderate sampling effort (Blüthgen *et al.* 2006; Schleuning *et al.* 2014a). These three descriptors (NODF, weighted NODF, and H_2') showed high site-specific trends, and while they seem to convergence to a narrower range during the five sampling days (Fig. 5), this sampling effort is insufficient to provide a clear pattern. The breakpoints identified by the piecewise regression (i.e. those that maximise the variability explained by the regression lines) should also be regarded as conservative given that quasi-asymptotic values are often reached a few days before the estimated breakpoint (Fig. 6).

From Fig. 6, and considering the sampling effort and data quality trade-off, a minimum of eight sampling days are required to adequately sample our seed dispersal network (detection of 67% for seed species, 88% for disperser species, and 71% for links). After this day, all descriptors either reach an asymptote or have a highly reduced slope and show a consistent pattern of significance with respect to null models. Our results are restricted to seven sites in Portugal and to a particular sampling method (identification of seeds on the droppings of mist-netted birds), so further studies testing different sampling strategies and different interaction types, particularly in hyper-diverse ecosystems, are important to test the generality of these results.

An assessment of each descriptor's quality is critical to derive meaningful network descriptors that reveal true ecological attributes of biological communities and not mathematical artefacts resulting from poor sampling or exclusively from species abundances. Since rare species and links require a higher sampling effort to be recorded, the typically low evenness of species abundances alone can drive some observed properties of the structure of some networks (Vázquez & Aizen 2004). Network descriptors can be more or less sensitive to these sampling artefacts (Blüthgen *et al.* 2008; Blüthgen 2010; Rivera-Hutinel *et al.* 2012), and thus its values may either reflect sample effects or real nature processes. The use of appropriate null models is a way to disentangle the processes structuring the observed networks (Vázquez & Aizen 2004; Vázquez *et al.* 2007; Vázquez *et al.* 2009b). These analyses surpass the scope of this study but are crucial if one wants descriptors that mirror true biological patterns.

The efficiency of mist nets to capture birds depends on several factors, such as habitat structure, weather, or bird behaviour and size (Pagen *et al.* 2002; Estades *et al.* 2006). Nevertheless, mist-netting is likely the best method to construct quantitative seed dispersal networks as it is largely free of observer bias, allows great taxonomic resolution (species level with few exceptions), the detection of inconspicuous dispersers and interactions, makes the study of individual food choice possible through individual marking (e.g. rings), and allows the evaluation of the effect of seed ingestion on its viability (Heleno *et al.* 2011; Traveset *et al.* 2014; Escribano-Avila *et al.* 2018).

Sampling completeness

Five sampling-days detected consistently > 71% of the dispersed seed species and > 92% of the disperser species across the seven sites. We estimate that during the same period, the majority of the links (79%) were also detected, but the sampling completeness was lower (down to 57%) on the most diverse site (Larçã).

As expected, the thorough detection of links required higher sampling intensity than the detection of the species. A similar effect was already described for pollination networks (Chacoff *et al.* 2012). However, due to the generally lower size of seed dispersal networks, with lower diversity of dispersers, i.e. animals/plants ratio lower in seed dispersal than in pollination networks, one may expect that seed dispersal studies require less effort to attain a similar sampling completeness (Blüthgen *et al.* 2007; Guimarães *et al.* 2007).

In Larçã, 25 sampling days resulted in the detection of a higher richness of seeds, dispersers, and links than that estimated by Chao 2 with only five sampling days. However, since this estimator computes the minimum expected richness (Chao 1984; Colwell & Coddington 1994) and the seeds and link richness detected after 25 days lay within the confidence interval of that estimated after five days, we consider that this estimator performed relatively well for seeds and links. For dispersers, however, the richness detected after 25 days lay above the confidence interval estimated after five sampling days. This, at least apparent, underestimation of Chao 2 might reflect a poor performance of the estimator for species richness due to low sample size (five capture occasions) (Walther & Moore 2005). This apparent underestimation may be largely explained by an increase in the availability of ripe fruits (advancing fruit phenology) and new potential dispersers (bird migration) entering an open community.

We estimated that all species and 73% of the links between plants and avian dispersers were detected in Larçã with 25 sampling days. Interestingly, some species pairs that are present at this site, but apparently not interacting, are known to interact elsewhere: e.g. *Sylvia atricapilla* with *Daphne gnidium*, *S. melanocephala* with *Smilax aspera* (Olesen et al. 2011a). We believe that the independent study of species and link distributions (e.g. interaction distribution modelling) holds a large potential to understand community assembly rules in ecology.

Our results suggest that while some descriptors can be accurately estimated with lower sampling effort, at least eight sampling days were needed to accurately describe the structure of our seed dispersal network based on the droppings of mist-netted birds. More studies on the effect of sampling intensity on network descriptors are needed to allow a generalization of the conclusions on the effort required to get a realistic overview of the seed dispersal networks' structure and to critically assess sampling limitations in previous studies. The reproducibility of the results is a central tenet of experimental science, and there is no reason why this should not be applied to ecological networks studies, for which the identification of what is a sound sampling effort is paramount.

Chapter III

Rewiring of experimentally disturbed seed dispersal networks might lead to unexpected network configurations

Costa J.M., Ramos J.A., da Silva L.P., Timóteo S., Andrade P. Araújo P.M., Carneiro C., Correia E., Cortez P., Felgueiras M., Godinho C., Lopes R.J., Matos C., Norte A.C., Pereira P.F, Rosa A. & Heleno R.H. (2018). Rewiring of experimentally disturbed seed dispersal networks might lead to unexpected network configurations. *Basic and Applied Ecology*, 30: 11-22. https://doi.org/10.1016/j.baae.2018.05.011

Abstract

The consequences of species extinctions in ecological communities may be buffered through the rearrangement (rewiring) of the interactions between the remaining species. The structural and functional consequences of such extinctions can be explored by means of computer simulations that try to predict secondary extinctions and the degradation of ecosystem services. However, to improve the accuracy of these simulations, it is pivotal to evaluate their performance in predicting changes observed in natural communities. In this study, we first described the avian seed dispersal networks in 17 sites throughout Portugal and found that blackberry (*Rubus ulmifolius*) was the most dispersed species in 13 out of the 17 sites. Second, we performed a manipulative experiment to evaluate the effect of removing the most dispersed plant species and compared the observed outcome in the structure of the network with computer simulations with and without rewiring. Observed changes were consistent with some rapid network rewiring, with dispersers shifting to alternative fruit species. Although the observed network topology after the experimental removal was not accurately predicted, the extinction simulation with rewiring performed considerably better than that without rewiring. Individual species roles were even harder to predict than emergent network properties on both types of models. We show that incorporating rewiring rules can considerably increase the accuracy of species extinction models, however, the functional consequences of losing important resources might not be easily anticipated, and rewiring might occur in unexpected directions.

Keywords: Ecological networks; frugivory; passerines; *Rubus ulmifolius*

Introduction

The loss of any species from a community entails a concomitant loss of all its interactions with other species, whether they are prey, predators, hosts, parasites or mutualists (Bond 1994). In some cases, the extinction of these ecological interactions can lead to secondary species extinctions (Brodie *et al.* 2014), which are hard to predict (Ives & Cardinale 2004; Brodie *et al.* 2014; Timóteo *et al.* 2016) as communities may undergo a structural rearrangement through the establishment of new interactions among the remaining species, i.e. rewiring (Brosi & Briggs 2013; Goldstein & Zych 2016; Timóteo *et al.* 2016; CaraDonna *et al.* 2017; Hallett *et al.* 2017). Recently, the complex web of interactions that sustain long-term survival of co-occurring species has been visualised and analysed in the form of interaction networks, whose structure can be described by topological descriptors, such as connectance, nestedness, or specialization (Petanidou *et al.* 2008; Ramos-Jiliberto *et al.* 2012; Poisot *et al.* 2014; Trøjelsgaard *et al.* 2015).

Under the ongoing global biodiversity crisis, gaining predictive capacity regarding the consequences of species extinctions is arguably one of the most pressing needs in ecology (Isbell *et al.* 2017). Extinction simulations of species interaction networks can be particularly useful to predict consequences of species extinctions at the community level (Rumeu *et al.* 2017). Several studies have now shown that the extent of secondary extinctions is affected by the original structure of the networks, for example with more connected mutualistic communities being more robust to such perturbations (Thébault & Fontaine 2010). However, such simulations have been increasingly criticised for being too conservative regarding the establishment of new interactions (Blüthgen 2010). Although most studies to date do not allow network rewiring (i.e. the emergence of new interactions that compensate for lost ones) (Memmott *et al.* 2004; Santamaría *et al.* 2014; Correa *et al.* 2016), some began to incorporate different algorithms that allow some type of network rearrangement (Carvalheiro *et al.* 2008; Kaiser-Bunbury *et al.* 2010; Ramos-Jiliberto *et al.* 2012; Schleuning *et al.* 2016). To prevent the virtual creation of interactions that are actually impossible due to morphological, temporal or spatial mismatches, i.e. forbidden links (Jordano 2016), some of these studies constrain rewiring to previously observed interactions (e.g. Kaiser-Bunbury *et al.* 2010; Timóteo *et al.* 2016). The incorporation of rewiring in extinction simulations showed ecological networks to be more robust to secondary extinctions than when no rewiring was accounted (Kaiser-

Bunbury *et al.* 2010). Nevertheless, since the potential interactions considered in simulations with rewiring are often constrained by the locally observed interactions, these may be biased towards the most abundant interactions (Fründ *et al.* 2016; Plein *et al.* 2017). Therefore, the potential of natural communities to originate new interactions that have not been previously recorded in the target network remains unclear, rendering most simulations highly speculative as they may not reflect the real consequences of species extinctions (Timóteo *et al.* 2016). To overcome this limitation and increasing the spectrum of potential interactions being established during rewiring simulations, one can incorporate information of interactions observed on other locations.

Given the ongoing threat that frugivores and their habitats are facing worldwide (Farwig & Berens 2012), experiments on how seed dispersal networks behave after the extinction of fleshy-fruited plant species are needed to evaluate the potential consequences for the remaining species in these networks, which ultimately may affect plant recruitment and long-term vegetation dynamics (Traveset *et al.* 2014; Bello *et al.* 2015; Rumeu *et al.* 2017). On the one hand, the typical generalist nature of frugivorous bird-fruit interactions (Fuentes 1994; Blüthgen *et al.* 2007) is likely to render dispersal networks more robust to species extinctions than predicted through constrained extinction simulations that do not allow the establishment of previously unobserved interactions (Rumeu *et al.* 2017). On the other hand, there are important morphological, temporal, and spatial limitations to the interactions between fruits and their bird dispersers (Olesen *et al.* 2011a), and unconstrained rewiring might lead to a dangerous overestimation of network resistance to secondary extinctions (Ramos-Jiliberto *et al.* 2012; Rumeu *et al.* 2017). Therefore, identifying potential interactions is essential for meaningful extinction simulations.

There are four objectives to this study: (1) Characterize the range of potential pairwise interactions between fruits and their avian dispersers during the peak of the fruiting season in Portugal; (2) Identify the most important fleshy-fruited plant species for birds during that period; (3) Evaluate the structural plasticity of a seed dispersal network after the experimental removal of the most abundant fruit resource; (4) Evaluate which *in silico* extinction simulations, with or without rewiring, provide better predictions of the observed network structure of a manipulated community. We predict that frugivorous birds, after the removal of the most important fruit resource, will start to disperse new seed species thus diverting their seed dispersal services to co-occurring plants, as well as increase the frequency of previously dispersed seed species.

Materials and Methods

This work is divided in two parts. First, we compiled fruit-frugivorous bird interactions at a national scale to identify the most important fleshy-fruited plant species, their dispersers, and the potential interactions between the two groups (objectives 1 and 2). Second, we manipulated a seed dispersal network and compared the observed changes in the network's topology with those predicted by secondary extinction simulations with and without rewiring (objectives 3 and 4).

Characterization of the seed dispersal networks

We sampled 17 sites across mainland Portugal (Fig. 7) for five consecutive days in the first half of September 2012, 2013, and 2014. This period corresponds to the peak of abundance and richness of fleshy fruits and coincides with the passage of many migrant birds (Costa *et al.* 2016). At each site, approximately 100 m of mist nets were operated per day (with some small adjustments to comply with logistic constraints and bird safety) for five hours after dawn to capture birds. The nets were visited every 30 minutes and any bird captured was kept inside a cotton bag for up to 30 minutes to obtain a dropping (see Heleno *et al.* 2013a). Under a dissecting microscope, all undamaged seeds retrieved from bird droppings were counted and identified to the lowest taxonomical level possible by comparison with a comprehensive seed reference collection at the Centre for Functional Ecology of the University of Coimbra. We defined interaction frequency of a bird-plant pairwise interaction as the number of droppings of each bird species containing at least one intact seed from any plant species (i.e. frequency of occurrence) (see Vázquez *et al.* 2005; Heleno *et al.* 2013a; Correia *et al.* 2016). To assess the availability of fleshy fruits, we counted all ripe fruits along three linear transects, running parallel to the mist-nets at each site, each of them consisting of a rectangle of 2 m x 25 m (50 m^2). Although we likely have missed some rare fleshy-fruiting species at each site, they represent a very large sampling area (150 m^2 per site), and they were considered representative of the local fruit availability by the observers.

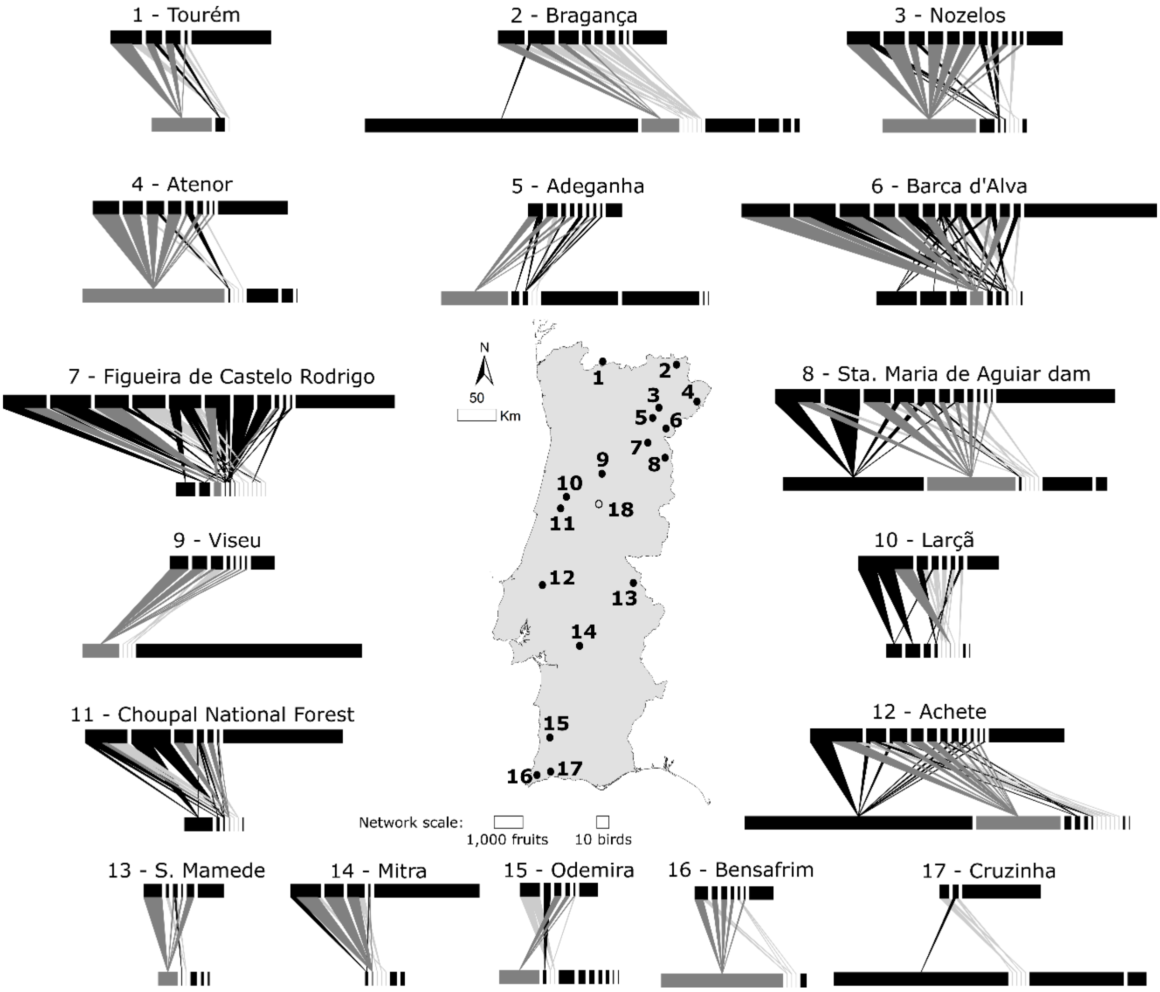

Figure 7 - Location and structure of the 17 seed dispersal networks assembled in this study and drawn to the same scale. The experimental site where blackberries were removed corresponds to site 18 (open circle). In each network, the upper and lower levels represent bird and plant species, respectively, and the width of the links between the levels is proportional to the number of droppings of each bird species that contained intact seeds. The width of each bird's and plant's box depicts, respectively, the number of captured birds and the abundance of fruits in the transects. The bird boxes without any links include all captured bird species that did not disperse any seeds. Interactions with the blackberry are represented in dark grey; plants whose fruits were found in the transects are represented in black; and plants whose seeds were found in the droppings but not in the transects are represented in light grey.

Experimental removal of the most dispersed plant species

To experimentally evaluate the rewiring capacity of plant-frugivore interactions, we performed a manipulative experiment in which we manually removed all fruits of the most dispersed plant species (*Rubus ulmifolius*, hereafter blackberry, see results) from one site, and compared the composition and structure of the seed dispersal network before and after the removal. This experiment was performed between the 1st and 14th of September 2015, in a secondary native woodland in central Portugal (site 18, Fig. 7) dominated by maritime pine (*Pinus pinaster*), Pyrenean oak (*Quercus pyrenaica*), and English oak (*Q. robur*), with an understory of strawberry tree (*Arbutus unedo*), hawthorn (*Crataegus monogyna*), and narrow-leaved mock privet (*Phillyrea angustifolia*). This site was selected due to the high local abundance of blackberry fruits (the overall most important fruit resource across the 17 sites), the large abundance of other fruit resources, and the concentrated distribution of blackberry fruits which made their removal logistically possible in a single afternoon. During seven consecutive days, we assessed seed dispersal interactions by retrieving seeds from the droppings of mist-netted birds. On the afternoon of the 7th day, we manually removed all ripe and unripe blackberries within a radius of 100 m from the mist nets. After that removal, we continued to sample in the same way for another seven consecutive days. Fruit availability was also estimated by counting all ripe standing fruits along a 2 m x 75 m transect running parallel to the mist-nets on day 5 (before) and day 10 (after the removal). The variation in the total number of captured birds and number of retrieved droppings (total, with seeds overall, with blackberry's seeds, and with seeds from other species), before and after the removal of blackberries, was assessed with G-tests.

Secondary extinction simulations

We evaluated the performance of secondary extinction simulations with and without rewiring, against the observed structure of the manipulated network. For these comparisons, the following descriptors were used: (1) weighted connectance, the ratio between the mean number of interactions per species and the total number of species in the network (Bersier *et al.* 2002); (2) network specialization H_2', which measures the community level selectiveness of partners as a departure from a random (i.e. abundance-based) interaction pattern (Blüthgen *et al.* 2006); (3) Weighted Nestedness metric based on Overlap and Decreasing Fill (WNODF, Almeida-Neto & Ulrich 2011), a measure of the nestedness of the network, quantifying to what degree interactions are organized around a core of highly generalist species; (4) bird species strength, a measure of the importance of each bird species as a disperser to the overall plant community (Bascompte *et al.* 2006); (5) bird specialization d', which quantifies the species' selectiveness for resources (Blüthgen *et al.* 2006); and (6) number of plant species dispersed by each bird species (other than blackberry). In the simulations that allowed interaction rewiring, the blackberry was deleted from the interaction matrix and the observed frequency of these interactions was randomly assigned to other plant species dispersed by the remaining bird species in the network. To avoid the occurrence of forbidden links, we only allowed new pairwise interactions if they were previously detected in any of the 17 networks. Since the random redistribution of the interactions produces a different interaction matrix in each run, we repeated this process 100 times, after which the mean and 95% bias-corrected and accelerated (BCa) bootstrapped confidence intervals (10,000 replicates) (Efron 1987) were mostly constant regardless of the number of runs (see Supplementary Materials: Figs. S2 and S3). In the extinction simulation without rewiring, the blackberry was deleted from the interaction matrix along with those bird species that only dispersed this species. This procedure resulted in a single matrix that was a reduced form of the full empirical network. The descriptors predicted by the extinction simulations, with and without rewiring, were then compared with those of the observed network after the experimental removal of blackberries.

Networks were visualized using specific code implemented in Mathematica 9.0 (Wolfram Research 2012), and all data analysis was performed in R (R Core Team 2017) using packages "RVAideMemoire" (Hervé 2017) to compute G-tests, "bipartite" (Dormann *et al.* 2008; Dormann *et al.* 2009) to compute network- and species-level descriptors, and "boot" (Canty & Ripley 2014) to compute 95% BCa bootstrapped confidence intervals.

Results

Characterization of the seed dispersal networks

Overall, we captured 2,381 birds belonging to 64 species in the 17 sites, of which 587 birds from 24 species and 13 families dispersed 3,974 seeds from 35 plant species. Of all bird species that dispersed seeds, two are partially migratory (i.e. those with both resident and migratory populations), namely the European robin (*Erithacus rubecula*, hereafter robin) and blackcap (*Sylvia atricapilla*), while 12 species are migratory. Each network included on average 7.4 (± 2.9 S.D.) bird species and 6.2 (± 2.7) plant species. The large majority (80%, 28 species) of all dispersed seed species were from fleshy fruits. Overall, five bird species – robin, pied flycatcher (*Ficedula hypoleuca*), blackcap, garden warbler (*S. borin*), and Sardinian warbler (*S. melanocephala*) – accounted for more than half of all pairwise interactions (55%) and the vast majority of the droppings with seeds (76%). The blackcap was the most important disperser species, being present on 14 sites, and dispersing on average 65% of the plant species at each site. Other relevant dispersers were the Sardinian warbler, garden warbler, European robin, and pied flycatcher, dispersing 45%, 38%, 37%, and 24% of the plant species at each site, respectively. Blackberry seeds were present in 16 of the 17 networks and were the most commonly dispersed species in 13 of those sites (Fig. 7), being present in 43% of all droppings with seeds. These seeds were dispersed by 17 out of 24 disperser species, and by most disperser species at each site (mean = 73% ± 30%).

Experimental removal of the most dispersed plant species

Before the experimental removal of blackberries, eight bird species dispersed seven species of plants, with all of them dispersing blackberry seeds (Fig. 8). Of all droppings with blackberry seeds, 56% were droppings from robin. Three bird species: blue tit (*Cyanistes caeruleus*), pied flycatcher, and blackbird (*Turdus merula*), dispersed only blackberry before the removal of its fruits (Fig. 8). Blackberry had the second highest standing fruit crop (31 % of all available fruits, only surpassed by hawthorn, which is only consumed later in the season), and was by far the most dispersed species at this site (56% of all interactions) (Figs. 8 and 9).

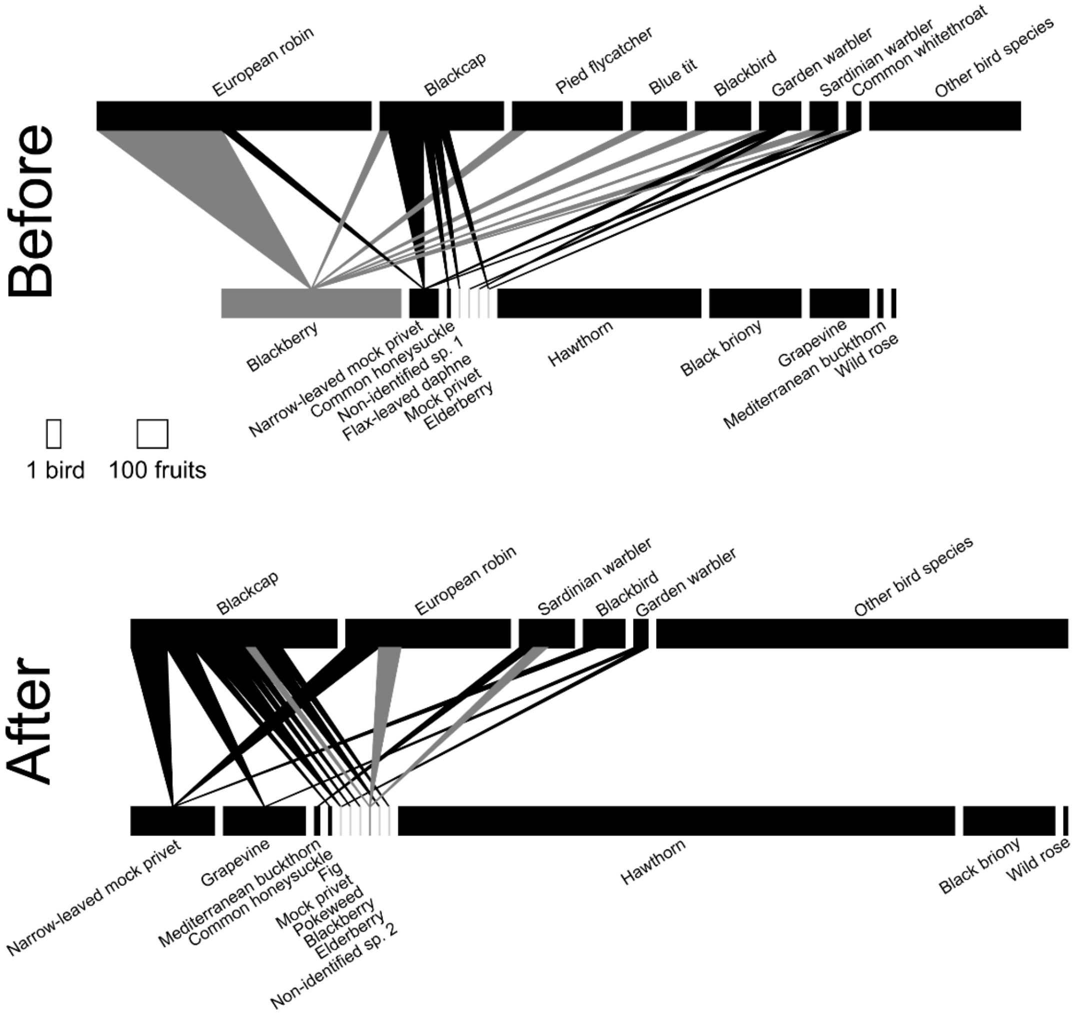

Figure 8 - Structure of the seed dispersal network before and after the experimental removal of blackberries. The upper and lower levels of each network, drawn to the same scale, represent bird and plant species, respectively, and the width of the links between the levels is proportional to the number of droppings of each bird species that contained intact seeds. The width of each bird's and plant's box depicts, respectively, the number of captured birds and the abundance of fruits in the transects. The bird boxes without any links include all captured bird species that did not disperse any seeds. Interactions with the blackberry (*Rubus ulmifolius*) are represented in dark grey; plants whose fruits were found in the transects are represented in black; and plant species with seeds found in the droppings but not in the transects are represented in light grey; robin (*Erithacus rubecula*), blackcap (*Sylvia atricapilla*), pied flycatcher (*Ficedula hypoleuca*), blue tit (*Cyanistes caeruleus*), blackbird (*Turdus merula*), garden warbler (*Sylvia borin*), Sardinian warbler (*S. melanocephala*), common whitethroat (*S. communis*), narrow-leaved mock privet (*Phillyrea angustifolia*), common honeysuckle (*Lonicera periclymenum*), flax-leaved daphne (*Daphne gnidium*), mock privet (*P. latifolia*), elderberry (*Sambucus nigra*), hawthorn (*Crataegus monogyna*), black briony (*Tamus communis*), grapevine (*Vitis vinifera*), Mediterranean buckthorn (*Rhamnus alaternus*), wild rose (*Rosa* sp.), fig (*Ficus carica*), pokeweed (*Phytolacca americana*).

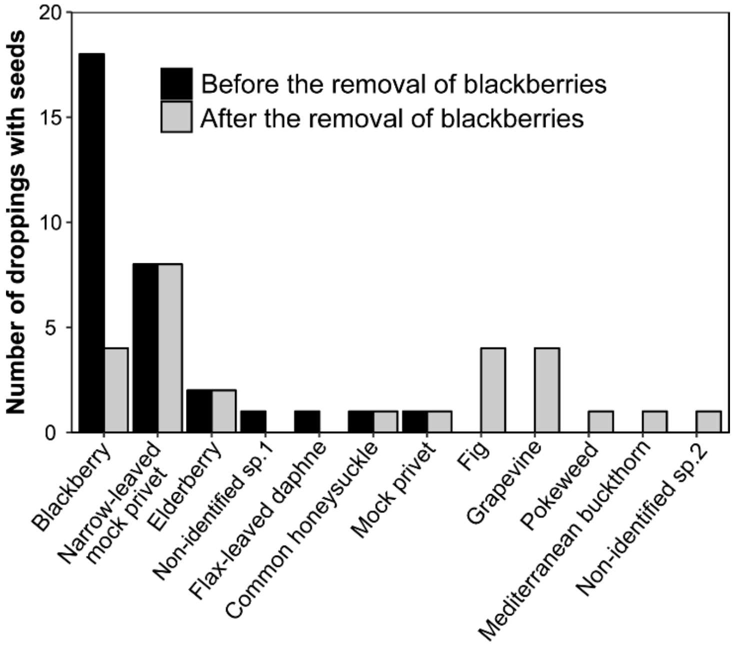

Figure 9 - Number of bird droppings with seeds (interaction frequency) of each plant species before and after the experimental removal of blackberries.

There were no differences in the overall number of birds captured before and after the removal of blackberries (before vs. after = 62 vs. 65; G-test = 0.035, d.f. = 1, p = 0.852), in the total number of droppings collected (53 vs. 57; G-test = 0.071, d.f. = 1, p = 0.789), nor in the number of droppings with seeds (23 vs. 19; G-test = 0.2, d.f. = 1, p = 0.655). As expected, the number of droppings with blackberry seeds declined significantly after the removal of its fruits (18 vs. 4; G-test = 6.223, d.f. = 1, p = 0.013), whereas there was a considerable, yet non-significant increase in the number of droppings with other seed species (10 vs. 17; G-test = 0.789, d.f. = 1, p = 0.374). The removal of blackberries did not lead to the dominance of any other plant species in the network (Figs. 8 and 9). Five plant species were dispersed only after the removal, most notably fig and grapevine, with four interactions each, but also the Mediterranean buckthorn (*Rhamnus alaternus*), pokeweed (*Phytolacca americana*), and an unidentified seed species (Fig. 9). The main blackberry disperser before its removal (robin) did not disperse any new plant species but increased the dispersal frequency of the previously dispersed species (narrow-leaved mock privet) present in 5% and 25% of the droppings of robins before and after the removal of blackberries, respectively (Fig. 8). Of the three bird species that dispersed only blackberries before its removal, two species (blue tit and pied flycatcher) did not disperse any seed while one (blackbird) started to disperse the narrow-leaved mock privet after the removal of blackberries (Fig. 8).

In general, extinction simulations with rewiring provided better predictions of the observed network topology than those without rewiring (Fig. 10). Both observed network specialization H_2’ and weighted connectance were relatively well predicted by the simulations with rewiring (Fig. 10A and B). On the contrary, nestedness (weighted NODF) was underestimated, with both simulation scenarios rendering similar estimations (Fig. 10C).

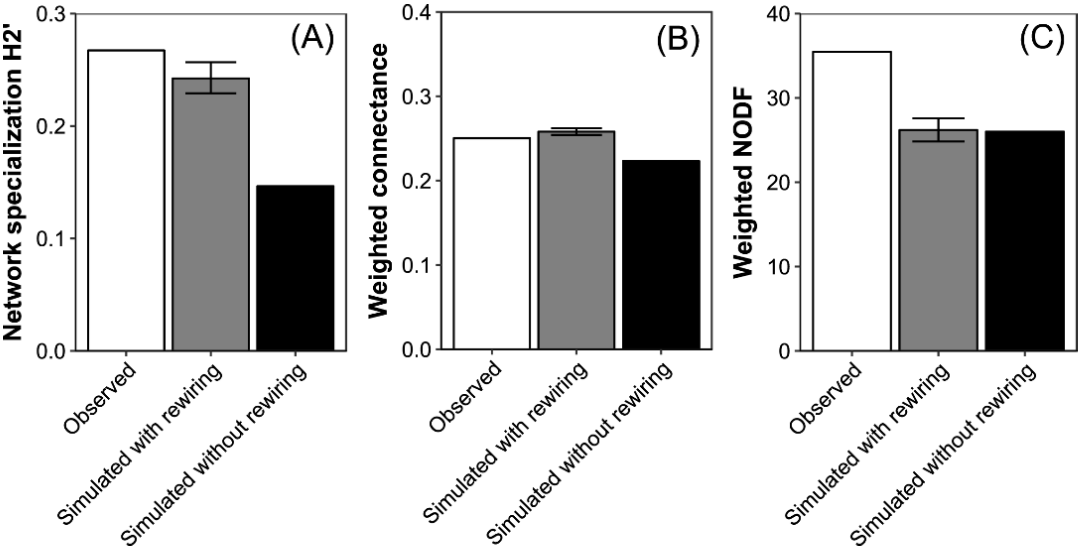

Figure 10 - Network-level descriptors of the observed network after the removal of blackberry fruits and of secondary extinction models with and without rewiring: (A) network specialization H_2’, (B) weighted connectance, and (C) nestedness (weighted NODF). The figures corresponding to the simulation with rewiring depict the mean of 100 runs with the error bars indicating the 95% bias-corrected and accelerated (BCa) bootstraped confidence intervals of the simulations with rewiring.

Overall, both simulation scenarios provided poor predictions for the observed species-level descriptors (Fig. 11). Predicted species strength was highly inaccurate for most species, with simulations without rewiring performing slightly better than those with rewiring for three bird species (Fig. 11A). Specialization d’ was accurately predicted for only two species: blackcap with rewiring and garden warbler without rewiring (Fig. 11B). Simulations without rewiring also performed better in the estimation of the number of dispersed seed species other than the blackberry, producing good estimates for three bird species (Fig. 11C).

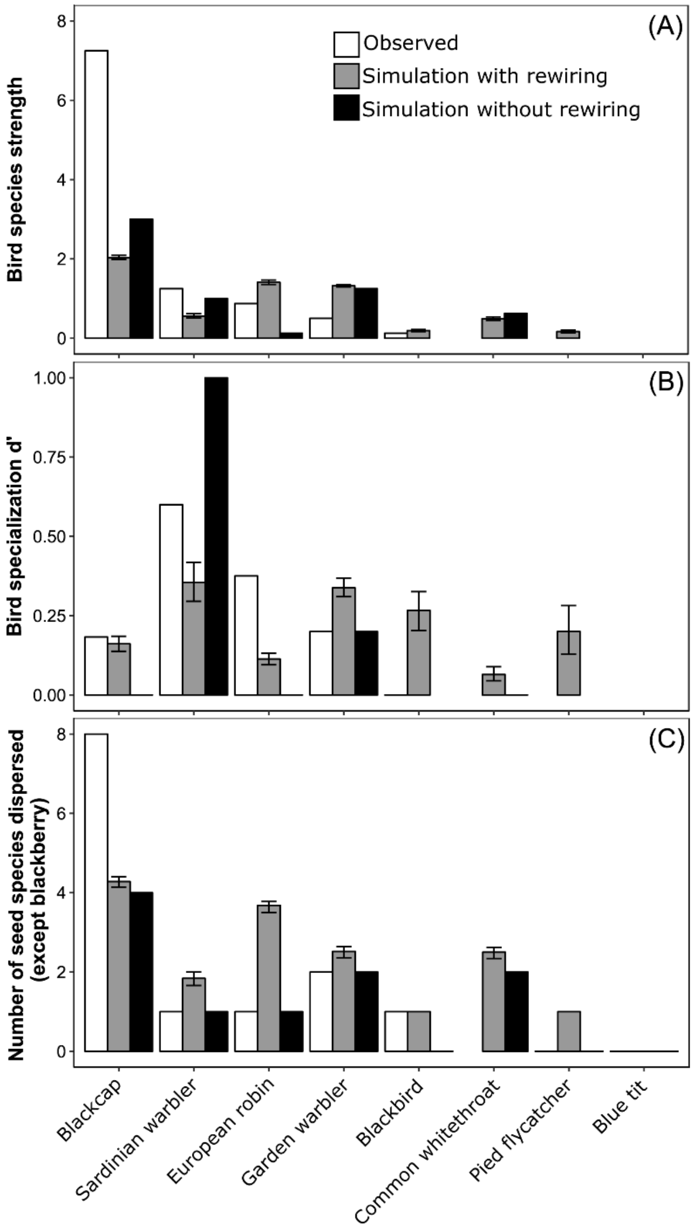

Figure 11 - Species-level descriptors of the observed interactions after the experimental removal of blackberry fruits and of secondary extinction models with and without rewiring: (A) bird specialization d’, (B) species strength, and (C) number of dispersed seed species, other than blackberry. Bird species are ordered according to their observed species strength. The figures corresponding to the simulation with rewiring depict the mean of 100 runs with the error bars indicating the 95% bias-corrected and accelerated (BCa) bootstraped confidence intervals of the

simulations with rewiring. Missing bars correspond to missing data while those represented by a horizontal line correspond to observed values of zero.

Discussion

Here, we characterized avian seed dispersal during the peak of the fruiting season which coincides with the autumn bird migration through Portugal and show that blackberry, a native species, is by far the most important fruit resource across the country. The experimental removal of blackberries from one experimental site did not significantly affect bird community composition and induced a small increase on the dispersal of co-occurring fleshy-fruits. The changes at the community-level were better predicted by a simulation model that allowed interaction rewiring, although the disparate responses of each bird species to the removal of blackberries hampered the accurate prediction of species-level roles.

Characterization of the seed dispersal networks

The vast majority of the interactions were performed by five bird species that were recorded in almost all sites, four of these are known for being among the most important seed dispersers in the Mediterranean region: robin, blackcap, and the garden and Sardinian warblers (Herrera 1995). We also detected a number of typically non-frugivorous bird species dispersing fleshy-fruited seeds (e.g. the insectivorous great spotted woodpecker *Dendrocopos major* and the granivorous chaffinch *Fringilla coelebs*) as well as granivorous species dispersing undamaged seeds from herbaceous plants (e.g. Spanish sparrow *Passer hispaniolensis*). These findings add up to those of other studies that have empirically demonstrated the existence of a continuum in the contribution of frugivorous and non-frugivorous birds to seed dispersal (Heleno *et al.* 2011; Costa *et al.* 2014).

The 17 seed dispersal networks reported here clearly show that the blackberry is consistently the most important fruit resource for birds during the autumn migration across Portugal. The consumption and dispersal of its seeds by birds has been often documented (e.g. Jordano 1982; Jordano 1984; Debussche & Isenmann 1989; Fuentes

1994), but the spatial consistency of its importance (sampling sites with a longitudinal distribution of more than 500 km across the Iberian Peninsula) was still unappreciated. Blackberry seeds were dispersed by a variety of bird species displaying a gradient of dependence on fruits, including typical frugivores, e.g. garden warbler, mainly granivores, e.g. rock bunting (*Emberiza cia*), and mostly insectivores, e.g. blue tit. Such importance may be partly explained by the composition of its fruits, rich in water and non-structural carbohydrates that are easily metabolized, making it a valuable water and energy source for many birds (Jordano 1982; Herrera 1987). Additionally, its abundance and morphology, composed of small drupelets, make the fruits of blackberry a valuable resource for a variety of bird species.

Experimental removal of the most dispersed plant species

Five plant species were only dispersed after the removal of blackberries, chiefly the fig (*Ficus carica*) and the grapevine (*Vitis vinifera*). These plants were dispersed by blackcap, and the garden and Sardinian warblers, all of which dispersed blackberry seeds before their removal, suggesting rewiring towards these plant species. Contrary to our expectations, the main blackberry disperser in this site (robin) did not disperse any new plant species after the removal of its fruits but increased the dispersal frequency of other previously dispersed species. This result suggests that the proportion of the robin's diet previously filled by blackberries was slightly diverted to fruits of narrow-leaved mock privet. The robin is an omnivore species that includes a large fraction of invertebrates in its diet along with fleshy fruits (Debussche & Isenmann 1985; Heleno *et al.* 2013b) and it did not leave the study site, so it probably compensated the loss of blackberries also by increasing the consumption of the previously consumed fruit species and probably also non-fruit resources. Similarly, all the other bird species found in this study are not exclusively frugivorous, they may also divert their foraging effort not only to other fruit species but also to non-fruit items, namely invertebrates. If this was the case, their functional role as seed dispersers may have been reduced even though bird population were largely unaffected by the loss of an abundant fruit resource. Unfortunately, we did not quantify the non-fruit component of the bird diet in this study. Nevertheless, ignoring the ecological plasticity of omnivores is likely to undermine the predictions of secondary extinction models as they can supply their energetic demands by shifting into alternative sources (Blüthgen 2010; Schleuning *et al.* 2016; Timóteo *et al.* 2016).

Both specialization H_2' and weighted connectance after the removal of the blackberries were considerably better predicted by extinction simulation models that incorporate rewiring. Nestedness (weighted NODF), however, was highly underestimated by both types of models, only with a negligible improvement in models that allowed interaction rewiring. In ant-seed dispersal networks, other observed descriptors such as network robustness and seed species richness were largely underestimated by extinction simulations with rewiring (Timóteo *et al.* 2016). Extinction simulations without rewiring also failed to predict network specialization H_2' in a pollination study, where flowers had been manually removed (Goldstein & Zych 2016). These results suggest that when accounting for rewiring, some network-level descriptors may be better predicted than others, while without rewiring, all network descriptors are poorly predicted.

Regarding the individual role of the species (i.e. species-level descriptors), simulations with and without rewiring performed similarly. Such poor performance is also a reflection of the difficulty in estimating specific feeding choices with simple algorithms and that more complex rewiring rules might be necessary for greater accuracy in estimating species roles after environmental disturbances (Olito & Fox 2015; Trøjelsgaard & Olesen 2016). The rearrangement of interactions after one species declined is intrinsically difficult to predict since it requires more data than just the presence of a potential partner species. An important limitation of current rewiring protocols is that they often only redistribute the lost interactions through resources that were already known to be consumed locally, not allowing the establishment of new interactions (Rumeu *et al.* 2017). Here we constrained rewiring to any of the pairwise interactions that had been observed in any of the 17 networks sampled across the country. Such an extensive background pool of potential interactions is an improvement over models that simply redistribute the lost interactions across the local resources without accounting for the identity of potential interacting partners. However, at the experimental site after the removal of blackberries, we recorded interactions that, despite being observed at any of the 17 sites, were not considered in rewiring simulations because those plant species were not observed at that site. Accordingly, we observed new, previously unrecorded plant species being dispersed after the experimental treatment by some important disperser species (e.g. blackcap), while others (e.g. robin) exhibited a lower plasticity than expected by not dispersing any new plant species. This constraint may have hindered more accurate predictions of bird species roles after the removal of blackberries and highlight the need of an exhaustive list of all local available plant species. Factors that are also known to shape bird-fruit interactions and ultimately rewiring dynamics include availability of alternative food resources, inter-specific competition, both bird and plant species abundances, bird preferences for some fruit species over others due to characteristics such as fruit nutritional content, and temporal, spatial, and morphological trait matching (Martin 1985; Levey & Martínez del Rio 2001; Burns 2006; Jordano 2014; Jordano 2016). Moreover, these factors may vary across space (Poisot *et al.* 2014), such as the intra-specific variability in fruit size or bird gape width, constraining potential interactions across the landscape (González-Varo & Traveset 2016). The incorporation of all these interaction drivers, e.g. by the inclusion of more refined rewiring probabilities based on species traits or abundances (Ramos-Jiliberto *et al.* 2012), hold a great potential to improve the realism of rewiring simulations, and particularly to predict species topological roles after disturbances (Stang *et al.* 2006; Santamaría & Rodríguez-Gironés 2007; Kaiser-Bunbury *et al.* 2014).

The second part of our study (manipulative experiment) was limited to one site due to the logistical constraints of the experiment, namely to accumulate sufficient sampling hours with enough mist-nets and to remove all blackberries within 100 metres from the nets. A two-week sampling period was chosen to optimize the trade-off in data quality regarding the effort to satisfactorily sample the network to overcome potential differences caused by the advance of plant fruiting phenology and bird migration. Despite the short sampling period, we are confident that the two networks (before and after) reliably show the effect of the removal of blackberries. A similar constraint occurred in the first part of our study, where a sampling effort of five days, which was selected based on a previous study in this system showing that five days was enough to record most species of birds and dispersed seeds (Costa *et al.* 2016), might have missed some rare interactions, such as those with abundant fleshy fruits that were not dispersed at any site (blackthorn *Prunus spinosa*, hawthorn, prickly juniper *Juniperus oxycedrus*, and *Rosa* spp.). However, these fruits are hardly consumed until the end of the fruiting season, making them unimportant resources in early September (when sampling took place) for frugivorous birds. Nevertheless, our sampling allowed the detection of several rare interactions involving non-frugivorous bird species and small dry fruits.

Although similar studies are still scarce, there is an emerging consensus that accounting for interaction rewiring increases the accuracy of species extinction simulations. Recent evidence shows that network architecture can respond very differently depending on the identity and abundance of the extinct and remaining species (Rumeu *et al.* 2017). Network descriptors are known to be relatively stable to species turnover (Petanidou *et al.* 2008), rendering network-level descriptors easier to estimate with simple rewiring rules. Nevertheless, studies where alien species were experimentally removed showed different levels of plasticity at the level of network structure (Heleno *et al.* 2010; Ferrero *et al.* 2013; Kaiser-Bunbury *et al.* 2017). Therefore, detailed knowledge of each study system is invaluable for accurate predictions regarding community responses to local extinctions. To date, the few studies that experimentally evaluated community rearrangement are limited to the removal of a single species (Heleno *et al.* 2010; Brosi & Briggs 2013; Goldstein & Zych 2016; Timóteo *et al.* 2016; Hallett *et al.* 2017, this study). Collectively, these studies show that rewiring occurs when a highly connected species goes extinct, but also that rewiring patterns are hard to model, and this difficulty is likely to increase greatly when simulating the consequences of multiple species extinctions.

Chapter IV

Species activity promotes the stability of fruit-frugivore interactions across a five-year multilayer network

Costa J.M., Ramos J.A., Timóteo S., da Silva L.P., Timóteo S., Ceia R.S. & Heleno R.H. Species activity promotes the stability of fruit-frugivore interactions across a five-year multilayer network. Published as a preprint in bioRxiv. https://doi.org/10.1101/421941

Abstract

While biological communities are intrinsically dynamic, with species and interactions changing over time, interaction network analyses are mostly static. We implemented a temporally explicit multilayer network approach to explore the changes on species roles and on the emergent structure of a seed dispersal network over five years. Network topology was relatively constant, with four interaction modules spanning across all years. Species that were present on more years were also disproportionally important on each year, forming a core of temporally reliable species that are critical to the cohesiveness of the multilayer network structure. We propose a new descriptor termed *species activity* reflecting the number of temporal, spatial or functional layers that each species integrates, providing a simple and powerful index of species importance for multilayer network cohesion.

Keywords: birds, ecological networks, fleshy fruits, mist-netting, multilayer networks, seed dispersal, species activity

Introduction

The structure of ecological communities, reflected in the complex network of biotic interactions that connects all organisms and species, is intrinsically dynamic. Such dynamics may directly emerge from temporal changes on species composition (species turnover), switch in animal feeding preferences (rewiring), relative species abundances, and availability of suitable resources (phenological matching), such as flowers and fruits (Olesen *et al.* 2010; Burkle & Alarcón 2011; Trøjelsgaard & Olesen 2016). Although the dynamic nature of species interactions is widely recognized (Olesen *et al.* 2010; Trøjelsgaard & Olesen 2016), most studied networks to date are constrained to relatively short time scales. Several studies started to explore the temporal variability of species interaction networks across seasons and years, mostly focusing on plant-pollinator (Petanidou *et al.* 2008; Dupont *et al.* 2009; Chacoff *et al.* 2018) or on plant-frugivore interactions (Carnicer *et al.* 2009; González-Castro *et al.* 2012b; Ramos-Robles *et al.* 2016). Although these studies provide useful information about structural community changes across time, they still inevitably aggregate all observed interactions into a few formally disconnected time-slices, likely providing an incomplete perception of true temporal dynamics (Blonder *et al.* 2012; Pilosof *et al.* 2017). The tool to circumvent this limitation has recently became available, particularly with the implementation of a multilayer network approach where interdependencies between time-ordered layers (i.e. networks) are explicitly incorporated in the analysis by quantifying the strength of interlayer links that connect the same nodes (species) across multiple layers (Pilosof *et al.* 2017; García-Callejas *et al.* 2018; Timóteo *et al.* 2018). By independently quantifying intra- and interlayer strength, multilayer networks are a most powerful tool to explore meta-community dynamics, improving the realism of species interaction networks naturally spanning across multiple spatial, temporal or functional (e.g. herbivory, parasitism) layers (Pilosof *et al.* 2017; Timóteo *et al.* 2018).

For most plants, seed dispersal represents a key stage in their life-history, through which they can move away from mother plants and colonize new habitats (Traveset *et al.* 2014). Birds are critical seed dispersers across most terrestrial ecosystems, largely influencing habitat structure and long-term vegetation dynamics (Jordano 2014; Wenny *et al.* 2016). Over the recent decades our understanding of the organization of plant-frugivore interaction networks has improved tremendously (Jordano *et al.* 2007; Donatti *et al.* 2011). In Southern Europe, a peak in seed dispersal intensity occurs during late-summer and early-autumn, where the production of fleshy fruits coincides with the post-breeding bird migration (Herrera 1984). Although both fruit production and bird migration occur every year, allowing the regular encounter between fruits and dispersers, there might be important fluctuations in their abundance and diversity (Herrera 1998). Surprisingly, we still do not know how these fluctuations affect the persistence of plant-frugivore interactions across years, hindering current understanding of long-term community dynamics (Estes *et al.* 2018). Furthermore, the lack of such a long-term perspective is unanimously recognized as a major limitation of current understanding of biodiversity – ecosystem services relationships as a whole (Tilman *et al.* 2014).

Here, we implemented a temporal multilayer network approach to explore the variability of seed dispersal interactions across five consecutive years in central Portugal. We aimed to (A) characterize and identify the drivers of species and interactions turnover; (B) investigate the relationships between short- and long-term species topological roles; and (C) explore the drivers of temporal changes in emergent network properties.

Material and Methods

Experimental approach

For five consecutive years, we reconstructed the network of interactions between birds and fleshy-fruited plants on a secondary native forest in Central Portugal (40º19’N; 8º24’W). The site is under a typical Mediterranean climate and is dominated by *Quercus faginea, Arbutus unedo*, and *Pinus pinaster* with a dense and diverse understory dominated by native fleshy-fruited shrubs, such as *Pistacia lentiscus*, *Crataegus monogyna*, *Rhamnus alaternus*, *Rubus ulmifolius*, and *Viburnum tinus*.

Interaction networks were assembled by identifying entire seeds on the droppings of mist-netted birds captured under two complementary designs: (A) sampling all days with favourable weather conditions during the month of September between 2012 and 2016 (corresponding to the predicted peak of seed dispersal); and (B) sampling fortnightly between January and December 2013 to evaluate the intra-annual richness of pairwise seed-bird interactions. On each day, birds were captured with mist-nets (total length = 102 m), operated during the first five hours after dawn, and placed in individual cotton bags until they defecate or up to 30 minutes. All droppings retrieved from the bags were air-dried and the undamaged seeds were later extracted, counted and identified under a dissecting microscope with the aid of a seed reference collection. Interaction frequency was quantified as the number of droppings of bird species *i* containing undamaged seeds of plant species *j*. This measure was used because it is more likely to reflect recruitment probability after post-dispersal density-dependent mortality of seeds deposited in the same dropping. The overall effort resulted in 635 sampling-hours distributed along 25, 17, 20, 21, and 20 days in September 2012 to 2016, respectively, and 120 hours in 24 additional days across the entire year of 2013. Sampling completeness was estimated for each year as the proportion of plant and bird species observed relatively to those estimated by the Chao2 richness estimator (Chao 1987) implemented in program EstimateS 9.1 (Colwell 2013). Additionally, fleshy-fruit availability was estimated by counting all ripe standing fruits along three linear transects (each: 25m x 2m) running parallel to the mist-nets and resampled each year in early-, mid-, and late-September. Unless stated otherwise, all results are presented as mean ± standard deviation.

Interannual turnover of species and links

The interannual turnover of bird and plant species was assessed with the Whittaker beta diversity index (β_W) adapted by Koleff *et al.* (2003), which varies between 0 (similar) and 1 (completely dissimilar species composition). The turnover of links was evaluated with package betalink (Poisot 2016) in R (R Core Team 2017), following the approach outlined in Poisot *et al.* (2012), which allows the partition of link turnover (β_{WN}) between networks into two driving mechanisms: those attributed exclusively to species turnover (β_{ST}) and those attributed to the rewiring of interactions between co-occurring species (β_{OS}).

Relationship between intra- and interannual species topological roles

To characterize the regularity of species across the five years, we propose a new species-level index in the context of ecological multilayer networks, which we coined "species activity", and quantifies the number of layers (here: years) in which each species interacts (i.e., the number of layers with activity of each species). This descriptor is a direct extension of the concept of "node activity" used in physics to reflect the number of layers where the nodes of multiplex networks are "active" (Nicosia & Latora 2015). We then evaluated how species activity is related with species topological importance in each year, by computing three monolayer species-level descriptors: (A) degree, i.e., the number of mutualistic partners; (B) species strength, an estimation of the cumulative importance of each species for the species on the other trophic level (Barrat *et al.* 2004); and (C) specialization d', quantifying species selectivity in relation to resource availability (Blüthgen *et al.* 2006). Additionally, we also evaluated the relationship between species activity and species versatility, a descriptor of multilayer centrality, expressing the sum of the importance of the partners of species *i*, both within and between layers (De Domenico *et al.* 2015b; Timóteo *et al.* 2018). Species versatility was computed using the PageRank algorithm (Brin & Page 2012) adapted to a multilayer scenario (De Domenico *et al.* 2015b) and available in program muxViz (De Domenico *et al.* 2015a). This was done separately for bird and plant species, based on unipartite projections of the original networks using the Newman's method (Newman 2001) adapted for weighted networks (Opsahl 2013) with the R package tnet (Opsahl 2009).

In order to assess if plants are dispersed proportionally to their abundance on each year, we calculated the Kendall's tau rank correlation test, available from the R package Kendall (McLeod 2011), between the abundance of fleshy-fruits of each species in the transects and their respective interaction frequency. The effect of species activity on mean species degree and strength was assessed with generalized linear mixed models (GLMM) with Poisson and Gamma distributed errors, respectively. In order to control for the effect of variable network sizes, the number of species on the other trophic level (i.e. number of plant species for bird degree and vice-versa) was included as an offset variable in the Poisson GLMM. The relationship between species activity and species specialization d' was modelled with linear mixed models (LMM). All mixed models were fitted with the R package lmer4 (Bates *et al.* 2015). and included year as a random factor. The relationship between species versatility and species activity was assessed with generalized linear models (GLM) with Gamma distributed errors.

Interannual community structure

Changes in the emergent structure of the seed dispersal network were evaluated by calculating four key network-level descriptors: (A) connectance, the proportion of observed/ possible links (Jordano 1987); (B) network specialization H_2', measuring the community-level selectiveness of the observed interactions as a departure from a random (i.e., abundance-based) association pattern (Blüthgen *et al.* 2006); (C) weighted-interaction nestedness (WIN) (Galeano *et al.* 2009), quantifying how interactions are hierarchically organized (i.e. nested) around a core of the most generalist species (Bascompte *et al.* 2003); and (D) modularity, which identifies and quantifies the existence of groups of tightly interacting species, loosely linked to the remaining network (Olesen *et al.* 2007). To compute modularity, we employed an explicit multilayer approach where we included interlayer links connecting the same species occurring in consecutive years. These links were quantified as the change in each species relative abundance between consecutive layers (i.e. abundance i_{t+1} / abundance i_t) (see also Pilosof *et al.* 2017), where bird abundances correspond to the mean number of birds captured, and plant abundances corresponds to mean fruit availability in the transects. When plant species were found in the bird droppings but not in transects, these were attributed the lowest availability score (i.e. 1 fruit/transect), under the rationale that those fruits need to be available in order to be consumed but are probably locally rare. Modularity was maximized with a generalized Louvain algorithm (Blondel *et al.* 2008), implemented in MATLAB (The MathWorks, Inc., Natick, Massachusetts, USA) using code provided in Jutla *et al.* (2014) and modified by Pilosof *et al.* (2017) to account for the bipartite nature of the multilayer network (see also Timóteo *et al.* 2018). The significance of each descriptor was then assessed by comparing it with those obtained for 1000 randomized networks generated by a null model based on the Patefield´s algorithm (Patefield 1981), which randomly reshuffles the interactions across the matrix while constraining marginal totals. Each descriptor was considered significantly different from a random expectation if the respective z-score was lower than -1.96 or higher than 1.96, corresponding to a significance level of 0.05 (Trøjelsgaard *et al.* 2015). The randomized networks to compute modularity significance were obtained with the R package vegan (Oksanen *et al.* 2015). All other network-level descriptors and respective null-models were obtained with package bipartite (Dormann *et al.* 2008; Dormann *et al.* 2009).

Results

Throughout 2013 (fortnight sampling) we captured 671 birds from 30 species, whose 202 droppings contained 537 undamaged seeds from 16 plant species. September was the month with a greater diversity of links between fleshy-fruited plants and birds, with 15 out of the 40 links being detected in this month (Supplementary Materials: Fig. S4).

Overall, the daily sampling throughout September from 2012 to 2016 resulted in the capture of 1620 birds (30 species), of which 454 (12 species) dispersed 2133 undamaged seeds from 17 plant species, rendering a total of 75 links (Fig. 12). Estimated sampling completeness was very high for both plants and birds, with an annual mean of 93% (Min.= 90%; Max.= 98%) and 92% (Min.= 89%; Max.= 100%) of species detected, respectively.

Five plant species (*Ficus carica*, *Rhamnus alaternus*, *Rubus ulmifolius*, *Smilax aspera*, and *Vitis vinifera*) and six bird species (*Erithacus rubecula*, *Ficedula hypoleuca*, *Sylvia atricapilla*, *S. borin*, *S. melanocephala*, and *Turdus merula*) were detected on the five years of the study (Figs. 12 and 13). These species accounted respectively for 29% of the fruit production and 50% of the individual birds captured in September across the five years. Mean species turnover between consecutive years (β_W) was higher for plants than for birds (0.31 ± 0.12 and 0.16 ± 0.07, respectively).

Nine out of the 75 links detected (12%) were observed in all years, accounting on average for 49% (Min.= 30%; Max.= 63%) of the interactions detected in each year. The turnover of links was greater than that of plant and animal species (β_{WN}= 0.53 ± 0.10) and mostly driven by interaction rewiring, i.e. by the detection of new links between species already co-occurring in previous years (β_{OS}/β_{WN}= 70% ± 14%) with a lower contribution of species turnover (β_{ST}/β_{WN}= 30% ± 14%).

There was no significant correlation between fruit abundance and the frequency of interactions in any year (τ_{2012}= 0.12, p= 0.74; τ_{2013}= 0.60, p= 0.18; τ_{2014}= 0.53, p= 0.07; τ_{2015}= 0.44, p= 0.17; τ_{2016}= -0.32, p= 0.63). Both plant and bird species activity were positively related to their respective mean degree (β_{plants}±SE= 0.37 ± 0.09, χ^2= 14.76, p < 0.01; β_{birds}±SE= 0.68 ± 0.14, χ^2= 24.45, p < 0.01; Fig. 13), mean species strength (β_{plants}=

0.23 ± 0.04, χ^2= 40.17, $p < 0.01$; β_{birds}= 0.94 ± 0.11, χ^2= 75.56, $p < 0.01$; Fig. 13), and versatility (β_{plants}= 0.41 ± 0.06, χ^2= 52.77, $p < 0.01$; β_{birds}= -1.62 ± 0.18, χ^2= 78.81, $p < 0.01$; Fig. 13). In contrast, plant and bird specialization d' were not associated with species activity (β_{plants}= 0.04 ± 0.02, χ^2= 3.68, p= 0.06; β_{birds}= 0.01 ± 0.02, χ^2= 0.19, p= 0.66; Fig. 13).

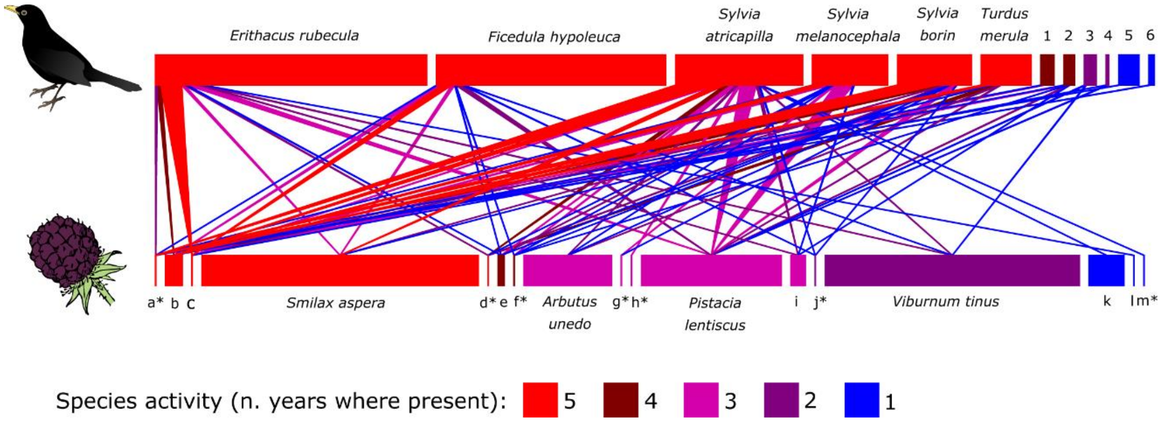

Figure 12 - Overall seed dispersal network recorded during September across five consecutive years on a secondary native forest in central Portugal. Species are ordered from highest to lowest species activity, i.e. number of years where present. The width of the boxes representing plant and bird species is proportional to the number of fruits counted along linear transects and to the number of birds captured with mist-nets, respectively. Asterisks (*) represent seed species recovered from bird droppings but not detected in the transects. 1 – *Cyanistes caeruleus*, 2 – *S. communis*, 3 – *Muscicapa striata*, 4 – *S. undata*, 5 – *Chloris chloris*, 6 – *Dendrocopos major*; Plants: a – *Ficus carica*, b – *Rhamnus alaternus*, c – *Rubus ulmifolius*, d – *Vitis vinifera*, e – *Phillyrea latifolia*, f – *Solanum nigrum*, g – *Daphne gnidium*, h – *Lonicera periclymenum*, i – *Rubia peregrina*, j – *Phytolacca americana*, k – *Crataegus monogyna*, l – *Olea europaea*, m – *Phillyrea angustifolia*.

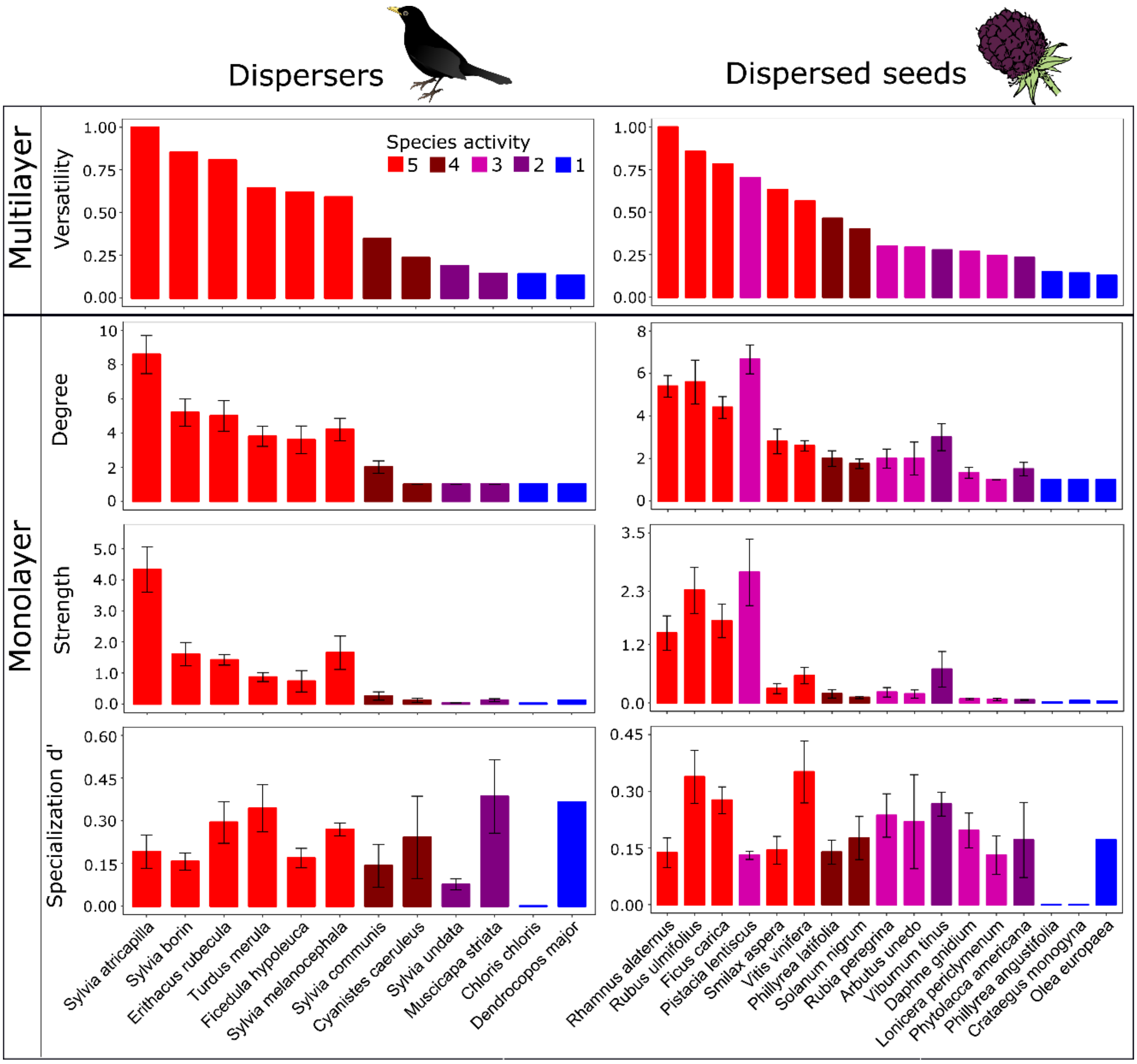

Figure 13 - Topological descriptors of species roles on avian seed dispersal networks compiled over five years. The top panel corresponds to species roles on a temporal multilayer network, while the monolayer panels reflect average species roles across the yearly networks where each species occurs. Error bars represent the standard error for each descriptor across the five years. Bars without error bars correspond to species with no interannual variation to a given descriptor. Species are ordered according to their multilayer versatility (top).

Overall, the topology of the networks was relatively constant throughout the study (Supplementary Materials: Fig. S5). The network was significantly less connected (z_{2012}= -5.12; z_{2013}= -2.97; z_{2014}= -2.15; z_{2015}= -3.31; z_{2016}= -4.51) and more specialized (z_{2012}= 9.67; z_{2013}= 3.90; z_{2014}= 3.16; z_{2015}= 5.86; z_{2016}= 8.14) than predicted by the null models in all years. The network also tended to be significantly nested, which happened in all years except in 2014, when observed nestedness was indistinguishable from a random interaction pattern (z_{2012}= 4.22; z_{2013}= 3.43; z_{2014}= 0.93; z_{2015}= 3.91; z_{2016}= 5.41). The multilayer network was significantly more modular than expected by chance (Q_{obs}= 0.50, mean Q_{null}= 0.43, z= 10.3), and formed by four interaction modules (Fig. 14) that spanned across the five years of the study. Most bird species (8 out of 10 species, 80%) were consistently allocated into the same module across all years. Plants had a lower temporal constancy regarding their module affiliation, with 9 out of the 14 plant species (64%) remaining in the same module across all years.

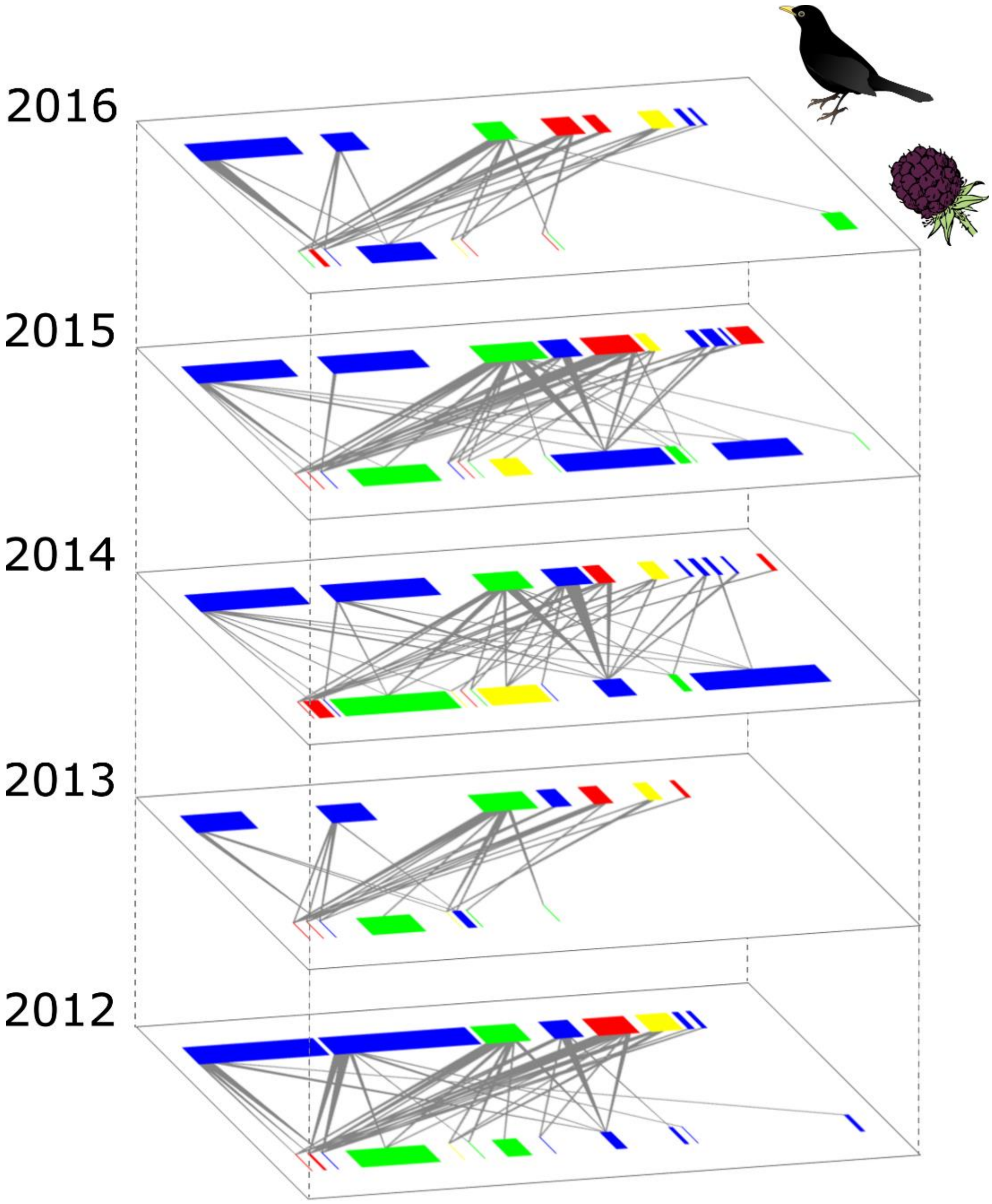

Figure 14 - Interannual module affiliation of species across a five-year temporal multilayer seed dispersal network. Colours represent different interaction modules. All networks are represented

on the same scale and species are ordered as in figure 12. The width of the boxes representing plant and bird species is proportional to the number of fruits counted along linear transects and to the number of birds captured with mist nets, respectively.

Discussion

Here, we show that the diversity of seed dispersal interactions between birds and fleshy-fruited plants in Portugal is particularly high in September, when a peak in fruit production coincides with the passage of most migratory bird species. We compiled seed dispersal interactions during this activity peak for five consecutive years to reconstruct a temporal multilayer network, quantifying intra- and interlayer (i.e. year) link strength. We found that the species present in more years (here said to have a greater species activity) also tend to be more important in each year, independently of their abundance. The emergent structure of the seed dispersal network was very stable through time and included four well-defined interaction modules spanning across all years of the study. Altogether, our results suggest that the temporally most reliable species, which are not necessarily the most abundant, tend to occupy central roles in the seed dispersal network across multiple temporal scales, thus providing a mechanism for network stability and increasing the reliability of the seed dispersal service.

Species activity is a species-level multilayer descriptor that quantifies the role of each species as connectors in the multilayer network system, although it can also be used in a monolayer context (i.e. when inter-layer link strength is undefined). For example, this index has been implicitly used to explore the persistence of species across years in flower-visitor networks (Petanidou *et al.* 2008; Olesen *et al.* 2011b), across months in bird-fruit networks (Yang *et al.* 2013; Ramos-Robles *et al.* 2016), and across multiple habitat layers in seed dispersal networks (Timóteo *et al.* 2018). However, until now it was not properly described. This simple descriptor, naturally related to what Olesen *et al.* (2011b) named “temporal persistence”, is very intuitive and might prove useful in situations where interaction networks are structured across not only temporal, but also spatial or functional multiple layers. Species activity is likely to prove most advantageous given the strong emphasis to integrate multiple ecological processes, such as bellow-aboveground interactions, multitrophic interactions, or mutualistic-antagonistic networks (García-Callejas *et al.* 2018).

The most important bird and plant species in terms of species strength, number of links (i.e. degree), and multilayer centrality (i.e. versatility), were those with higher species activity. These birds (e.g. *Sylvia* spp., *E. rubecula*, and *T. merula*) are highly frugivorous during this period of the year and are among the most important avian seed dispersers in the Mediterranean basin (Herrera 2001). Therefore, their importance was not surprising as birds with a high degree of frugivory are fundamental to the structure of bird-fruit networks (Sebastián-González 2017). The birds with lower species activity and lower importance to the seed dispersal network mainly include granivorous and insectivorous species that occasionally dispersed seeds. As for plants, although the most important species in each year also tended to be those with higher species activity, one topologically important species in the seed dispersal network was not temporally reliable: *Pistacia lentiscus* (Fig. 12). This species produces small and abundant lipid-rich fruits which are highly consumed by several bird species (Olesen *et al.* 2011a), but its fruits were only ripe during the study period in three of the years. Its absence in two networks was the result of a delay in the maturation of its fruits because it had unripe fruits during the sampling period. Nevertheless, when available, they were one of the most important fruit resources for birds. As some plants are known for highly variable fruit crops or fluctuations in the timing of fruit ripening (Herrera 1998), species activity may be a more accurate indicator of species importance for birds than for plants. Overall, these results indicate that bird species with high species activity tend to be more reliable seed dispersers. In turn, plants with high species activity provide reliable resources for several bird species, namely migrants that rely on fruits to increase their body fat reserves for the migratory flights (Bairlein 2002). Interestingly, there seems to be an independent relationship between species activity and plant and animal specialization d’. This is probably due to the generalist nature of most seed dispersal communities (Blüthgen *et al.* 2007), suggesting that the greater importance of temporally reliable species is not a reflection of a lower selectivity for resources.

Only six (50%) bird species and five (29%) plant species were present in all years; a similar relationship was observed in species turnover between years. These results contrast with previous findings of pollination studies interannual turnover which tends to be greater for animal than for plants (Petanidou *et al.* 2008; Dupont *et al.* 2009; Cirtwill *et al.* 2018). Part of this turnover might be related with the timing in fruit ripening of certain plant species, as already described here for *P. lentiscus*. The bird and plant species with higher species activity tend to dominate the network in terms of diversity and frequency of interactions. Consequently, the establishment of new interactions between temporally persisting species (i.e., rewiring) seems the main driver of interannual interaction turnover. However, it is at this point difficult to distinguish true rewiring (i.e., new interactions taking place) from a normal undersampling of rare interactions in certain years (i.e., interactions that do occur but are not detected). Only nine links (12%) were observed on all years, indicating a low temporal link persistence. As observed in pollination studies (Chacoff *et al.* 2018), those few links were disproportionally frequent, suggesting that birds might predictably prefer to interact with the most temporally reliable plant species.

Our study revealed a relatively stable interannual network structure, with the noteworthy exception of 2014, when the network was not significantly nested. While the stability of seed dispersal network structure across seasons has already been noted (Plein *et al.* 2013). Our study suggests that such stability can also be extended to interannual network structure, despite an appreciable species turnover, following the patterns observed in pollination networks (Petanidou *et al.* 2008; Dupont *et al.* 2009). However, our study suggest that species roles tend to be temporally conserved in seed dispersal networks, in contrast with pollination systems (Cirtwill *et al.* 2018). Our five-year study also shows that, as expected on any ecological process, not all years are exactly equal and that extrapolations based on temporally restricted sampling (such as nestedness in 2014) may lead to a biased characterization of network structure (Estes *et al.* 2018).

The identification of tight interaction modules within the relatively loose interaction networks has been one of the most insightful advances in community ecology of the last decade (e.g. Olesen *et al.* 2007; Schleuning *et al.* 2014b). However, most ecological processes, including seed dispersal, are continuous and not constrained by rigid temporal or spatial windows, likely affecting module detection. A multilayer modularly detection algorithm that is not constrained to seasonal or yearly data and where modules can span across multiple temporal or spatial layers, is likely to bring us much closer to the reality of natural communities (Mucha *et al.* 2010; Pilosof *et al.* 2017). This approach was used here for the first time to detect temporal seed dispersal modules that are very stable across years, with most species, especially birds, maintaining their module affiliation throughout time. This stability suggests that not only birds from different modules tend to have distinct fruit preferences, but these preferences tend to be temporally consistent and independent of fruit abundance. Indeed, some abundant plant species were rarely dispersed (e.g. *Arbutus unedo*), suggesting that birds likely select fruits based on other intrinsic traits such as their nutritional composition (Schaefer *et al.* 2003; Morán-López *et al.* 2018). Furthermore, under a multilayer approach, the incorporation of a "historical interaction information" for module detection minimizes the influence of transient species roles and allows the detection of long-lasting modules which may be highly informative for conservation efforts (Blonder *et al.* 2012).

Here, we implemented an innovative multilayer approach to understand the interannual dynamics of seed dispersal networks and identified four temporally coherent interaction modules spanning across five consecutive years. The structure of the seed dispersal network was relatively stable across years despite a significant turnover of species and interactions. Interestingly, the highly mobile birds, some of which migratory, presented a lower interannual turnover than their sessile mutualistic partners (i.e. fruiting plants). More importantly, our results revealed that species present across more years (defined here as having higher species activity) are also the most important on each year, both in terms of link richness and species strength, in a relationship independent from fruit availability and bird or plant specialization d'. Our results suggest that fruit-frugivore interactions are structured around a core of temporally reliable species, with which transient species tend to interact. By formally integrating species interacting on multiple spatial, temporal, or functional layers, multilayer networks are a most promising tool to approximate network analysis to the intrinsic complexity of natural communities.

General Discussion

General Discussion

Baseline knowledge on how ecological communities are structured is crucial to predict and tackle disturbances that may disrupt important ecological functions. In the previous chapters, this thesis focused on exploring the structure of avian seed dispersal networks and their temporal and spatial dynamics. In the current section, I will integrate the main findings of this thesis and propose particularly promising research avenues to expand our knowledge on the structure, functioning, and resilience of seed dispersal networks.

While endozoochory (internal dispersal) by birds is well documented in the literature, data on avian seed dispersal via epizoochory (dispersal by external adhesion) is scarce and almost inexistent for passerines, despite their widespread distribution (Payevsky 2014). The results of the concurrent assessment of both seed dispersal mechanisms by passerines suggested that epizoochory is much rarer than epizoochory, at least during their post-breeding migratory period. These result supports previous evidences that epizoochory is likely rare on passerines (Choi *et al.* 2010; Nogales *et al.* 2012), in contrast with that of water birds, where epizoochory is relatively frequent (Vivian-Smith & Stiles 1994; Figuerola & Green 2002; Aoyama *et al.* 2012). These differences might result, to some extent, from different types of vegetation between habitats preferred by passerines and water birds (e.g. abundance of epizoochorous plants) and also due to the effective mechanism of seed retention on the mud attached to their feet. Interestingly, a seed from a fleshy-fruit (*F. alnus*) was found attached to the feathers of one bird (*T. merula*), which shows that seeds with no adaptations to such dispersal mechanism may also be dispersed via epizoochory and consolidate previous evidences for the ecological relevance of non-standard seed dispersal mechanisms (Higgins *et al.* 2003; Vargas *et al.* 2012). One epizoochorous seed was also found on a migratory bird (*L. naevia*), indicating the possibility of long-distance epizoochory by migratory birds. Previous research showed that migratory birds may disperse other organisms such as fungi (Alfonzo *et al.* 2013) and seeds either via endozoochory (Viana *et al.* 2016a) or epizoochory (Choi *et al.* 2010). These events are likely rare, however c. 2.1 billion birds, mostly passerines, are estimated to migrate during late summer/early autumn from Europe towards Africa (Hahn *et al.* 2009). Therefore, even if only 0.33%

(results from chapter I) of these birds disperse seeds externally, this still represents a large potential for long-distance epizoochory. Clearly, that potential is contingent on the time that seeds remain attached to birds. As such, experimental studies evaluating for how long seeds remain attached to birds, similarly to those assessing seed retention time on mammals' fur (e.g. Couvreur *et al.* 2005), could help to disclose any potential for long-distance dispersal. Additionally, molecular tools may provide valuable information regarding the potential origin of epizoochorous seeds retrieved from birds. Such potential might have important biogeographical and ecological implications such as increasing gene flow between geographically distant plant populations, range expansions, and the dispersal of invasive species (Viana *et al.* 2016b).

The use of a network approach to study ecological communities, such as avian-seed dispersal networks, has become extremely popular in the last few decades due to the development of multiple descriptors that can capture different attributes of emergent network properties. In this thesis, it was assessed how 10 commonly used network descriptors are affected by different levels of sampling intensities when assembling avian seed dispersal interactions. The results show that five days of mist-netting operation are sufficient to detect most bird species and the seeds they disperse. However, eight sampling-days were the minimum sampling effort required to properly characterize the emergent structure of the seed dispersal network. For higher levels of sampling effort, either there was no change in the network descriptor value (connectance, weighted connectance, and network specialization H_2') or that change was minimal (modularity and nestedness). Obviously, such minimal sampling thresholds should not be seen has rigid, as they are expected to vary according to the main habitat and species composition, but they provide an important rule-of-thumb to plan future seed dispersal studies. These results conform to previous findings in pollination interactions, highlighting the need to explicitly consider sampling effort when comparing network structure (Nielsen & Bascompte 2007; Rivera-Hutinel *et al.* 2012).

The results of chapter III suggest that seed dispersal networks are relatively robust to external disturbances due to the capacity of interaction rewiring – i.e. the capacity to establish new interactions to compensate for the lost ones - which buffers against secondary extinctions and the degradation of ecosystem functions (Goldstein & Zych 2016; Timóteo *et al.* 2016). The experimental removal of the most important fleshy-fruited resource resulted in some rapid interaction rewiring. Accordingly, the species extinction simulations that allow interaction rewiring generally predicted the observed network structure more accurately than those without rewiring. However, some descriptors, noticeably nestedness, were harder to predict than others with the current rewiring assumptions of *in silico* simulations. This finding conforms with a previous study in ant-seed dispersal interactions where network robustness was still underestimated even after the incorporation of interaction rewiring in the species extinction simulations (Timóteo *et al.* 2016). Overall, these results highlight the need to incorporate interaction rewiring in extinction simulation models (Goldstein & Zych 2016). However, the current thesis also suggests that species-level descriptors are harder to predict than network-level structure, even in models that allow rewiring. Such result proposes that the observed dynamics at the species-level was not captured by the rewiring assumptions considered in the simulations. Multiple factors drive the role of a species in ecological communities (e.g. species' abundance, food preferences, trait matching). Incorporating such information in interaction rewiring probabilities for the full range of potential interacting partners for each species is highly challenging, but likely to significantly improve the predictive capacity of these *in silico* simulations.

While most networks described to date are restricted to relatively short sampling periods (characteristically ≤ one year), in this thesis one avian-seed dispersal network was sampled during five consecutive years. This is, to the best of my knowledge, the longest study to date on avian-seed dispersal at the community level. Furthermore, this study implements an innovative multilayer approach, which allowed to explore the temporal dynamics of the overall network structure. The yearly networks were dominated by a few disproportionally frequent and temporally persistent species and interactions. Network structure was relatively stable across time, with the presence of four consistent interaction modules spanning across all years. Interestingly, the most important species in the overall multilayer network were recurrently the most important species in any given year, and also the ones more often present in multiple years. In this thesis, the number of years where a given species is present was defined as a new species-level multilayer descriptor coined as *species activity*. However, the applicability of the concept of species activity is not restricted to temporal seed dispersal networks and can be used in any ecological multilayer network where the layers represent for example, years, habitats, sites or types of interactions. The application of a multilayer approach in ecological networks is still in its infancy but holds great promise in the study of multiple interconnected ecological networks, such as temporal, spatial, or multiplex networks where each layer represents a distinct type of interaction, as it allows the quantification of ecological processes connecting the different subnetworks that structure natural ecosystems (Hutchinson *et al.* 2018; Timóteo *et al.* 2018). Overall, the results presented here suggest that avian-seed dispersal networks are consistently dominated by a group of temporally reliable generalist species to which more transient species tend to interact. These results contrast with those described for pollination networks where there seems to be a lower interannual predictability in the identity of the available interaction partners (Petanidou *et al.* 2008; Herrera 2018). Such partial redundancy in the seed dispersal service provided across species is likely to increase the robustness and resilience of avian-seed dispersal networks against external perturbations. However, different bird species differ in the treatment (e.g. deposition patterns, depulping, scarification, etc.) conferred to the seeds of the various plants influencing seed germination and seedling recruitment (Traveset *et al.* 2001; Traveset *et al.* 2007). Therefore, it is important to assess not only the quantitative (i.e. number of seeds dispersed) but also the qualitative (i.e. the treatment conferred to seeds by dispersers) component of seed dispersal in order to better estimate seed dispersal effectiveness (Schupp *et al.* 2010).

Final remarks

Over the last decades, our understanding of the drivers of ecosystem functioning has increased dramatically, especially with the advent of ecological networks, which provided a quantitative analytical tool to clarify the seemingly chaotic complexity of nature’s entangled bank of interactions. Seed dispersal, of which birds are one of the most important animal vectors, is one key ecosystem service that benefited greatly with the incorporation of a “network way of thinking”. Birds may disperse seeds via endozoochory and endozoochory, but the latter mechanism is much more frequent than the former, constituting a network of interactions requiring at least eight sampling-days of mist-netting to be properly characterized. The structure of avian-seed dispersal networks tends to be relatively constant across years, with a set of consistently important species that are fundamental to the cohesiveness of the multilayer network structure and confers temporal reliability to the seed dispersal service. Furthermore, the generalist nature of seed dispersal interactions is likely to confer an additional buffer against external stressors, and species extinction simulations should improve the way that interaction rewiring in incorporated into their predictions. Several particularly promising research avenues stem from the results of this thesis, which need to be addressed in order to take full advantage of networks in the study of seed dispersal. These include the explicit consideration of sampling intensity, the development of better rewiring algorithms, and the wider implementation of multilayer networks. In a rapid changing world where ecological communities are under increasing pressure due to anthropogenic activities, the combination of ecological networks to the study of seed dispersal still holds a great potential for future findings.

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Supplementary Material

Supplementary Material

Table S1 (next page) - Number of bird droppings containing at least one intact seed of each plant species. Values marked with * indicate an epizoochory interaction. Non-passerine species are indicated with (**NP**) after the species name. The migratory behaviour of passerine species is classified as: migratory (**Migr.**), partially migratory (**P-Migr.**), or resident (**Res.**).

Birds	**Migratory behaviour**	**Nº of captures**	*Asparagus acutifolius*	*Bryonia dioica*	*Daphne gnidium*	*Ficus carica*	*Frangula alnus*	*Galium aparine*	*Linicera implexa*	*Lonicera periclymenum*	*Myrtus communis*	*Pistacia lentiscus*	Poaceae sp. 1	Poaceae sp. 2	*Portulaca oleracea*	*Rhamnus alaternus*	*Rubia peregrina*	*Rubus ulmifolius*	*Sambucus nigra*	*Solanum nigrum*	*Tamus communis*	*Torilis arvensis*	*Vitis vinifera*
Acrocephalus schoenobaenus	Migr.	1																					
Acrocephalus scirpaceus	Migr.	11																					
Aegithalos caudatus	Res.	17																					
Alcedo atthis (NP)	Res.	6																					
Anthus trivialis	Migr.	1																					
Aquilla pennata (NP)	Migr.	1																					
Caprimulgus ruficollis (NP)	Migr.	2																					
Carduelis carduelis	Res.	3																					
Certhia brachydactyla	Res.	11																					
Cettia cetti	Res.	9																1					
Chloris chloris	Res.	18																					
Cisticola juncidis	Res.	2																					
Coccothraustes coccothraustes	Res.	1																					
Cyanistes caeruleus	Res.	62									1							14					
Cyanopica cyanus	Res.	8																1					1
Dendrocopos minor (NP)	Res.	1																					
Emberiza calandra	Res.	1																					
Emberiza cia	Res.	9																1					
Emberiza cirlus	Res.	5																					
Erithacus rubecula	P-Migr.	66		1	1	2						2				3	1	20		1			
Estrilda astrild	Res.	4																					
Ficedula hypoleuca	Migr.	138				1				1						10	1	28	4				
Fringilla coelebs	Res.	21													3								
Hippolais polyglotta	Migr.	10																					
Lanius meridionalis	Res.	1																					
Locustella naevia	Migr.	3						1*															
Lophophanes cristatus	Res.	2																					
Lullula arborea	P-Migr.	1																					
Motacilla cinerea	P-Migr.	1																					
Muscicapa striata	Migr.	8																1					
Oriolus oriolus	Migr.	1																					
Parus major	Res.	12																					
Passer domesticus	Res.	17				1								1									
Passer montanus	Res.	1																					
Petronia petronia	Res.	1																					
Phoenicurus phoenicurus	Migr.	5														1		1					
Phylloscopus bonelli	Migr.	1																1					
Phylloscopus collybita	Migr.	1																					
Phylloscopus ibericus	Migr.	16																					
Phylloscopus trochilus	Migr.	87																1					
Saxicola rubicola	Res.	4																					
Serinus serinus	Res.	14																				1*	
Sitta europaea	Res.	1																					
Strix aluco (NP)	Res.	1																					
Sturnus unicolor	Res.	2																					1
Sylvia atricapilla	P-Migr.	46		4	2	7			1			2				3		16	7	8	1		
Sylvia borin	Migr.	60		3		10	1		1			1				14		23	8	1			
Sylvia cantillans	Migr.	48			1											1		4	2				
Sylvia communis	Migr.	24														3		10					
Sylvia melanocephala	Res.	83	1		12	3			1			6				5		31	1				
Troglodytes troglodytes	Res.	7																					
Turdus merula	Res.	56				3	1*						1					19		2			2
Upupa epops (NP)	P-Migr.	1																					

Table S2 - Number of bird species (Spp.) individuals (Ind.) captured and droppings (Drop.) in each study site. There were no significant differences in the number of droppings collected per day across the seven sites (Generalized Linear Mixed Model with negative binomial error distribution and site as a random effect: $\chi^2 = 6.298$, d.f. = 4, $p = 0.178$).

Day	Achete			Atenor			Bensafrim			Sta. Maria de Aguiar dam			Freixo do Meio			Larçã			Nozelos		
	Spp.	Ind.	Drop.	Spp.	Ind.	Drop.	Spp	Ind.	Drop.	Spp	Ind.	Drop.	Spp	Ind.	Drop.	Spp	Ind.	Drop.	Spp	Ind.	Drop.
1	18	34	31	11	28	15	5	9	9	20	81	46	10	24	16	8	11	12	12	28	26
2	13	42	33	12	28	22	3	4	3	12	33	24	7	10	6	4	9	8	14	43	38
3	14	26	23	10	27	15	10	17	11	13	40	21	10	19	14	8	20	17	11	20	16
4	12	23	22	13	19	12	5	10	7	10	34	14	5	10	8	6	11	11	7	23	19
5	13	34	32	15	24	14	3	4	3	14	37	24	6	9	3	10	29	30	10	23	19
6																3	16	15			
7																9	26	22			
8																8	21	20			
9																6	17	16			
10																5	20	19			
11																7	12	10			
12																4	11	11			
13																7	24	21			
14																9	23	19			
15																8	20	17			
16																6	14	11			
17																4	10	9			
18																7	24	16			
19																7	18	13			
20																8	20	17			
21																6	15	11			
22																5	17	12			
23																1	3	2			
24																7	20	16			
25																5	8	8			

Table S3- Statistics of piecewise regressions used to assess the temporal stability of seed and disperser species, links, weighted NODF, and network specialization H_2’ with increasing sampling effort. Each breakpoint [with standards error (SE)] indicates the day when the trend of a given variable changes. Regression coefficients that are significantly different from zero [95 % confidence intervals (95% C.I.) not overlapping zero] are marked with an asterisk. Since connectance and weighted connectance regression lines had no breakpoints, the results for this descriptor are those of an ordinary linear regression.

		Seed species	Dispersers	Links	Connectance	NODF	Qualitative Modularity	Weighted Connectance	Weighted NODF	Quantitative Modularity	H_2’
Trend 1	day interval	1 - 5	1 - 8	1 - 2	1 - 25	1 - 2	1 - 7	1 - 25	1 - 3	1 - 8	1 - 3
	slope β (95% C.I.)	1.00 (0.72 to 1.29)*	0.65 (0.59 to 0.72)*	1.00 (-1.77 to 3.77)	0.0008 (-0.002 to 0.0005)	-11.11 (-23.98 to 1.75)*	-0.036 (-0.042 to - 0.029)*	-0.0005 (-0.001 to 0.0002)	1.57 (-1.41 to 4.56)	-0.03 (-0.03 to - 0.02)*	0.15 (0.10 to 0.20)*
	Breakpoint (SE)	5.27 (0.55)	8.89 (0.28)	2.39 (0.49)		2.41 (0.35)	7.02 (0.39)		3.76 (0.43)	8.43 (0.71)	3.96 (0.28)
Trend 2	day interval	6 to 16	8 – 25	2 - 7		2 - 7	7 - 25		3 - 7	8 - 25	3 - 7
	slope β (95% C.I.)	0.18 (0.09 to 0.26)*	-1.1x10^{16} (-0.02 to 0.02)	4.30 (3.68 to 4.92)*		10.14 (7.26 to 13.01)*	-0.0007 (-0.002 to 0.0008)		9.26 (7.38 to 11.15)*	-0.002 (-0.004 to 1.68x10^{5})	-0.04 (-0.07 to - 0.01)*
	Breakpoint (SE)	16.17 (3.04)		7.16 (0.25)		7.61 0.46			7.37 (0.31)		7.49 (0.83)
Trend 3	day interval	16 to 25		7 - 25		7 - 25			7 - 16		7 - 25
	slope β (95% C.I.)	0.30 (0.19 to 0.42)*		0.71 (0.62 to 0.80)*		0.32 (-0.10 to 0.73)			1.68 (1.14 to 2.23)*		-0.001 (-0.004 to 0.001)
	Breakpoint (SE)								16.71 (1.15)		
Trend 4	day interval								16 - 25		
	slope β (95% C.I.)								0.03 (-0.51 to 0.58)		
R^2		0.97	0.98	0.99	0.06	0.95	0.95	0.09	0.99	0.93	0.89

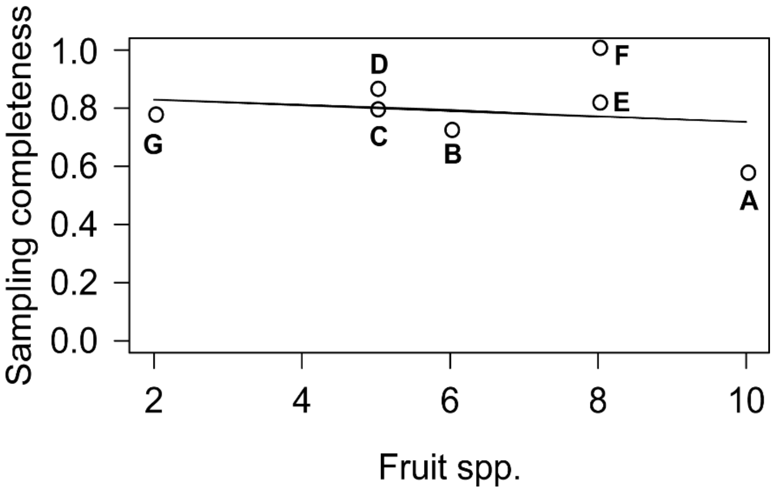

Figure S1 - Effect of the number of fruit species counted in transects on the link's sampling completeness (proportion of detected links) for each of the seven study sites. The line represents the fit of a linear logistic regression (quasibinomial error distribution, slope = - 0.05969, t = - 0.464, p = 0.662). A – Larçã, B – Atenor, C – Nozelos, D – Santa Maria de Aguiar dam, E – Achete, F – Freixo do Meio, G – Bensafrim.

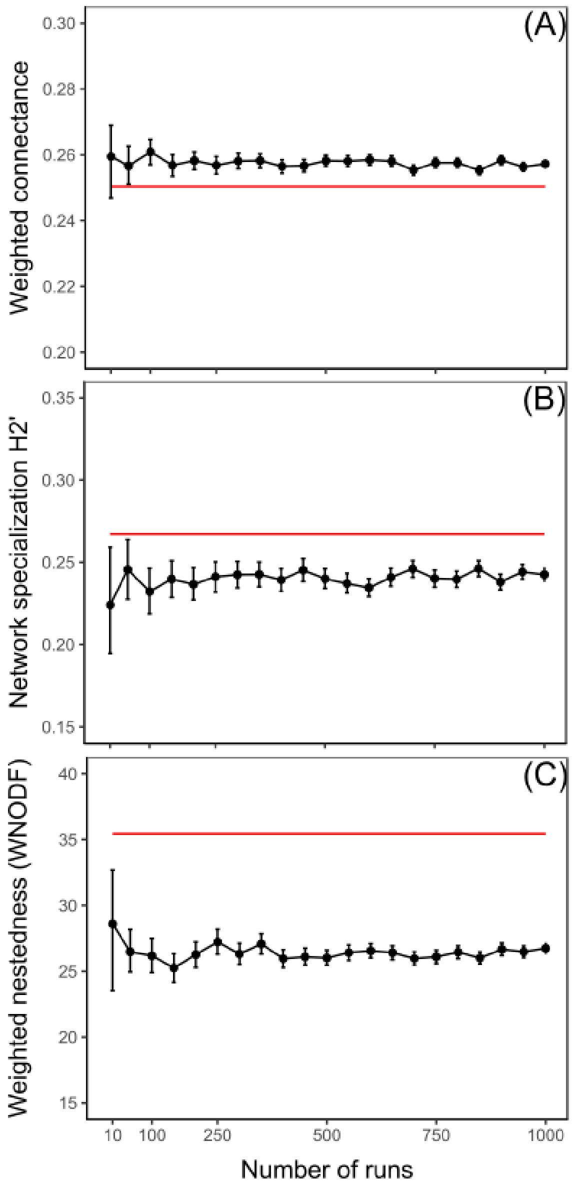

Figure S2 - Relationship between the estimated mean of three main network-level descriptors and the number of simulations (runs) of species secondary extinction models with rewiring. (A) weighted connectance, (B) weighted nestedness (WNODF), and (C) network specialization H_2'. Error bars correspond to the 95% BCa bootstrapped confidence intervals around the mean. The horizontal red line corresponds to the observed descriptor after the empirical removal of blackberry fruits.

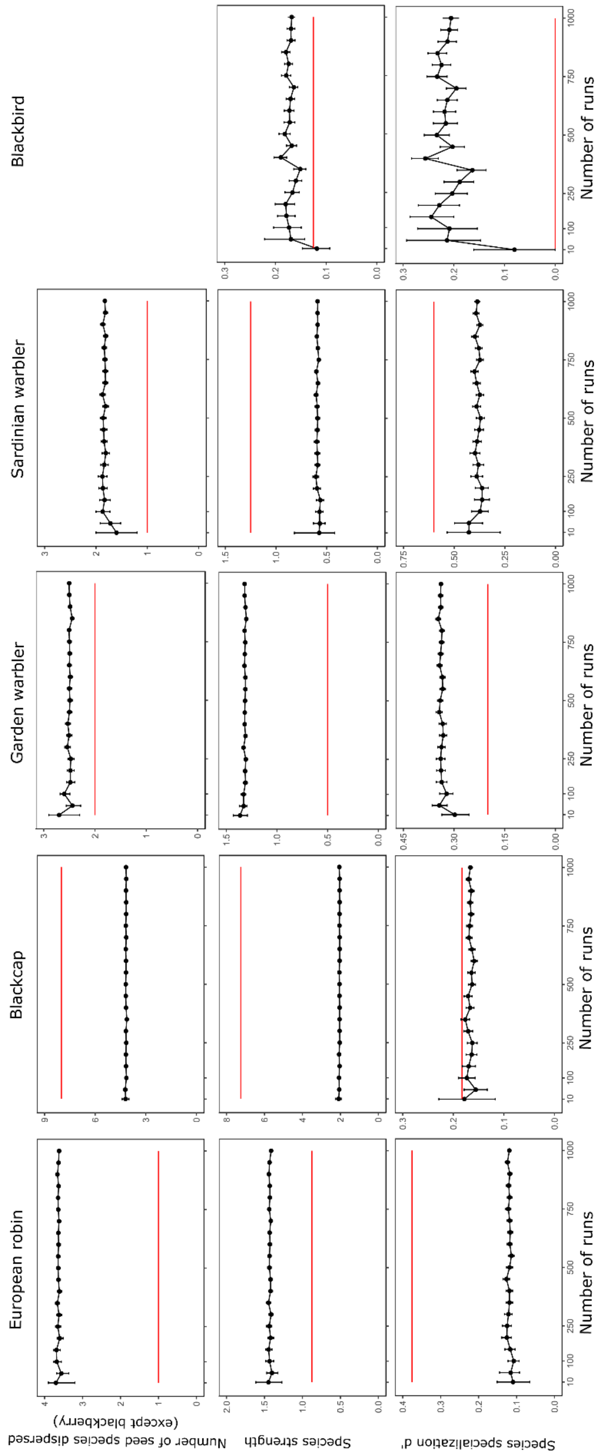

Figure S3 - Relationship between the estimated mean of three main species-level descriptors and the number of simulations (runs) of species secondary extinction models with rewiring: number of seed species dispersed (top row), species strength (middle row), and specialization (bottom row). The relationship between the first descriptor and the number of runs is not shown for blackbird because it had only one interaction to rewire. Bird species that did not disperse any seed after the removal of blackberry fruits are also not shown (blue tit, pied flycatcher, and common whitethroat). Error bars correspond to the 95% BCa bootstrapped confidence intervals around the mean. The horizontal red line corresponds to the observed descriptor after the empirical removal of blackberry fruits.

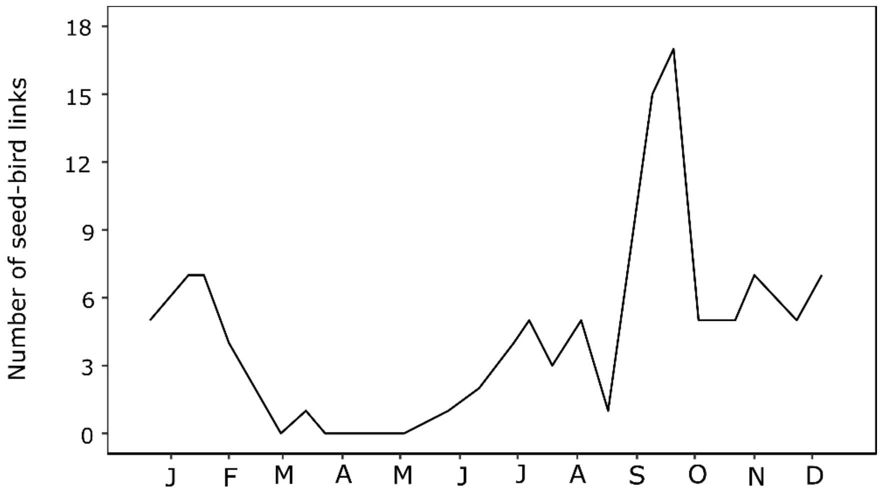

Figure S4 - Richness of pairwise links between seeds and their bird dispersers. Data recorded fortnightly throughout one year (2013) on a native forest in central Portugal.

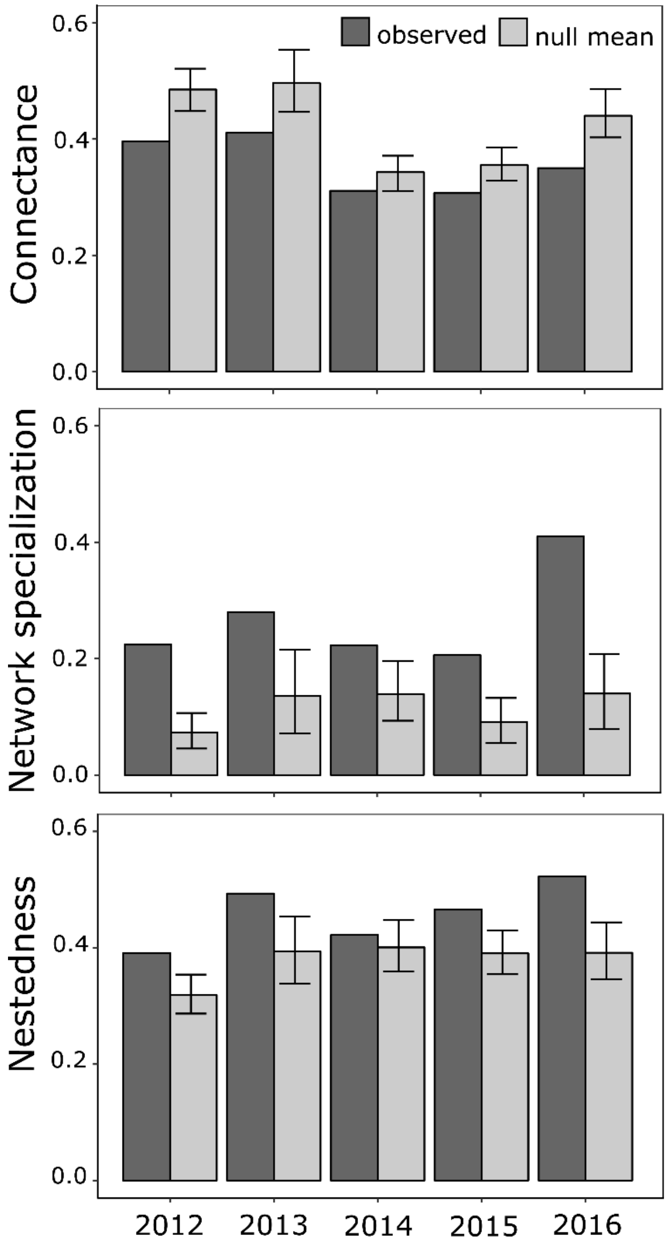

Figure S5 - Interannual variability in Connectance, Network specialization (H_2'), and Nestedness (WIN) of the seed-dispersal network. The observed descriptor (“observed”) is compared with the mean of 1000 Patefield null models (“null mean”). Error bars correspond to the 95% percentile of the null models’ distribution.