Advancing metabarcoding techniques for the study of trophic interactions and ecosystem services in small vertebrates

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Nota Prévia

Na elaboração desta dissertação, e nos termos do número 2 do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada.

Lista de artigos

Capítulo 2 – **Mata V.A.,** Rebelo H., Amorim F., McCracken G.F., Jarman S., & Beja P. (2019) How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, **28**(2), 165–175. https://doi.org/10.1111/mec.14779.

Capítulo 3 – da Silva L.P., **Mata V.A.**, Lopes P., Pereira P., Jarman S., Lopes R.J., & Beja P. (2019) Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists. Molecular *Ecology Resources*, **19**(6), 1420-1432. https://doi.org/10.1111/1755-0998.13060. L.P. da Silva e V.A. Mata são primeiros autores em conjunto.

Capítulo 4 – **Mata V.A.**, Amorim F., Corley M.F. V., McCracken G.F., Rebelo H., & Beja P. (2016) Female dietary bias towards large migratory moths in the European free-tailed bat (*Tadarida teniotis*). *Biology Letters*, **12**(3), 20150988. https://doi.org/10.1098/rsbl.2015.0988.

Capítulo 5 – **Mata V.A.**, da Silva L.P., Veríssimo J., Horta P., Raposeira H., McCracken G.F., Rebelo H., & Beja P. (*In prep*). DNA metabarcoding illuminates pest control services in complex multifunctional landscapes.

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People do PhDs for different reasons. In my case, I don't think being a Doctor was something that I ambitioned at all. Science for me has always been more about the challenge and the fun than anything else. Especially the fun. Unfortunately, Academia seems to be a strange place in which for people to be in it, they need to be constantly moving up this ladder. It is not exactly clear for me where it goes, or what will I gain by climbing it, but I guess that as long as I am enjoying the science and the life it provides me, I don't see any reason to drop out of it.

These past four and a half years of PhD have been a crazy ride, and three days away from the delivery deadline I can only say that I do not believe that it is coming to an end. Fortunately, I can say that I enjoyed every bit of it, except perhaps having to "stop" to actually write down this thesis. Along the way, I have travelled more than I ever thought I would (or could afford), met amazing people (and scientists!) across the globe (so many to name!), did more field-work for non-PhD related projects than I probably should have (don't regret any bit of it though, thank you Dina for the *T.teniotis* tracking!), learned more about insects than I ever thought possible, and engaged in probably way too many side-projects for my own good.

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Abstract

The natural world has been changing at speeds never seen in human history. The current toll of humanity on Earth is unsustainable, with far-reaching consequences on biodiversity, the environment, and humanity itself. Urgent and concerted actions are thus desperately needed to halt the current crisis. In particular, nature friendly solutions to the management of multifunctional landscapes to sustain the production of food, fibre and energy required by a growing human population, while maintaining biodiversity and their inherent ecosystem services, are among the major challenges facing scientists and stakeholders today.

Small insectivorous vertebrates are particularly widespread and abundant in multifunctional landscapes, provided minimal conditions of shelter, food, and connectivity are met. These organisms can deliver important ecosystem services such as pest control, thereby reducing the need of agrochemical inputs. The flow, stability and resilience of such services are critically dependent on the networks of trophic interactions between small vertebrates and their arthropod prey, which at present are still poorly understood. Recent molecular tools like metabarcoding can offer unprecedented detail into the intra- and inter-specific variation in the diet of small insectivorous vertebrates, providing key understanding on their role in the provision of ecosystem services. However, the application of these techniques is still in its infancy, and the impact of their technical caveats and limitations on the estimate of trophic interactions still needs to be fully understood.

The overall goal of this thesis is to advance the use of metabarcoding techniques in the study of species interactions, focusing on small insectivorous vertebrates, thereby enhancing its applicability in the management of complex landscapes towards multifunctionality. Specifically, the thesis aims at: i) understanding the impacts of technical and biological replication on the results of metabarcoding dietary analysis; ii) testing the use of multiple overlapping markers in metabarcoding dietary analysis, as well as to define criteria for integrating these data; iii) assessing the power of taxonomically resolved dietary data obtained through metabarcoding to reveal subtle intraspecific variations in predator-prey relationships; and iv) evaluating the role of individual species in sustaining pest regulation services, thereby illustrating the potential of metabarcoding as a tool for improving the management of multifunctional landscapes.

The results of this thesis stress that maximizing biological replication is critical in dietary metabarcoding studies and that the integration of multi-marker data provides far more detailed dietary information than any single marker approach. Yet, high levels of secondary predation can be detected in the diet of small generalist vertebrates, and thus other sources of information are recommended to help guiding the interpretation of metabarcoding results in

these cases. Moreover, metabarcoding proved to be a powerful tool, capable of detecting subtle intra- and inter-specific variations in the diet of bat species. The combined use of metabarcoding with ecological network analysis further allowed the description of the first predator-pest interaction network of bats and insect agricultural pests, along with the identification of candidate bat species that could be favoured to intensify the control of pests. The observed interaction patterns underline the functional importance of conserving diverse vertebrate communities in multifunctional landscapes.

Overall, this thesis underlined the value of metabarcoding to unravel the functional role of small insectivorous vertebrates in multifunctional landscapes, while providing guidance on best practice to minimise the potential caveats and limitations of this technique. Together with a growing number of studies advancing the use of molecular techniques to describe species interactions, this thesis thus opens exciting opportunities to build ever more comprehensive and taxonomically-resolved ecological networks, where more and different components of biodiversity and its interactions can be put together to build entire ecosystem food-webs. This will open up the way to enhance productivity in multifunctional landscapes while safeguarding biodiversity and ecosystem services via better decision-making.

Keywords: metabarcoding, sampling design, replication, molecular diet analysis, trophic ecology, multi-marker, secondary predation, resource partitioning, pest control, ecological networks, predator-prey interactions, food-webs, bats, birds.

Resumo

O mundo natural tem vindo a alterar-se a velocidades nunca antes vistas na história humana. O custo atual da humanidade à Terra é insustentável e com largas consequências na biodiversidade, ambiente e à própria humanidade. Ações sérias e urgentes são desesperadamente necessárias de modo a travar a crise atual. Especificamente, medidas amigas da natureza para a gestão de paisagens multifuncionais que sustenham a produção de comida, fibra, e energia necessária à crescente população humana, e ao mesmo tempo mantenham a biodiversidade e os seus serviços de ecossistema inerentes, são um dos maiores desafios atuais dos cientistas e políticos.

Os pequenos vertebrados insectívoros são particularmente comuns e abundantes em paisagens multifuncionais, assim as condições mínimas de abrigo, comida e conectividade estejam asseguradas. Estes organismos podem oferecer importantes serviços de ecossistemas como controlo de pragas, reduzindo assim a necessidade de aplicação de agroquímicos. O fluxo, estabilidade e resiliência destes serviços estão criticamente dependentes da rede de interações tróficas entre pequenos vertebrados e os seus insetos presa, a qual é atualmente muito pouco conhecida. Ferramentas moleculares recentes como códigos de barra de ADN em massa (daqui em diante denominadas *metabarcoding*), podem oferecer um detalhe sem precedentes na variação intra- e inter-específica da dieta das espécies de pequenos vertebrados insectívoros, fornecendo um conhecimento chave dos seus papeis na provisão de serviços de ecossistema. No entanto, a aplicação destas técnicas está ainda na sua infância, e o impacto das suas limitações técnicas nos descritores de interações tróficas ainda precisa de ser melhor conhecido.

O objetivo geral desta tese é avançar a utilização de ferramentas de *metabarcoding* no estudo das interações das espécies, focando em pequenos vertebrados insectívoros, e assim melhorando a sua aplicabilidade na gestão de paisagens complexas com vista à sua multifuncionalidade. Especificamente, esta tese pretende: i) compreender os impactos da replicação técnica e biológica nos resultados das análises de dieta por *metabarcoding*; ii) testar o uso de vários marcadores moleculares em análises de dieta por *metabarcoding*, assim como definir critérios para a integração destes dados; iii) testar o poder de dados taxonomicamente finos, obtidos através de *metabarcoding,* na identificação de padrões subtis de variação intra-específica nas relações predador-presa de pequenos vertebrados; e iv) avaliar o papel de diferentes espécies em suster serviços de regulação de pragas agrícolas, ilustrando assim o potencial do *metabarcoding* como ferramenta para o melhoramento da gestão de paisagens multifuncionais.

Os resultados desta tese realçam a importância da replicação biológica nos estudos de *metabarcoding*, e que a integração de vários marcadores moleculares fornece dados de

dieta muito mais detalhados do que qualquer abordagem baseada em apenas um único marcador. No entanto, níveis elevados de predação secundária podem ser detetados na dieta de pequenos vertebrados generalistas, e como tal, outras fontes de informação são recomendadas para guiar a interpretação dos resultados obtidos por esta técnica. Além disso, o *metabarcoding* provou ser uma ferramenta poderosa, capaz de detetar subtis variações intra- e inter-específicas na dieta de morcegos. O uso combinado de *metabarcoding* com redes ecológicas, permitiu ainda a descrição da primeira rede de interações predador-presa entre morcegos e pragas agrícolas, assim como a identificação de espécies de morcegos cuja presença poderá ser favorecida para intensificar o controlo de pragas. Os padrões de interações observados sublinham a importância funcional de conservar comunidades diversas de vertebrados em paisagens multifuncionais.

Em geral, esta tese sublinhou o valor do *metabarcoding* em descobrir o papel funcional de pequenos vertebrados insectívoros em paisagens multifuncionais, orientando simultaneamente para as melhores práticas que reduzam eventuais advertências e limitações desta técnica. Em conjunto com um crescente número de estudos que avançam o uso de ferramentas moleculares para a descrição de interações entre espécies, esta tese abre excitantes oportunidades para construir redes ecológicas cada vez mais compreensivas e taxonomicamente resolvidas, onde mais e diferentes componentes da biodiversidade e as suas interações podem ser integradas para construir redes-tróficas dos ecossistemas. Este conhecimento permitirá a tomada de decisões mais informadas, abrindo assim o caminho para o melhoramento da produtividade das paisagens multifuncionais, em simultâneo com o resguardo da biodiversidade e os serviços que ela oferece.

Palavras chave: *metabarcoding*, desenho experimental, replicação, análise molecular de dieta, ecologia trófica, multi-marcadores, predação secundária, partição de recursos, controlo de pragas, redes ecológicas, interações predador-presa, redes-tróficas, morcegos, aves.

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List of Abbreviations

AIC	Akaike Information Criteria
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data System
bp	base-pair
CBD	Convention on Biological Diversity
COI	Cytochrome Oxidase I
DNA	Deoxyribonucleic Acid
DOI	Digital Object Identifier
e.g.	exempli gratia, for example
ESV	Exact Sequence Variant
et al.	et alii, and others
ETRS89	European Terrestrial Reference System 1989
FAO	Food and Agriculture Organization
GLM	Generalized Linear Model
GLMM	Generalized Mixed Linear Models
i.e.	id est, that is
IPBES	Science-Policy Platform on Biodiversity and Ecosystem Services
k	kilo
km	kilometre
km^2	square kilometre
logLik	log Likelihood
MEA	Millennium Ecosystem Assessment
mg	milligram
min	minute
mm	millimetre
MOTU	Molecular Operational Taxonomic Unit
MSS	Mean Sum of Squares
NCBI	National Center for Biotechnology Information
nM	nanomolar
NODF	Nestedness based on Overlap and Decreasing Fill
ºC	degree Celsius
PCR	Polymerase Chain Reaction
PerMANOVA	Permutational Multivariate Analysis of Variance
pM	picomolar
qPCR	quantitative Polymerase Chain Reaction
rpm	rotation per minute
s, sec	second
SD	Standard Deviation
SE	Standard Error
USA	United States of America
wNODF	weighted Nestedness based on Overlap and Decreasing Fill
µl	microlitre

Chapter 1

General Introduction

1.1 Global change and biodiversity decline

In 1859, Charles Darwin elegantly demonstrated that the incredible biological diversity surrounding us is constantly changing and being renewed into new species. Natural selection acts upon populations and drives evolution, determining which species are better adapted to their environmental conditions. The ones unable to survive eventually end up extinct and are soon replaced by better fitting ones. A rapid look at the fossil record makes it easy to see how life on Earth has changed over the course of its history (Alroy, 2008). It has been calculated that about 99% of all species known to science are now extinct, and that only a small fraction of the species that have lived are still around these days (Raup, 1991). Besides the slow and gradual mutation and extinction of species, life on Earth has experienced a small number of planet-wide mass extinction events. Since the Cambrian period, around 542 million years ago, there were at least 5 mass extinctions that usually involved the disappearance of around 70% of the occurring species (Jablonski, 1994; Hallam & Wignall, 1997). Event after event, natural selection led surviving species to diversify, re-occupying previous niches and creating new ones.

Today, about 65 million years after the last great extinction that led to the disappearance of dinosaurs and to the rise of mammals and birds, we are experiencing the 6th mass extinction and entering a new era called the Anthropocene (Barnosky et al., 2011; Dirzo et al., 2014). For the first time in our planet history, a single species (our own) is changing the natural balance of the planet in ways never observed before (Ceballos et al., 2015). Technological advances in agriculture and medicine have allowed human populations to grow at exponential rates with far-reaching consequences on the environment and biodiversity (Henderson & Loreau, 2019). We are now over 7.7 billion and still increasing every day (Worldometers.info, 2020). Natural resources are needed to fulfil our energy and lifestyle demands, adding a considerable strain on the environment. It has been estimated that humans are currently using natural resources 1.75 times faster than the regenerating capacity of Earth (Footprintnetwork.org, 2019). This blind and irrational belief (or hope?) that natural resources are infinite and/or that humanity will be able to find a way to cope with infinite growth in a finite world, has led to a generalized lack of action across the globe. As the scale of the processes governing people's lives and Earth's ecological and regulation patterns are quite distinct, it is hard for us to conceptualize how something as large as the Earth, with such extensive natural areas, could be running out of resources to sustain human populations.

To tackle these challenges, a coordination between policy and scientific knowledge-based decision-making is thus of the utmost importance. Within this scope, the first United

Nations Conference on Environment and Development was held in 1992, which led to the creation, of the Convention on Biological Diversity (CBD; UN, 1992). Since then, countries meet regularly to define global goals and targets and to develop national strategies to reach them. In particular, the CBD meeting in 2002 adopted the '2010 Biological Diversity Target', that proposed "to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national level as a contribution to poverty alleviation and to the benefit of all life on Earth" (UNEP, 2002). However, the targeted goals were far from met, with problems like habitat degradation, ecosystem fragmentation, shrinking vertebrate and invertebrate populations and high species extinction risk still ongoing (Butchart et al., 2010; CBD, 2010a). A new set of goals was thus redefined in 2010, this time to be met by 2020, along with a proposal to create an Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES; CBD, 2010b). Its goal was to improve the interface between science and policy on issues related to biodiversity and ecosystem services, this way leading to the conservation and sustainable use of biodiversity, long-term human well-being and sustainable development.

On May 2019 IPBES released the Global Assessment Report on Biodiversity and Ecosystem Services, which was the first major assessment on changes in Earth's biodiversity occurring in the past 50 years, after the Millennium Ecosystem Assessment (MEA) released in 2005. The news were grim and exploded on social media: one million species, including 40% of amphibians, 30% of reef-building corals and marine mammals, along with 10% of all insects are threatened with extinction; 85% of the world's wetlands have been lost; total biomass of wild mammals has decreased 82%, with humans and farm animals now making 96% of mammalian biomass on Earth; tropical rainforests continue to be cleared for cattle at accelerating rates; 25% of ice-free land is used for cattle; 23% of the land is now ecologically degraded and no longer usable; among with major problems of overfishing, pollution, and invasive species. The summary report for policymakers highlighted four key and clear messages: (i) Nature is vital to people and its contributions are deteriorating worldwide; (ii) Global change is unprecedented in human history and has accelerated in the past years, with land-use change being the largest driver of nature decline; (iii) Conservation and sustainability goals for 2030 and beyond may only be achieved through transformative changes across economic, social, political and technological factors; and (iv) Nature can be conserved, restored and used sustainably, if urgent and concerted efforts fostering transformative change are implemented. If there is one thing that we can be sure of, is that life on Earth will continue and natural selection and evolution will keep generating biological diversity. What will happen with humans on this 6th mass extinction however is still to unfold. Previous mass extinction events did not turn very well for dominant species, so humanity's impact on nature is mostly an impact on humanity itself and its ability to survive. The future of our species is thus

dependent on urgent and responsible political decisions and adequate actions based on sound scientific knowledge.

1.2 Multifunctional landscapes as a tool for sustainability

The conversion of natural habitats to agricultural land and pastures is the largest driver of terrestrial ecosystem change (MEA, 2005; IPBES, 2019). Most of this land is used for livestock grazing and production of animal feed, while the remaining 23% is used to grow crops for human consumption (FAO; Figure 1.1). Besides the expansion of farmland, there is also a rapid intensification process within the land currently cultivated, either for direct human consumption or for animal feeding, which will likely increase even further in the future (Ramankutty et al., 2018). This intensification process has dramatically changed agricultural landscapes in places like North America and Europe in the past 60 years (Robinson & Sutherland, 2002). Homogenization, mechanization and irrigation of the fields, along high input of agrochemicals like pesticides and fertilizers, have scaled-up production of farms, while reducing the need of human-labour input. However, there is accumulating evidence that the simplification of landscapes, with little or no presence of natural habitats, along with high levels of agrochemicals, are linked to rapid decreases in biodiversity and the services they provide, as well as soil degradation and water pollution (e.g. Benton et al., 2003; Bianchi et al., 2006; Ekroos et al., 2010; José-María et al., 2010; Cardinale et al., 2012; Gámez-Virués et al., 2015; Dainese et al., 2017.

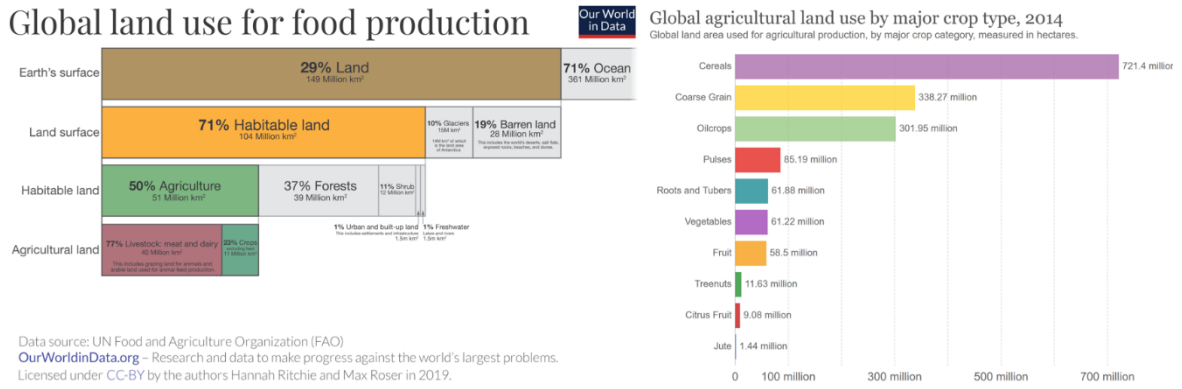

Figure 1.1 – Global land use for agriculture and major crops planted (source: FAO, OurWorldinData).

The way to reconcile agriculture and biodiversity has been long debated by researchers and conservationists, with people often dividing into two traditionally opposing views of land-sharing vs land-sparing approaches (Phalan et al., 2011; Kremen, 2015). On the one side, land-sharing defendants argue that biodiversity conservation should happen by using

environmentally friendly agricultural methods that allow the co-existence of natural and agricultural patches of land, which in practice may translate into biodiversity rich, low-yield, large land footprint agricultural systems. On the other hand, land-sparing defendants argue that by using highly productive agricultural practices in small areas of land, large wild natural lands can then be left out for species conservation. This has been the most popular way of doing nature conservation, with 14.7% of the world's land area being covered by protected areas as of 2016 (UNEP-WCMC & IUCN, 2016). Notwithstanding, protected areas have neither halted habitat destruction nor species extinctions, although they do sustain particularly high levels of biodiversity and may be key to the conservation of specialists species that require large wilderness areas to survive (Gray et al., 2016).

Although not the central point in the discussion of land-sharing/-sparing approaches, land-sharing favours species that are able to use the semi-natural agricultural matrix and that are often key in the provision of ecosystem services like pollination and biological control (Grass et al., 2019). In contrast, land-sparing approaches confine biodiversity to restricted natural areas, likely limiting their provision of ecosystem services to agriculture. More recently, half-way approaches have been proposed by arguing that land-sharing is complementary to land-sparing, and that their interwinding can lead to the multifunctionality of agricultural landscapes (Grass et al., 2019; Figure 1.2). Designing multifunctional landscapes, or in other words, landscapes that can provide food, water, fibre, fuel, and forest products, while maintaining biodiversity and their inherent ecosystem services, such as pest control, are thus one of the main challenges for scientists and stakeholders to solve.

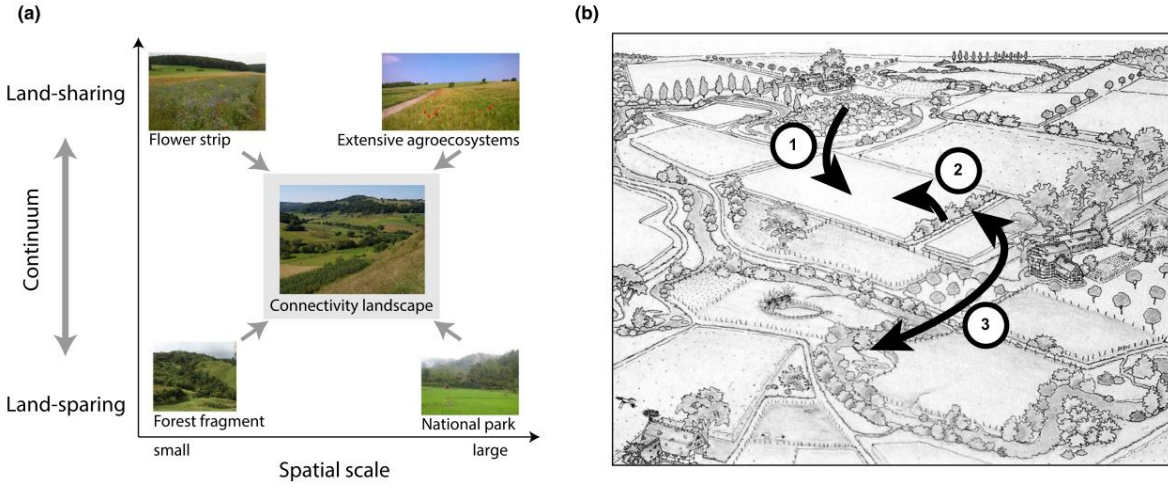

Figure 1.2 – Land-sharing/-sparing connectivity landscapes. (a) Land-sharing and land-sparing measures cover multiple spatial scales and fall along a sharing–sparing continuum. Their combination in land-sharing/-sparing connectivity landscapes promotes both biodiversity conservation and the provisioning of ecosystem services. (b) High connectivity across the agricultural landscape matrix is needed for land- sharing and land-sparing to be successful. The connectivity matrix ensures (1) spillover from (spared) natural habitats to agroecosystems as well as (2) spillover from (shared) crop boundaries to agroecosystems. In addition, (3) landscape connectivity facilitates immigration and species dispersal, counteracting possible extinctions in spared habitats and providing response diversity in changing environments. Source: Grass et al., 2019.

One of the key problems with highly intensive agricultural land is the massive use of chemical pesticides, which is neither economically nor ecological sustainable. A recent report on pesticide sales showed that in 2012 over 50,000 million euros were spent across the globe on pesticides, corresponding to about 2.7 million tonnes of pesticides applied worldwide (Atwood & Paisley-Jones, 2017). Still, losses of 10-30% due to pests and pathogens are observed on most cultivated crops (Savary et al., 2019). Ecological intensification has been proposed as a nature-based alternative that complements or (partially) replaces external chemical inputs (Kleijn et al., 2019). This process, is based on managing service-providing organisms that can contribute directly or indirectly to agricultural production (Bommarco et al., 2013). The ecosystem services provided by these species are thus incorporated into agricultural and forestry systems, so that production is maximized while environmental impacts are minimized. Natural enemies of agricultural pests, for example, can offer a sustainable solution to the economic and environmental costs of pesticide use, by reducing pest populations in the fields (Bianchi et al., 2006). This should have positive consequences not only on the level of damage by pests, but also on the need to apply pesticides, thereby improving water quality, health safety, pollinator abundance and even crop yields (Kovács-Hostyánszki et al., 2017; Catarino et al., 2019). The overall abundance and diversity of natural enemies, as well the level of pest control services, has been often linked to landscape complexity (Rusch et al., 2016). This way, just by promoting increasingly complex landscapes could help enhancing natural pest control. Nevertheless, agricultural landscapes might have different levels of crop diversity and therefore it might be more interesting to enhance the presence of certain predators instead of the overall diversity. This requires a detailed understanding of the function each species delivers along with the role that it plays in delivering such function, so that appropriate management actions can be implemented. For example, predatory insects are known to benefit from the presence of natural or semi-natural habitats, such as woodlands, field margins, permanent grasslands, and hedgerows (Rusch et al., 2010). However, if field margins are introduced in order to enhance a certain important crop pest predator, but that specific predator does not benefit from field margins, then, although overall predator diversity and abundance might increase in the area, the targeted service might not increase (Wilson et al., 2017).

1.3 Small vertebrates as insect pest suppressors

Most of the studies focusing on natural enemies have looked at insect predators, but little attention has been given to small insectivorous vertebrates. These animals are often top predators on terrestrial arthropod communities and due to their body size and high metabolic

rate can consume a substantially high number of insects per day. Their role as natural enemies has traditionally been disregarded as of the belief that their effect on insect pests would be counterbalanced by the simultaneous predation on predatory insects (Mooney et al., 2010). Nevertheless, a growing body of literature has found that vertebrates can exert important top-down regulation services on insect pests, not only reducing their numbers, but also their damage on crops (Mooney et al., 2010; Maas et al., 2016).

Bats and birds, in particular, share important traits that help them deliver vital ecosystem services. Due to their flight capacity, they are highly mobile and can thus freely move across complex landscapes and forage both opportunistically and by tracking resources. Also, due to their biological traits (birds usually need to feed a large number of offspring, while bats have an extremely high metabolism), these flying vertebrates need to predate on a high number of insects, especially during their breeding season, when most insects and insect pests are also available.

The effect of flying vertebrates on crops has been often assessed through the means of enclosure experiments (Maas et al., 2016). In these experimental setups, plots of crops are usually protected by nets in order to exclude bat and/or bird activity and compared to control plots where no enclosures are placed. Measured variables vary by study, but often include crop damage and yield after a determined period of enclosure, along with pest abundance and predatory insects' abundance. These types of studies have been conducted on corn and cotton plantations in the USA, and coffee, cocoa and macadamia orchards in the tropics, and have shown that the biocontrol services by these animals can sum up to billions of euros annually (Maine & Boyles, 2015; Maas et al., 2016), but have otherwise failed to identify the individual role of species in providing such services. This knowledge mismatch between the overall function of a group of organisms and the individual contribution of each species to the service might have important consequences in management decisions.

More recently, studies have linked bat and bird's activity to the predation of pests through the means of molecular analysis (Brown et al., 2015; Aizpurua et al., 2018; Krauel et al., 2018; Baroja et al., 2019; Kemp et al., 2019; Weier et al., 2019). By using arthropod specific DNA markers targeting standard barcode regions, along with polymerase chain reaction (PCR) and in many cases high-throughput sequencing, they have been able to identify exactly which species are being predated by these vertebrates. These important studies have further highlighted the ability of small vertebrates to prey on a wide and diverse number of different insect pests, but have mainly consisted on isolated evidence of one or few vertebrate species predating on pests of certain crops, without providing any insight into what happens at the community level.

1.4 Ecological networks for the study of ecosystem services

An alternative way to disentangle the functional role of species in the provision of ecosystem services is by the means of ecological networks. Since ecological networks include both species and the interaction strength among them, they provide an understanding of species' ecological roles and the mechanisms through which biodiversity influences ecosystem function, stability and resilience (Thompson et al., 2012; Heleno et al., 2014). These have been increasingly used to study and understand the complexity of ecosystems and their intrinsic interaction patterns (e.g. Schleuning et al., 2016; Strona & Lafferty, 2016; Hackett et al., 2019; Ma et al., 2019). Predator-pest interactions, for example, can be represented in bipartite networks, where one layer composed by predator species interacts with a second layer composed by insect pests. Network metrics can help identifying key species in communities by identifying nodes (species) in critical network positions that might have disproportional high effects in network functioning (Figure 1.3; Ebadi et al., 2017; Delmas et al., 2019). The loss of these key nodes might lead to cascading effects, so the management of these species is important to maintain the community functionality (Martín González et al., 2010).

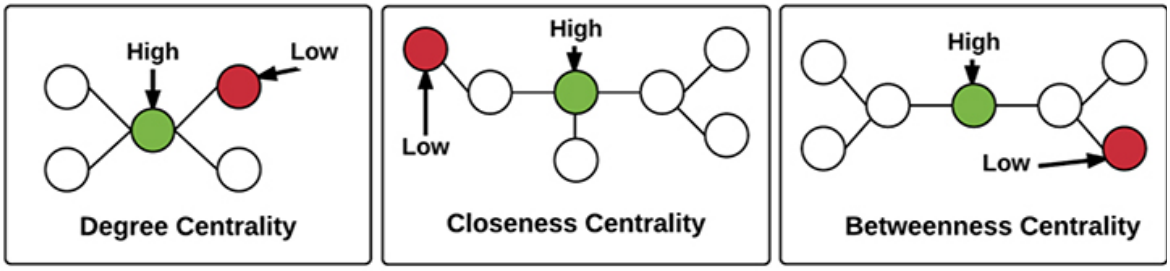

Figure 1.3 – Example of network metrics and how they vary with node position in the network: degree, closeness, and betweenness centrality. Degree centrality is the most commonly used index of positional importance in a food web and represents the number of species having trophic interactions with a particular species. This is mostly a local index that only reflects direct interactions of species and does not take into account the overall network structure and length. On the other hand, closeness centrality can measure the proximity of a node to all the other nodes in the network, while between centrality describes the importance of a node as a connector between different parts of the network. Figure adapted from Ebadi et al., 2017.

The use of ecological networks to study the provision of ecosystem services by pollinator and seed-dispersal species along with host-parasitoid interactions has a relatively long history (e,g, Memmott, 1999; Bascompte et al., 2003). Some of these studies have focused on identifying keystone species in the provision of such services (Dupont et al., 2009; Mello et al., 2015), however, studies targeting insect pest suppression services seem to be more unusual, most likely due to difficulties in accurately identifying prey remains to the species level in such predators (but see Roubinet et al., 2018; Feit et al., 2019; Sint et al., 2019). By

combining high species resolution techniques like metabarcoding with ecological network analysis, new insights might be uncovered on the role of small vertebrates in the suppression of insect pests (Evans et al., 2016; Vacher et al., 2016; Bohan et al., 2017; Clare et al., 2019).

1.5 Metabarcoding for the study of species interactions

Metabarcoding, or in other words, barcoding of complex biological or environmental samples, is a molecular tool that has emerged with the appearance of high-throughput sequencing (Taberlet et al., 2012). The technique has revolutionized biodiversity assessments across the globe by allowing species level identifications of virtually any type of sample that contains DNA. It has been successfully used in a vast array of applications, from detection of fish, amphibians, and many other organisms in water samples (Hänfling et al., 2016; Andruszkiewicz et al., 2017; Lopes et al., 2017), to the reconstruction of paleo environments from lake sediments (Pansu et al., 2015), the characterization of aquatic and terrestrial invertebrate communities from bulk samples (Yu et al., 2012; Emilson et al., 2017), as well as to the study of species interactions from faeces and pollen samples (De Barba et al., 2014; de Vere et al., 2017). In particular, the use of metabarcoding to study species interactions has renewed the interest of the scientific community in trophic ecology, with many species' diet now having been revisited and new detailed patterns of niche segregation having been found (Kartzinel et al., 2015; Arrizabalaga-Escudero et al., 2018).

The basic principles of the method are quite simple and exciting for any ecologist, as for whatever sample one takes from the field, a list of taxa contained in that sample can be obtained. Unfortunately, the simplicity of the technique probably ends there. The methods of

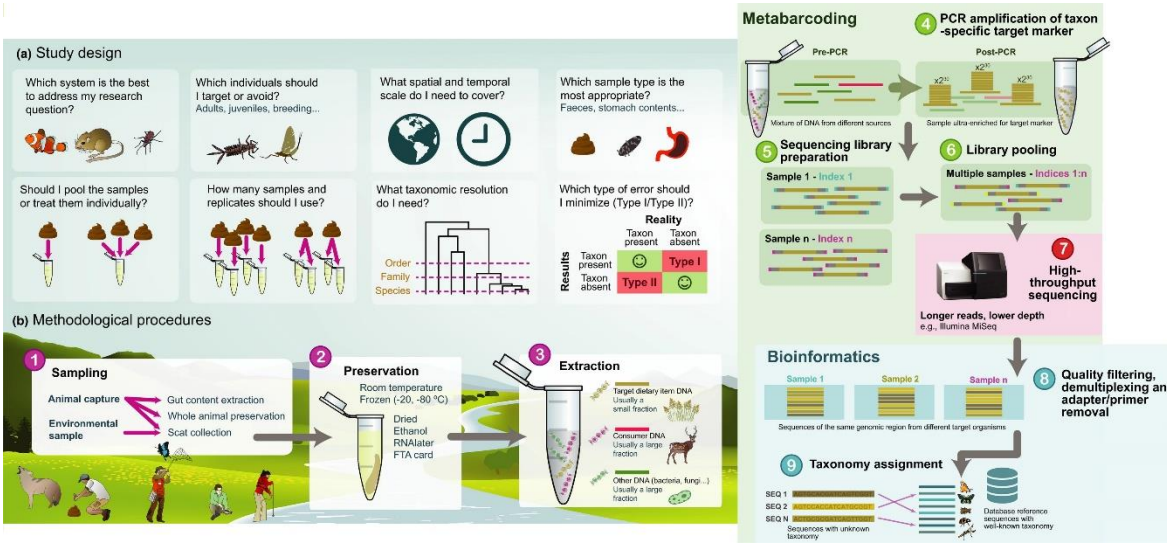

Figure 1.4 – Overall workflow in a metabarcoding study. Adapted from Alberdi et al., 2019.

a metabarcoding study can usually be divided in 4 main steps: sampling, molecular analysis, bioinformatic analysis, and finally statistical analysis. Each of these steps has a series of pitfalls and caveats that can strongly influence the final results and their interpretation, but due to the young age of the technique it is not very clear yet what are the consequences for each methodological decision that is made along the way (Alberdi et al., 2019; Figure 1.4).

As in any ecological study, sampling design has a major influence on the type of questions and answers that one can expect to make and get from the data. Studies based on unbalanced sampling schemes, and/or on a reduced number of samples, might be unable to find significant responses to ecological variables, even if they do exist (Ficetola et al., 2015). One of the problems that must be thought of by the start is that most metabarcoding analysis can only give reliable presence/absence results and not abundances (Elbrecht & Leese, 2015; Piñol et al., 2015, 2019; Krehenwinkel et al., 2017; Nichols et al., 2018). This limitation arises from issues like primer and enzyme bias, where not all species are amplified at equal rates, as well as differences between organisms' body mass and DNA content, resulting in skewed representations of species in samples. This has important consequences on the type of statistical analysis that can be applied in the end, with most abundance-based methods being left out, but also on the levels of biological replication needed. Although the number of dietary studies using metabarcoding have increased in the past few years, none have assessed how biological vs technical replicates can affect dietary descriptors. On the other hand, field collection of samples should consider possible sources of DNA contamination and employ measures to decrease them as much as possible (McInnes et al., 2017; Taberlet et al., 2018). For example, when collecting faeces from birds or bats, if the animals were kept in bags then these should be sterilized between uses to avoid cross-contamination. Samples should also be properly stored to avoid DNA degradation and minimize the risks of bacterial and fungal growth. This can be done by storing samples in ethanol or other DNA storing buffers, silica, or in a freezer.

As expected though, most of the problems and uncertainties of metabarcoding studies come from the molecular and bioinformatic analysis (Figure 1.5). DNA extraction methods are known to cause differences in species composition, with different sampling and homogenization protocols and extraction reagents leading to slightly different sampling disruption efficiencies, DNA recovery rates, as well as proportion of PCR inhibitors and thus amplification success rates (Deiner et al., 2015; Marquina et al., 2019; Martins et al., 2019). As with sampling, DNA extraction methods should try to minimize sources of contamination, as well as include negative controls in order to track possible sources of alien DNA (Taberlet et al., 2018). Amplification of the extracted DNA is probably the most sensitive step of the entire procedure. Here, the choice of appropriate primers can block researchers for months in

the

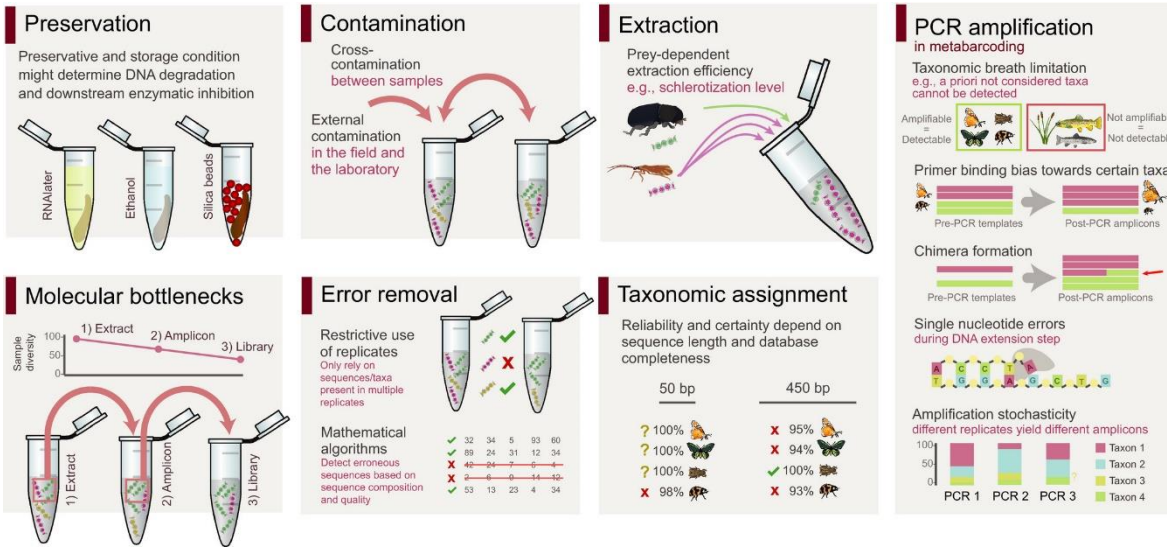

Figure 1.5 – Sources of technical distortion during molecular and bioinformatic analysis of samples. Source: Alberdi et al., 2019

ever-ending chase for the most non-biased primer pair, that allows both the amplification of the highest number of taxa with the highest taxonomic resolution and for which barcode reference databases exist. Unfortunately, the perfect barcoding marker does not exist, as the requirements that allow for high taxonomic resolution (highly variable region of DNA between different species) is often incompatible with highly conserved regions for primer design (Taberlet et al., 2018). Depending on which characteristic of the primer is selected (high species resolution vs universality), different problems might follow. In general, high taxonomic resolution primers will cause higher primer bias, while universal primers will have poorer taxonomic resolution (but often higher phylogenetic signal). The choice of one over the other will depend on the biological question, and in some cases the solution might be using multiple primer pairs and combining the information of all of them (De Barba et al., 2014; Zhang et al., 2018; Hajibabaei et al., 2019). This last option is often the only way to have a comprehensive characterization of the entire community under study, but brings additional methodological challenges, as how to integrate data with different taxonomic resolution.

Finally, bioinformatic analysis is probably the most challenging step for many researchers, as up to now there is no user-friendly software that can process next-generation sequencing data from raw sequencing reads to final taxa list per sample. Most of the developed pipelines only run on Linux based systems and by the means of command lines with no visual interface. This has led to a diversity of pipeline options, independently developed by different teams, working with different topics, and using different laboratorial procedures, with no established standards existing yet. Although not the focus of this thesis, bioinformatic pipeline decisions can have serious effects on the final data used for analysis (Coissac et al.,

2012; Alberdi et al., 2018). Things like PCR and sequencing errors, chimeric sequences, tag jumping events, as well as wrong species assignment can lead to overestimates of species diversity as well as wrong sample assignment of sequences. Although most studies do try to be transparent and thoroughly describe what was done, like in many other decision steps, it is not entirely clear what are the consequences of each option and the level of error that it introduces in dietary descriptors.

Albeit all these uncertainties, metabarcoding stands as a powerful tool that if used robustly can provide unprecedented information on species interactions. Further understanding of the consequences of each methodological decision will undoubtedly help in defining future standard procedures.

1.6 Objectives

The overall goal of this thesis is to advance the use of metabarcoding techniques in the study of species interactions, thereby providing a useful tool for managing complex landscapes towards multifunctionality. This general goal encompasses not only a methodological approach in which advancements in the experimental design and technical approach are sought, but also an applicated component where the technique is used to explore the trophic ecology and ecosystem services of species. In this context, this thesis specifically aims to:

1 – To understand the impacts of technical and biological replication on the results of metabarcoding dietary analysis. In particular, the thesis aims to evaluate how variability among (i) individuals, (ii) faecal pellets of the same individual, and (iii) PCRs of each pellet, affect estimates of diet diversity and composition, and on the frequency of occurrence of the prey items. It also aims to test the effects of analysing pools of samples versus separate samples per individual, as these two variants are often used in dietary studies.

2 – To test the use of multiple overlapping markers in metabarcoding dietary analysis, as well as to define criteria for integrating this data. Specifically, the thesis aims to assess differences between morphological, single marker and multi-marker approaches in the estimates of dietary descriptors, in terms of (i) taxonomic resolution, (ii) diet diversity, (iii) the identity of taxa recorded, and (iv) the composition of diet considering the taxa recorded and their representation in the samples.

3 – To show the power of taxonomically resolved dietary data made possible by metabarcoding to reveal subtle intraspecific variations in predator-prey relationships by small vertebrates, which may affect both their ecology and their role in ecosystems. The study focused on the European free-tailed bat *Tadarida teniotis*, evaluating how predation on

arthropod prey varied between sexes. While controlling for factors like age and sampling season, the thesis evaluated differences between males and females in relation to (i) prey species composition, (ii) prey species richness; (iii) prey size; and (iv) prevalence of migratory moth species.

4 – To evaluate the role of individual species in in sustaining pest regulation services, thereby illustrating the potential of metabarcoding as a tool for improving the management of multifunctional landscapes. By combining metabarcoding with ecological networks, the thesis aimed to understand how different bat species potentially contribute to the control of multiple agricultural and forest pests. The goal was to find a reduced subset of species that are particularly important for arthropod pest suppression, and that thus might need to be managed to achieve the ecological intensification of ecosystem services.

1.7 Thesis Outline

The thesis is organised in six chapters. The first chapter corresponds to the general introduction, setting the context and objectives of the thesis. The next four chapters correspond to papers published (three) or to be published (one) in international scientific journals, detailing the findings of the research carried out during the thesis. Chapters 2 and 3 are more technical, describing methodological developments that need to be considered when using metabarcoding to study insectivore diets and predation networks. Chapters 4 and 5 then use metabarcoding techniques to advance our knowledge on the interactions between insectivore predators and their prey, particularly focusing on insect pests. Finally, the last chapter provides a general discussion of the thesis, as well as further directions of research in the field. Below I present a short summary of chapter.

Chapter 1 describes the conceptual underpinning of the thesis, providing a general introduction on the effects of land-use change on biodiversity collapse and the provision of ecosystem services, particularly in agricultural landscapes. It also shows how metabarcoding can help address the challenges of sustainability in multifunctional landscapes, providing also a summarized view on its limitations and caveats, particularly in the scope of dietary studies. Finally, the chapter describes the main objectives of the thesis and the thesis structure.

Chapter 2 assesses the impacts of technical and biological replication on the results of metabarcoding dietary analysis, focusing on the European free-tailed bat (*Tadarida teniotis*). By using an orthogonal set up composed of 20 bat individuals, 15 individual pellets and a pool of 15 pellets, we investigated how diet descriptors as prey diversity, frequency of occurrence and diet composition, were affected by variability among (i) individuals, (ii) pellets of each individual, and (iii) PCRs of each pellet. In addition, we investigated the impact of (iv)

analysing separate pellets versus pellet pools. Overall, we found that most variation in diet comes from differences in individuals and that PCR replicates contain little variation compared to biological replicates. Also, analysing multiple individual pellets per individual, even if just two or three, provides higher prey diversity than using pools of pellets. In the end, our results stress that maximizing biological replication is critical in dietary metabarcoding studies. This paper was published in Molecular Ecology (Mata et al., 2019), and is already a highly cited paper (top 1% of its academic field).

Chapter 3 focuses on the problems of analysing the diet of generalist species through metabarcoding, in particular the need of using multiple markers to cover their dietary breadth. Specifically, the paper shows how to integrate multiple markers when they partly overlap in the taxa amplified, and vary in taxonomic resolution, biases and representation in databases. To answer this, we analysed the contents of 115 faeces from a generalist passerine, the Black Wheatear (*Oenanthe leucura*), using 4 molecular markers along with visual identification of prey fragments. We developed a python script to consistently merge the information obtained using each method and found that each individual method varied greatly in its capacity to detect prey. Integration of multi-marker data provided far more detailed dietary information than any single marker and estimated higher frequencies of occurrence of all taxa, stressing the value of integrating data from multiple, taxonomically overlapping markers. Yet, high levels of secondary predation of plants were detected with metabarcoding, and thus we recommend that for generalist species other sources of information are used to help guiding the interpretation of metabarcoding results. This paper was published in Molecular Ecology Resources (da Silva et al., 2019), with Luís P. da Silva and Vanessa A. Mata as joint first authors.

Chapter 4 uses metabarcoding to investigate interactions between a widespread bat species and its arthropod prey. Specifically, the paper focuses on gender-related variation in diet composition of the European Free-tailed bat (*Tadarida teniotis*), a moth specialist bat. For that we analysed guano pellets collected from 143 individuals mist-netted from April to October 2012 and 2013, in north-east Portugal, and indeed found that moths were by far the most frequently recorded prey, occurring in nearly all samples and accounting for most prey taxa. We also found significant dietary differences between males and females, irrespective of age and season. Compared to males, females tended to consume larger moths and more moths of migratory behaviour, known to be rich in fat reserves. Our study provides the first example of gender-related dietary variation in bats, illustrating the value of novel molecular tools for revealing intraspecific variation in food resource use in bats and other insectivores. This paper was published in Biology Letters (Mata et al., 2016), receiving 29 citations (Web of Science) as of December 2019.

Chapter 5 addresses the use of ecological networks coupled with DNA metabarcoding to assess the role of bats in pest control services. To do this, we sampled a community of bats composed by 19 species and identified the pest species that they fed on. Our approach revealed a complex interaction network involving 132 different pest species across the landscape. We found that just six generalist bats potentially regulated over three quarters of the pests, though functional redundancy within the community was high. Some pests were potentially regulated only by a few trophic specialists with high niche differentiation. Our approach underlines the functional importance of conserving diverse vertebrate communities in multifunctional landscapes, while identifying candidate species that could be favoured to intensify the control of pests. This paper is expected to be submitted in February 2020, after revision by all co-authors.

Chapter 6 presents the main conclusions of the thesis and discusses the main findings of both technical and ecological research chapters. In particular, the implications for further establishment of metabarcoding techniques in the study of species interactions is discussed, along with possible methodological alternatives that could slightly overcome its limitations. Finally, future research related to intra-specific variation in species' diet and the powerfulness of combining metabarcoding with ecological networks of species interactions for a better understanding of ecological communities and their contribution to agroecosystems is also discussed.

Chapter 2

How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis

Vanessa A. Mata, Hugo Rebelo, Francisco Amorim, Gary F. McCracken, Simon Jarman, Pedro Beja

Mata V.A., Rebelo H., Amorim F., McCracken G.F., Jarman S., & Beja P. (2019) How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, 28(2), 165–175. https://doi.org/10.1111/mec.14779

Keywords: metabarcoding; sampling design; replication; molecular diet analyses; bat ecology; trophic ecology

Abstract

DNA metabarcoding is increasingly used in dietary studies to estimate diversity, composition, and frequency of occurrence of prey items. However, few studies have assessed how technical and biological replication affect the accuracy of diet estimates. This study addresses these issues using the European free-tailed bat Tadarida teniotis, involving high-throughput sequencing of a small fragment of the COI gene in 15 separate faecal pellets and a 15-pellet pool per each of 20 bats. We investigated how diet descriptors were affected by variability among (i) individuals, (ii) pellets of each individual, and (iii) PCRs of each pellet. In addition, we investigated the impact of (iv) analysing separate pellets versus pellet pools. We found that diet diversity estimates increased steadily with the number of pellets analysed per individual, with seven pellets required to detect ~80% of prey species. Most variation in diet composition was associated with differences among individual bats, followed by pellets per individual, and PCRs per pellet. The accuracy of frequency of occurrence estimates increased with the number of pellets analysed per bat, with the highest error rates recorded for prey consumed infrequently by many individuals. Pools provided poor estimates of diet diversity and frequency of occurrence, which were comparable to analysing a single pellet per individual, and consistently missed the less common prey items. Overall, our results stress that maximizing biological replication is critical in dietary metabarcoding studies, and emphasize that analysing several samples per individual rather than pooled samples produce more accurate results.

2.1 Introduction

The study of animal predator diets has an old and rich history in ecology (e.g. Elton, 1927; Valverde, 1967), contributing to the understanding of species interactions, food web structure, and the mechanisms driving populations and ecosystem dynamics (Layman et al., 2015; Nielsen et al., 2018). The advent of DNA-based molecular tools for the identification of complex multi-taxa samples, i.e., metabarcoding, has greatly renewed the interest in dietary studies, particularly due to the high taxonomic resolution offered by this approach (e.g. De Barba et al., 2014; Kartzinel & Pringle, 2015; Lopes et al., 2015). This has been especially relevant to species whose diet is particularly difficult to study, either due to their secretive behaviour (e.g. Shehzad et al., 2012; Soininen et al., 2015) or to difficulties to identify prey in dietary remains such as stomach contents, regurgitates, and scats (e.g. Arrizabalaga-Escudero et al., 2015; Kaunisto, Roslin, Sääksjärvi, & Vesterinen, 2017; Mollot et al., 2014). However, despite its increasingly widespread use, uncertainties and potential biases associated with the quantification of diets based on metabarcoding are still not well understood, requiring a detailed enquiry on how results are affected by different methodological options (Alberdi et al., 2018; Nielsen et al., 2018).

Diet studies aim to answer three main types of question about animal populations: (i) dietary diversity, generally the number of different prey species consumed; (ii) dietary composition, i.e. the identity of the prey species consumed; and (iii) the contribution of each prey species to the diet, quantified as the proportion in numbers, biomass or energetic content (e.g. Baker, Buckland, & Sheaves, 2014; Klare, Kamler, & MacDonald, 2011; Whitaker Jr., McCracken, & Siemers, 2009). Surprisingly, there is a significant knowledge gap on the ability of metabarcoding-based studies to provide accurate estimates of dietary descriptors, particularly under field conditions and involving species with diverse diets (Nielsen et al., 2018). Despite this paucity of quantitative studies, researchers often recognise that metabarcoding can be strongly influenced by numerous factors, which should be accounted for in dietary studies. For instance, dietary descriptors can be strongly affected by amplification bias due to unequal primer binding, which leads to systematic over- or underestimation of the importance of some prey types relative to others (Clarke et al., 2014). Also, “universal” barcoding markers are not necessarily good metabarcoding markers, and one often has to trade taxonomic resolution for taxonomic range and vice-versa (Clarke et al., 2014; Deagle et al., 2014; Albaina et al., 2016), though this problem is ameliorated to some extent by recent degenerate primer versions (e.g. Alberdi et al., 2018). Taxonomic assignments of amplicon sequences are frequently limited by poor reference databases for most taxonomic groups and

localities (Bohmann et al., 2011), therefore hampering data interpretation. Another problem is the imperfect correlation between the proportions of sequencing reads and biomass, making it hard to establish the contribution of each prey item to the overall diet (Deagle et al., 2013; Elbrecht & Leese, 2015; Piñol et al., 2015). Because of this, metabarcoding studies generally quantify diet in terms of frequency of occurrence (e.g. Biffi et al., 2017; Kartzinel & Pringle, 2015; Mata et al., 2016), though this does not necessarily reflect the relative dietary intake of different prey items in terms of numbers, biomass or energy (e.g. Foster, Harmsen, & Doncaster, 2010; Greenstone et al., 2010; Sheppard et al., 2005).

An important aspect often missed in metabarcoding dietary studies is the impact of both technical and biological replication on final results. Technical replication, i.e. the number of extractions and PCRs carried out on each sampling unit, is important because both extractions and PCRs have a random component, and a given prey item may be missed in some replicates even if it was present in the original sample. These false negatives are expected particularly if an item's DNA is scarce or if there is a negative primer bias (Willerslev et al., 2014; Ficetola et al., 2015; Pansu et al., 2015). Biological replication, i.e., the number of sampling units analysed per species, including for instance the number of individuals or the number of samples per individual, is important because the number of prey species detected tends to increase with the number of samples analysed. Lack of sufficient biological replication can be detected by either rarefaction or asymptotic species richness estimators, which identify sample sizes as being too small to characterize the biodiversity in a sample (Gotelli & Colwell, 2001). Likewise, the precision of frequency of occurrence estimates is low when biological replication is low, and it varies with the prevalence of the prey items, and thus a poor description of diet may occur at low sample sizes as a mere consequence of binomial sampling (Trites & Joy, 2005). These problems are worse when there is high variation in diet composition among individuals according for instance to gender, age, or individual preferences (e.g. Mata et al., 2016; Pagani-Núñez, Valls, & Senar, 2015; Pleguezuelos & Fahd, 2004), and there may also be intra-individual variations due for instance to temporal changes in prey availability (Burgar et al., 2014; Clare et al., 2014b, 2014a).

Here we address the impacts of technical and biological replication on the results of metabarcoding dietary analysis, focusing on the European free-tailed bat (*Tadarida teniotis*). This species was considered suitable because previous studies (Rydell & Arlettaz, 1994; Mata et al., 2016) have shown that it is a specialist predator of moths (Lepidoptera), and thus may be less affected by problems of primer bias than species feeding on a wider range of taxonomic groups. Furthermore, moths are well represented in reference barcode databases, which reduces problems due to unidentified MOTUs. Finally, metabarcoding dietary studies have often focused on bats (e.g. Arrizabalaga-Escudero et al., 2015; Hope et al., 2014; Razgour et al., 2011), thus making it possible to evaluate the implications of our results in the

context of widely used replication options. In this study, we evaluate how variability among (i) individual bats, (ii) faecal pellets of each bat, and (iii) PCRs of each pellet affect estimates of diet diversity and composition, and on the frequency of occurrence of the prey items. Also, we tested the effects of analysing pools of samples versus separate samples per individual, as these two variants are often used in dietary studies (e.g. *pools*: Burgar et al., 2014; Clare, Symondson, Broders, et al., 2014; Clare, Symondson, & Fenton, 2014; Krauel, Brown, Westbrook, & McCracken, 2018; *individuals*: Hope et al., 2014; Mata et al., 2016; Vesterinen, Lilley, Laine, & Wahlberg, 2013). Our results were used to analyse the level of replication required to obtain accurate descriptions of predator diets using metabarcoding.

2.2 Materials and methods

2.2.1 Study design

This study was based on the dietary metabarcoding analysis of 20 European free-tailed bats (*Tadarida teniotis*), using both a 15-pellet pool and 15 separate pellets per bat, and three PCR replicates per each pool and pellet (Figure 2.1). The number of individuals analysed is within or close to the range used in previous studies investigating for instance trophic structure in bird and bat assemblages Razgour et al., 2011; Burgar et al., 2014; Emrich et al., 2014; Sedlock et al., 2014; Crisol-Martínez et al., 2016. The number of pellets analysed separately for each individual is much larger than that of previous studies, which analysed either a single pellet or a pool of pellets per bat. The number of PCRs per sample is within the range (2-4) of recent studies using multiple PCRs Biffi et al., 2017; Galan et al., 2018, though the large majority of dietary studies has been based on a single PCR per sample (e.g. Burgar et al., 2014; Crisol-Martínez et al., 2016; Emrich et al., 2014; Razgour et al., 2011; Sedlock et al., 2014). Metabarcoding was carried out separately for each combination of bat x pellet (or pool) x PCR, yielding 960 sampling units, for which we recorded the presence/absence of each prey species. To investigate the effects of pellet sample size on the results of dietary

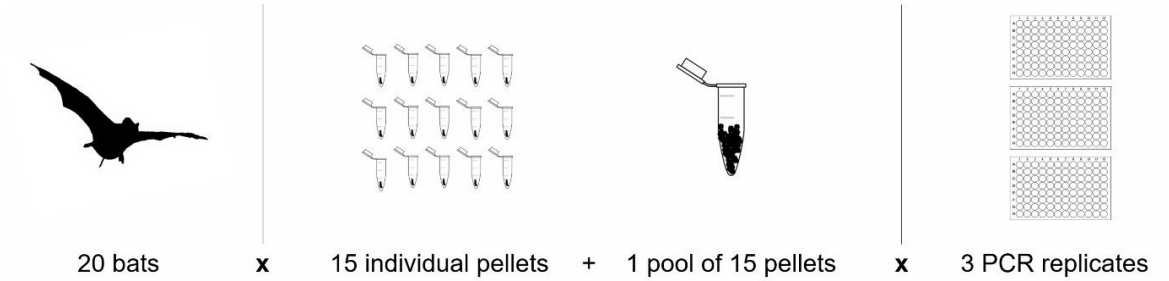

Figure 2.1 – Scheme of experimental design, indicating that analyses were based on faecal pellets collected from 20 bats, with 15 separate pellets and a pool of 15 pellets per bat, and three PCR replicates per pellet/pool (n=960 sampling units).

studies, we selected randomly one PCR replicate per pellet (320 sampling units), and quantified how increasing the number of pellets analysed affected estimates of both diet diversity and the frequency of occurrence (FO) of the most important prey species. Also, we compared diet diversity and FO estimates for separate pellets and pooled samples. Finally, we used the overall sample to quantify the contribution of variation among individual bats, pellets and PCR replicates to variation in diet composition.

2.2.2 Bat pellet sampling

European free-tailed bats (*Tadarida teniotis*) were mist-netted at their roosts in five bridges located in northeast Portugal (N41º09' – 42º00'), in April–October 2012 and 2013, under an on-going monitoring programme Amorim et al., 2015. Individual bats were placed in clean cotton bags, from where guano pellets were collected. We recorded gender, age (juveniles versus adults) and sampling date of each individual. Pellets were stored in tubes containing silica-gel and refrigerated at 4 ºC until DNA extraction. Pellets from a subset of 143 individuals were used in a previous study to describe the diet of European free-tailed bats Mata et al., 2016, while for the present study we selected the pellets from a different subset of 20 individuals that had left more than 30 guano pellets in the same capture event.

2.2.3 Molecular analysis

We extracted DNA from each sample using the Stool DNA Isolation Kit (Norgen Biotek Corporation) following the manufacturer's protocol. Samples were extracted in batches of 23 plus a negative control in which no sample was added. Samples and negative controls were distributed in four 96-well plates and kept in a freezer at -20 ºC until further use. DNA amplification was done using the COI primers ZBJ-ArtF1c and ZBJ-ArtR2c Zeale et al., 2011, modified to contain Illumina adaptors and a 5 bp identification barcode. Each plate was then amplified in three independent reactions (replicates) with amplification primers containing different barcode sequences. The PCR reactions were carried in volumes of 10 µl, comprised of 5 µl of QIAGEN Multiplex PCR Master Mix, with 0.3 µl of each 10 pM primer, and 1 µl of DNA extract. Cycling conditions used initial denaturing at 95 °C for 15 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel. Library preparation followed the manufacturer's protocol for metagenomic sequencing (Illumina). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter) and subsequently quantified using Nanodrop and diluted to similar concentrations. All the 12 cleaned PCR plates were then

pooled into a single plate, as each plate contained a different barcode. Illumina indexes were added to the cleaned PCR products using the Nextera XT Kit (Illumina), allowing individual identification of each amplified product. Indexed samples were again cleaned and then pooled at equimolar concentrations and sequenced using a whole v2 run of a MiSeq desktop sequencer (Illumina; ~0.1% coverage per sample). To test for the effect of sequencing depth on individual and pooled pellets, an additional MiSeq run was used, where one pellet and a pool were selected per individual and sequenced at “low coverage” (0.1%) and “high coverage” (1.5%). The actual coverages achieved are provided in Supplementary Table S2.1.

2.2.4 Bioinformatics and prey identification

We used OBITools (Boyer et al., 2016) for general sequence processing. Briefly, paired-end reads were aligned and assigned to samples, barcodes and primers were removed, and finally, sequences were collapsed into haplotypes. Singletons were removed, as well as sequences smaller than 155 bp and longer than 159 bp. The remaining haplotypes went through ‘obiclean’, a method that allows the removal of haplotypes differing 1 bp from each other, if one has a higher read count than the other in every sample. From each PCR, we further removed haplotypes representing less than 1% of the total number of reads and those containing stop codons. We then compared the haplotypes retained against known sequences within the BOLD database (www.boldsystems.org) and unpublished sequences of arthropods collected in northern Portugal. Haplotypes that best matched the same species were collapsed into a single taxon unit. For the haplotypes for which only family, order, or class level identification was possible, a neighbour-joining tree was built with all haplotypes in order to cluster similar sequences (> 98% similarity) into distinct taxa (e.g. Cerambycidae haplotypes with divergences above 98% among them were clustered into Cerambycidae 1, Cerambycidae 2, and so on). Although this approach may artificially increase the number of taxa present in some cases, it was taken to avoid removing from further analysis taxa that are less represented on BOLD and for which genus or species level identification is often not possible.

2.2.5 Data analysis

We analysed how pellet sample size affected estimates of diet diversity by building species accumulation curves per individual, as a function of the number of pellets analysed Colwell & Coddington, 1994. We used both the actual number of species recorded and the Chao2 estimator of species richness (Chao & Chiu, 2016). We then averaged estimates for each pellet sample size across the 20 bats analysed, to produce a mean species accumulation

curve per individual. Estimates along this curve were compared to richness estimates obtained from analysis of a pellet pool per individual. To evaluate the effects of sequencing depth, we tested for the difference in species richness in estimates based on one pellet and on a pool of 15 pellets, both at low and high coverage. We used generalized mixed linear models (GLMM) with logit link and binomial errors, specifying individual bats as the random component, to test whether the probability of detecting a given prey item in pools was related to its frequency of occurrence in the sample of separate pellets (FOpel). Accumulation curves were carried out using the 'iNEXT' package Hsieh et al., 2016a, and GLMMs were implemented using lme4 (Bates et al., 2015).

The contribution of biological and technical replication to variation in diet composition were analysed using PerMANOVA (Anderson, 2001). Specifically, we modelled the contribution of three independent components: (i) bats, (ii) pellets within bats, and (iii) PCRs within pellets within bats to the observed differences in species composition among sampling units. The contribution of each component while controlling for differences in degrees of freedom was estimated from the corresponding mean sum of squares (MSS). We used a nested design because we were interested in how analysing several pellets per individual contributed to variation in estimates of diet composition, and not in actual dietary variation among pellets. Likewise we were interested on the contribution of variation among PCRs of the same pellet, and not on variations among PCRs *per se*. As a measure of the statistical significance of each component we used an *F*-statistic estimated with a permutation procedure (10,000 permutations), based on randomizations of the residuals of the "reduced" model (randomized residual permutation procedure – RRPP). We also used PerMANOVA to test for significant differences in prey composition inferred from pools of 15 pellets and 15 separate pellets. PerMANOVA was implemented using the function 'procD.lm' of the 'geomorph' package (Adams et al., 2017).

The effects of the number of pellets analysed per individual on frequency of occurrence (FO) estimates of each prey species at the level of the overall sample (20 individuals) were evaluated using a simulation approach. Specifically, from each bat we randomly sampled from n = 1 to 14 pellets from the overall pellet sample, to generate the empirical distribution of FO estimates at each sample size. For instance, when n = 2 pellets, we sampled with replacement two pellets from the pool of 15 pellets analysed per bat, for all bats, and then estimated the FO of a given prey species from the proportion of bats in which that species was detected. Repeating this procedure 10,000 times produced the empirical distribution of FO estimates for n = 2 pellets. We then computed the estimation error for each n, as the simple difference between the FO estimated when using 15 pellets per bat, and the FO estimated using n pellets per bat. To further understand the sources of variability in FO estimates, we modelled the estimation error per pellet sample size and prey species, in relation to the number of pellets

analysed, the FO of that prey in the sample of 20 bats estimated using 15 pellets per individual (FOtot), the average frequency of occurrence of that prey species within individuals that consumed it (FOpel), and the first and second order interactions between the main effects, also using 15 pellets per individual. FOtot was used to investigate whether error rates tended to be systematically lower (or higher) in prey consumed frequently by the population, whereas FOpel was used to investigate whether error rates tended to be systematically lower (or higher) in prey that were frequently consumed by particular individuals, though not necessarily at the population level. We also used beta regression to estimate whether the error rates of FO estimates in pools varied in relation to FOtot and FOpel. Simulations were implemented in the R script described in Supplementary Material, while beta regression was carried out using the 'betareg' package (Cribari-Neto & Zeileis, 2010).

2.3 Results

Metabarcoding of free-tailed bat faecal pellets detected 153 taxa from nine insect orders, of which 65.4% were Lepidoptera (Supplementary Table S2.2). Most taxa (77.1%), including 95% of the Lepidoptera, were unambiguously assigned to a single species or to a group of two or three closely related species within the same genus. The seven species with the highest frequencies of occurrence (> 20% of pellets) were all moths of the family Noctuidae: *Mythimna vitellina* (70.3%); *Autographa gamma* (64.3%); *Agrotis segetum* (45.3%); *Peridroma saucia* (35.7%); *Noctua pronuba/janthe* (28.7%); *Phlogophora meticulosa* (25.3%); and *Hoplodrina ambigua* (23.7%).

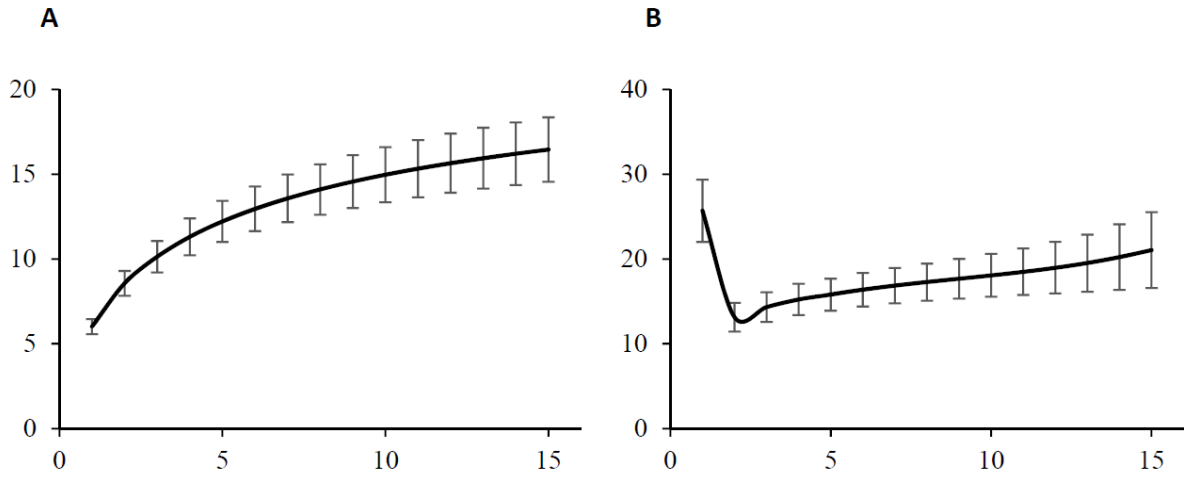

Figure 2.2 – Accumulation curves for the number of (A) detected and (B) estimated (Chao2) prey species per bat, when varying the number of pellets analysed from one to 15. The curves show averages across 20 individual bats analysed and error bars are the standard errors of mean estimates.

The estimates of diet diversity per individual were strongly affected by the number of pellets analysed, either when using the actual number of species detected or Chao2 species richness estimator (Figure 2.2). On average, it was necessary to analyse seven and 12 pellets to record about 80% and 95%, respectively, of the species detected in the overall sample of 15 separate pellets per bat. Estimates of diet diversity per bat were much lower (paired sample t-tests: t = 6.03, df = 19, P < 0.0001) in pooled samples of 15 pellets (mean ± SD: 5.0 ± 1.7) than in 15 pellets analysed separately (16.3 ± 8.4). Actually, either for low or high sequencing depth, the average number of species detected was not significantly different (*low*: t = 4.07, df = 19, P = 0.176*; high:* t = 1.26, df = 19, P = 0.222) in a pool of 15 pellets (*low:* 5.3 ± 1.8*; high:* 5.4 ± 1.8) and in a single pellet (*low:* 6.3 ± 3.9*; high:* 6.2 ± 3.7). The GLMM indicated that the probability of detecting a given prey item in a pool was strongly related to its frequency of occurrence in the diet estimated from the 15 separate pellets per individual (Regression coefficient [FOpel] = 5.958, SE = 0.6795, z = 8.768, *P* < 0.001; Figure S1 in Supplementary Material).

PerMANOVA showed that variation in species composition among sampling units was significantly affected by variation among individuals, pellets within individuals, and PCRs within pellets (**Table 2.1**). However, the highest variation in the identity of species consumed was associated with the individual bats (MSS = 8.63). Variation associated with pellets within individuals was much lower (MSS = 0.56), but still about thirteen times higher than variation associated with PCRs within pellets (MSS = 0.04), indicating that there was little variation in the identity of species retrieved from replicate PCRs of the same pellet. PerMANOVA also showed significant differences in diet composition between the pools of 15 pellets and the 15 pellets analysed separately (*F* = 2.20, R^2 = 0.0547*, P*=0.003).

Variation in the mean frequency of occurrence (FO) estimates in relation to the number of pellets analysed per individual showed a consistent pattern, being strongly underestimated when the number of pellets analysed was low, and progressively converging to the “true” value with increasing pellet sample size (Figure 2.3). Accordingly, the mean error rates of the

Table 2.1 – Summary results of PerMANOVA estimating the contributions of individuals, pellets within individuals, and PCRs within pellets, to overall variation in diet composition. Statistical significance was estimated from randomized residual permutation procedure, with 10,000 permutations.

Coefficient	df	SS	MS	R^2	F	p-value
Individual	19	163.93	8.6280	0.4703	21.6758	**0.0001**
Individual:Pellet	280	158.15	0.5648	0.4538	2.7493	**0.0001**
Individual:Pellet:PCR	600	26.45	0.0441	0.0759	1.4981	**0.0001**
Residuals	0	0				
Total	899	348.54				

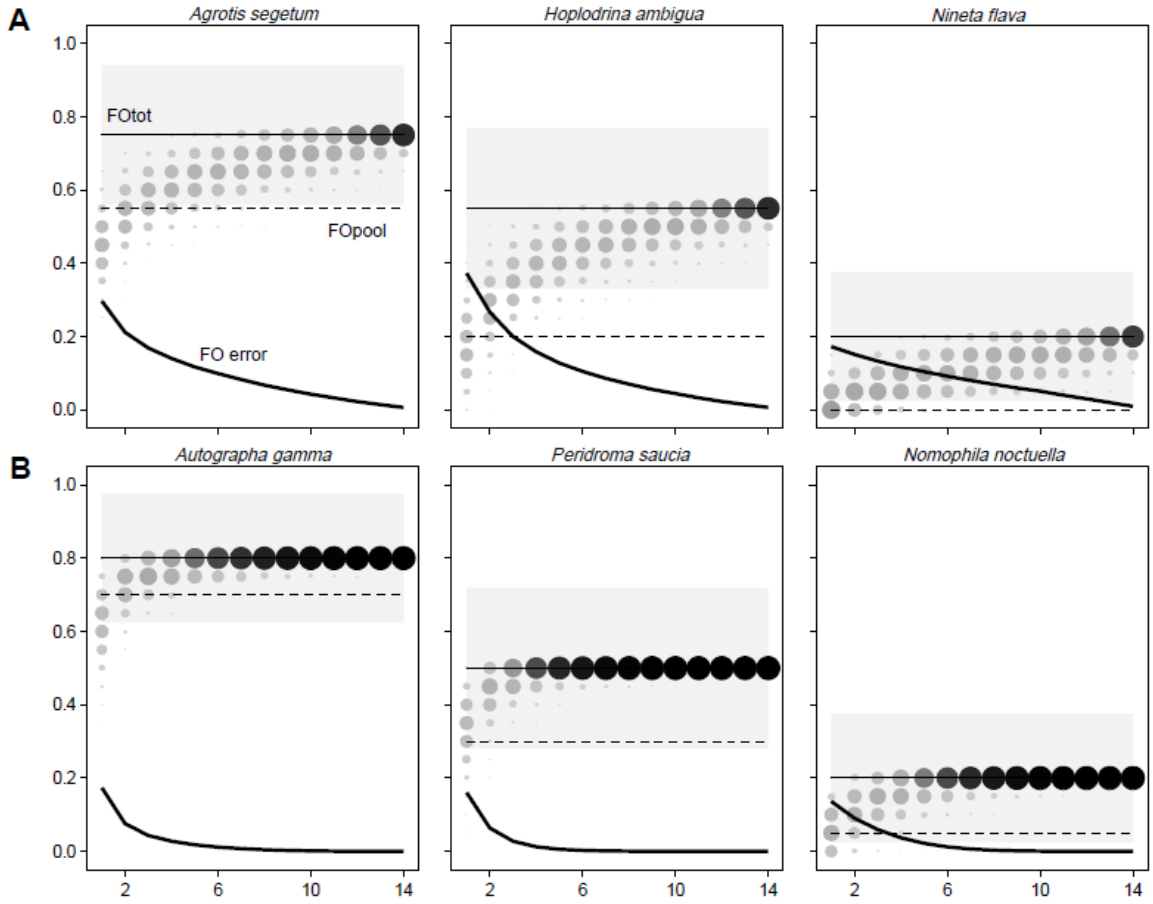

Figure 2.3 – Variation in the empirical distribution of frequency of occurrence (FO) estimates (circles), in relation to the number of pellets analysed per individual, for prey items with low (A) and high (B) intra-individual FO (FOpel). Thin black lines are the FO of prey items estimated from the analysis of 15 pellets per individual (FOtot), and light shaded areas the corresponding binomial confidence interval. Thick black lines represent the mean error of FO estimates. Dashed lines represent estimated FO from pools (FOpool).

estimates were particularly high when just one or two pellets were analysed per bat, but they declined thereafter. The Beta regression model indicated that variation in the error rates of FO estimates was largely accounted for (Pseudo R-squared = 0.84) by the significant effects of variation in the number of pellets analysed, and the frequencies of occurrence of the prey item in the sample of 20 bats (FOtot) and in the sample of 15 pellets per bat (FOpel) (Supplementary Table S2.3). The error rates always declined with the number of pellets analysed per individual, but for a given sample size the error rates tended to be higher for species with high FOtot (i.e., prey items consumed frequently by the population), and that they tended to be lower for species that had higher FOpel (i.e., prey items consumed frequently by particular individuals; Figure 2.4). The mean error rate of FO estimates was much higher ($t = -29.35$, $df = 134$, $P < 0.0001$) in pool samples of 15 pellets (82.4% ± 32.5%) than in 14 pellets analysed separately (2.2% ± 2.9%). Regarding sequencing coverage, either for low or high sequencing depth, the error rates were similar, but significantly higher (*low:* $t = -3.13$, $df = 134$,

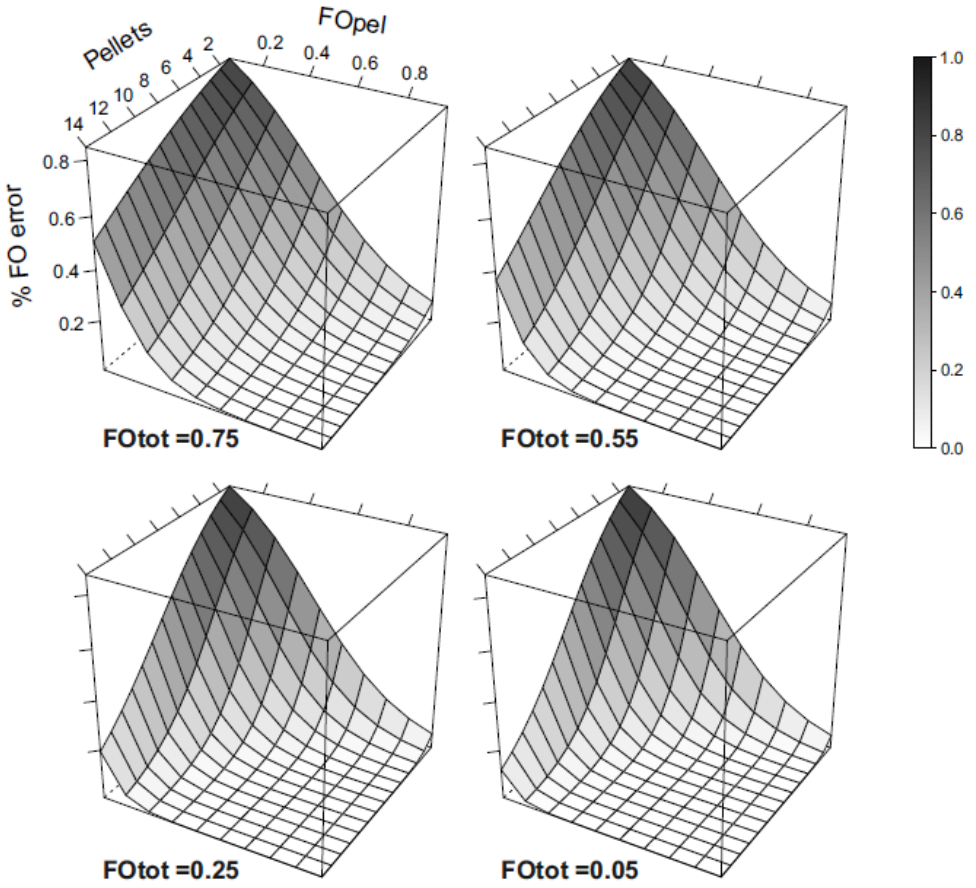

Figure 2.4 – Fitted responses surfaces inferred from a Beta regression model showing how the error rates of frequency of occurrence (FO) estimates of prey items in the diet of European free-tailed bats varied in relation to the number of pellets analysed and the mean frequency of occurrence of the prey items within individuals that consumed that prey (FOpel), at four levels of the frequency of occurrence of the prey items in the overall bat sample (FOtot, n = 20).

P = 0.002*; high:* t = -2.46, df = 134, P = 0.015) in a pool of 15 pellets (*low:* 80.5% ± 34.0%*; high:* 79.5% ± 34.5%) than in a single pellet (*low:* 69.5% ± 40.7%*; high:* 71.6% ± 40.0%). Beta regression indicated that FOpel was the main factor affecting variation in the error rate of pool FO estimates across prey items (Figure 2.5, Supplementary Table S2.4).

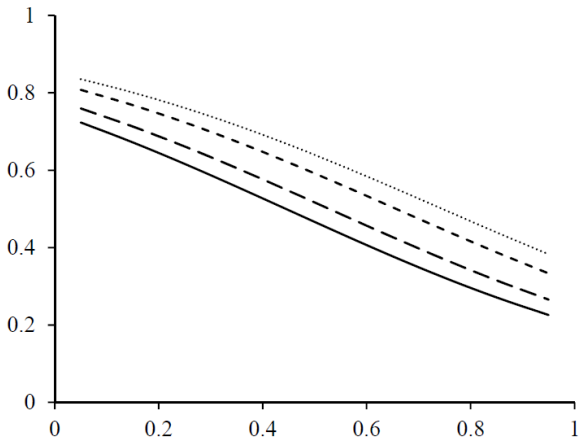

Figure 2.5 – Fitted responses curves inferred from a Beta regression model showing how the error rates of frequency of occurrence (FO) estimates of prey items in pooled samples varied in relation to the frequency of occurrence within the pellets of individual bats (FOpel), at four levels of the frequency of occurrence of the prey items in the overall bat sample (n = 20; FOtot = 0.75, 0.55, 0.25, 0.05 for black line, large dash line, small dash line, and point line, respectively).

2.4 Discussion

The results of our empirical case study focusing on the European free-tailed bat clearly shows the impact of technical and biological replication on the results of metabarcoding studies of animal predator diets. Specifically, we show strong effects of: (i) the number of samples analysed per individual on estimates of diet diversity; (ii) the number of individuals, samples per individual and, to a much lesser extent, the number of PCRs per sample, on estimates of diet composition; and (iii) the number of pellets per individual on estimates of frequency of occurrence of prey items. Also, we show that analysing pools of samples provide much poorer results than analysing separate samples to estimate diet descriptors. Therefore, our results demonstrate the importance of the levels of biological replication for adequately describing diets using metabarcoding. These results suggest that the small sample sizes in the range currently used by many studies may be insufficient to provide robust estimates of diet descriptors. However, when species are rare or otherwise difficult to sample, more limited sampling may still be useful to provide overviews of the prey consumed.

Although our results are based on a single case study that may be affected by some idiosyncrasies and limitations, this is unlikely to affect the generality of our conclusions to a significant extent. One possibility is that our results were largely driven by the particular species studied, as it consumes a wide range of different prey items (Mata et al., 2016; this study), and thus it may require higher levels of replication than species with less diverse diets. Although diverse diets may indeed be more difficult to estimate (Nielsen et al., 2018), there are many species such as insectivore bats and birds that feed on a very wide range of taxa, and thus may be as prone to insufficient biological replication as European free-tailed bats. Another limitation is that we did not have information on the “true” diet, against which our metabarcoding results could be compared. Previous field studies have circumvented this problem by comparing metabarcoding results with those from visual or stable isotope analysis (Nielsen et al., 2018), but this is not without problems, because all methods have their own errors and biases. Therefore, these comparisons do not show which method is closer to the “truth”, but only whether different methods provide consistent results. In these circumstances, we believe that our approach of assessing how estimates of diet descriptors vary with replication levels is warranted, though further research is needed on the extent to which the method provides accurate estimates of what is actually eaten by free-ranging animals. Finally, our study was based on the analysis of just 20 bats, with all pellets of each bat collected in the same night, and thus it might be argued that our own study had insufficient biological replication. Although this sample size is comparable to that of previous studies, we recognise

that it may be insufficient to describe in detail the diet of European free-tailed bats. However, it highlights the difficulties of accurately estimating what 20 individuals have eaten during a single night, thereby emphasising the challenges of inferring diets for entire populations over long time frames. This problem is not restricted to DNA metabarcoding studies of diet, however, except that their increased sensitivity of detection will make real biological variation in diet more detectable. Population diet is an inherently complicated ecological trait to characterize by any methodology and this has been noted in the past for many dietary studies using different methodologies (Nielsen et al., 2018).

Our results support the view that technical replication affects the estimates of diet descriptors (e.g. Alberdi et al., 2017; Pansu et al., 2015; Willerslev et al., 2014), though its impact was much lower than that of biological replication. Although there was variation among PCR replicates in the composition of prey items, this was about 13 times lower than variation among pellets of the same individual bat, and about 200 times lower than variation among bats. The low variation among PCR replicates suggests that prey DNA concentration was high and its degradation was low in bat faecal pellets, which are factors known to affect the amount of false positives and negatives, and thus technical reproducibility in metabarcoding studies (Ficetola et al., 2015). In contrast to PCR replicates, the magnitude of variation among individuals was particularly striking, suggesting that different individuals fed on different prey items. Reasons for this are unknown, but they may be related to the effects of season, gender, age, or foraging habitat. Random factors may also have played a major role, related to haphazard encounters between each foraging bat and a particular set of prey items in the night when pellets were collected. Variation among pellets of the same individual is also noteworthy, with the accuracy of diet diversity and frequency of occurrence estimates increasing markedly with the number of pellets analysed. These results seem surprising, because it might be expected that different pellets collected in the same time from a single individual would be representative of a single meal consumed in that night, thereby leading to low variability in dietary information among pellets. However, bats have an extremely rapid digestion and a high passage rate of food through the digestive tract (Staliński, 1994), and thus differences in pellet content within individuals may reflect prey consumed at different times during the same night. As a consequence, when the number of pellets analysed per individual is low there are many prey items missed and high error rates in frequency of occurrence estimates, particularly for prey items that are consumed by many individuals, but at low frequencies by each individual.

Pooling of samples before DNA extraction has been used to reduce processing time and costs by integrating variability among multiple samples or when individual samples were difficult to separate (Burgar et al., 2014; Clare et al., 2014b, 2014a; Jedlicka et al., 2017), but our results suggest that this strategy may lead to substantial errors in the estimation of dietary

descriptors. We found that pools strongly underestimated diet diversity and the frequency of occurrence of prey items, irrespective of sequencing depth, yielding results comparable to those obtained by analysing a single pellet. Prey items consumed less frequently were consistently missed when analysing pools, and there were high error rates of FO estimates for both common and rare prey items. The reason why pools did not detect more species, even with high sequencing depth, is not entirely clear as it seems somewhat counter-intuitive, because the DNA from species in individual pellets should also be present in a mix of the same pellets. However, common species in a mix will become proportionally more abundant, and rare species, which appear in low quantities in just a few pellets, will show an even smaller proportion. Therefore, the most likely explanation is that low abundance templates are not detected because of competition during PCR with proportionally more abundant templates. It is also possible that during DNA extraction, pooled samples might saturate the spin column and only the most common species get eluted. Nevertheless, the error in frequency of occurrence estimates are still slightly higher for pools even for common species. This is because pools seem to detect mostly what is highly abundant within individuals, meaning that the analysis of a single pellet is as likely to detect abundant species as is the analysis of a pool. It should be noted, however, that pooling may still be a necessary step when the initial DNA template is too low for extraction and amplification, though results need to be interpreted carefully given the errors associated with sample pooling revealed in our study.

Taken together, our results have important implications for the design of metabarcoding dietary studies, emphasizing the prominent role of biological replication to obtain robust estimates of diet diversity and composition, and the frequency of occurrence of prey items. In particular, the high variability reported here both among and within individuals point out that large numbers of individuals and sufficiently large numbers of samples per individual need to be analysed if the true diversity of the population's diet is to be recovered. Determination of sufficient levels of biological replication in general, however, will depend on the particular scientific questions being asked, and the dietary characteristics of the species being studied. For instance, although in conventional studies of bat diets it is generally agreed that 20-50 samples should be analysed for each ecological group under study (e.g. species, site, season, gender, age; Whitaker Jr. et al*.,* 2009), this may or may not be sufficient dependent on the levels of variability within groups, and the actual differences in the value of diet descriptors among groups. Larger sample sizes may thus be needed to detect differences in trophic niche between two species showing high intraspecific dietary heterogeneity due to gender, age or seasonal effects, than between adult males and females of the same species on a given season, for example. On the other hand, smaller sample sizes may be more acceptable in studies aiming to provide broad descriptions of dietary patterns in diverse

species communities, particularly when these include species that are rare or otherwise difficult to study, than when testing specific hypothesis in community ecology requiring precise dietary estimates. Therefore, scoping studies may need to be done before embarking in full scale projects, using power analysis to estimate the levels of biological replication required to detect a given effect size at a predefined probability level (Ferry & Cailliet, 1996). When this is impractical, researchers may need to take a precautionary approach and try to maximise the number of samples analysed, which is increasingly feasible due to the ever lower costs of high throughput DNA sequencing.

Acknowledgments

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Supporting Information

Table S2.1 - Average number of reads obtained per PCR after complete bioinformatic filtering and cleaning.

Run	Coverage	Pellet	Pool
1 - PCR replicates	Low	4,286 ± 95 (n=900)	4,548 ± 372 (n=60)
2 - Coverage experiment	Low	5,462 ± 650 (n=20)	5,400 ± 623 (n=20)
	High	90,456 ± 10,693 (n=20)	101,533 ± 8553 (n=20)

Table S2.2 – Prey species detected in the diet of 20 European free-tailed bats (*Tadarida teniotis*) based on the metabarcoding of either 15 individual pellets or pools of 15 pellets.

Order	Family	Species	No. Bats	No. Pellets	No. pools
Coleoptera	Cerambycidae	*Arhopalus ferus*	3	37	2
		Cerambycidae 1	1	2	0
		Cerambycidae 2	3	11	0
		Cerambycidae 3	1	8	1
		Cerambycidae 4	1	1	0
		Cerambycidae 5	2	8	0
	Chrysomelidae	*Sphaeroderma rubidum*	1	1	0
	Curculionidae	*Sitona discoideus*	1	1	0
	Tenebrionidae	*Tenebrio molitor*	3	8	0
Diptera	Cecidomyiidae	Cecidomyiidae 1	1	1	0
	Chironomidae	Chironomidae 1	1	1	0
		Chironomus sp. 1	3	3	0
		Cricotopus sp. 1	1	1	0
	Culicidae	*Culex pipiens/quinquefasciata*	11	33	0
		Culiseta subochrea/annulata	3	4	0
	Limoniidae	*Limonia nubeculosa*	1	1	0
	Scathophaga	*Scathophaga stercoraria*	1	1	0
	Tachinidae	*Meigenia* sp. 1	1	3	0
	Tipulidae	*Tipula oleracea*	6	28	2
		Tipula sp. 1	2	4	0
		Tipula sp. 2	1	2	1
		Tipulidae 1	1	1	0
	Unknown	Diptera 1	2	4	0
		Diptera 2	2	4	1
		Diptera 3	2	6	0
Hemiptera	Pentatomidae	*Acrosternum gramineum*	1	4	0
Hymenoptera	Ichneumonidae	*Campoplex* sp. 1	1	1	0
Lepidoptera	Coleophoridae	*Coleophora argenteonivea*	1	5	0
	Crambidae	*Diasemiopsis ramburialis*	1	3	0
		Udea ferrugalis	7	29	3
		Uresiphita gilvata	1	9	0
	Depressariidae	*Agonopterix capreolella/thapsiella*	3	20	1
		Agonopterix cnicella	2	3	1

Order	Family	Species	No. Bats	No. Pellets	No. pools
		Agonopterix heracliana	1	3	0
		Agonopterix scopariella	7	44	1
		Depressaria albipunctella	1	3	0
		Depressaria badiella	1	4	0
		Depressaria discipunctella	1	5	0
		Depressaria douglasella	1	1	0
	Epermeniidae	*Epermenia aequidentellus*	1	2	0
	Erebidae	*Autophila cataphanes*	1	2	0
		Eublemma ostrina	1	1	1
		Lymantria dispar	1	1	0
	Gelechiidae	*Teleiopsis lindae/diffinis*	2	2	0
	Geometridae	*Anarpia incertalis*	1	4	0
		Aplocera efformata	1	2	0
		Aspitates ochrearia	1	1	0
		Biston betularia	1	1	0
		Camptogramma bilineata	5	23	1
		Cataclysme uniformata	1	7	0
		Cyclophora puppillaria	5	24	1
		Eupithecia centaureata	1	1	0
		Eupithecia pantellata	2	2	0
		Gymnoscelis rufifasciata	2	9	1
		Idaea cervantaria	1	7	0
		Idaea degeneraria	1	3	0
		Idaea rhodogrammaria	1	2	0
		Idaea sardoniata	1	2	0
		Orthonama obstipata	1	5	0
		Rhodometra sacraria	6	37	2
		Scopula marginepunctata	1	6	0
		Stegania trimaculata	1	3	0
	Geometridae/Tortricidae	*Pachycnemia tibiaria/ Crocidosema plebejana*	1	6	0
	Gracillariidae	*Parornix torquillella*	1	3	0
	Noctuidae	*Agrotis bigramma*	2	17	1
		Agrotis ipsilon	5	21	1
		Agrotis puta/catalaunensis	4	32	2
		Agrotis segetum	15	136	11
		Agrotis segetum/clavis	1	4	0
		Agrotis segetum/ipsilon	1	1	0
		Autographa gamma	17	193	15
		Caradrina clavipalpis	1	9	0
		Caradrina flavirena	5	29	3
		Caradrina proxima	1	8	0
		Chloantha hyperici	3	18	0
		Cloantha hyperici	1	1	0
		Cryphia algae	1	1	0
		Cryphia algae/pallida	1	1	0
		Cryphia sp. 1	1	5	0
		Denticucullus pygmina	1	6	0
		Euxoa temera	1	13	1
		Hecatera dysodea	1	10	1
		Helicoverpa armigera	2	11	1
		Heliothis nubigera	1	5	0
		Hoplodrina ambigua	11	71	5
		Leucania loreyi	2	4	0
		Leucania zeae/ Mythimna litoralis	1	7	1
		Lophoterges millierei	1	5	0
		Mormo maura	1	1	0

Order	Family	Species	No. Bats	No. Pellets	No. pools
		Mythimna albipuncta	7	35	3
		Mythimna sicula	1	5	0
		Mythimna vitellina	17	211	15
		Noctua comes	4	22	0
		Noctua fimbriata	1	2	0
		Noctua orbona	4	26	0
		Noctua pronuba/janthe	9	86	4
		Noctua tirrenica	2	19	0
		Nomophila noctuella	4	25	1
		Nyctobrya muralis	1	5	0
		Ochropleura leucogaster	2	9	1
		Peridroma saucia	12	107	7
		Phlogophora meticulosa	6	76	7
		Rhyacia simulans	1	9	0
		Thalpophila vitalba	1	2	0
		Xestia agathina	1	8	0
		Xestia kermesina	1	3	0
		Xestia xanthographa	1	8	0
	Nolidae	*Nycteola columbana*	3	5	0
		Nycteola revayana	2	4	0
	Plutellidae	*Plutella xylostella*	2	6	1
	Praydidae	*Prays fraxinella*	2	4	0
		Prays oleae	3	12	1
	Pyralidae	*Acrobasis consociella*	1	6	0
		Acrobasis obliqua	5	16	1
		Ephestia elutella	2	3	0
		Etiella zinckenella	1	3	0
		Khorassania compositella	1	10	1
		Matilella fusca	1	2	1
		Synaphe punctalis	1	1	0
	Sphingidae	*Macroglossum stellatarum*	1	10	1
	Tortricidae	*Cydia fagiglandana*	1	1	0
		Cydia pomonella	1	1	0
		Cydia sp. 1	1	1	0
		Epagoge grotiana	1	1	0
	Yponomeutidae	*Zelleria oleastrella*	1	3	0
	Ypsolophidae	*Ypsolopha ustella*	1	1	0
	Unknown	Lepidoptera 1	3	7	0
Mantodea	Empusidae	*Empusa pennata*	1	3	0
Neuroptera	Chrysopidae	*Chrysopa viridana*	1	9	0
		Chrysoperla lucasina/agilis/carnea/pallida	9	58	2
		Chrysopidae 1	3	9	0
		Chrysopidae 2	2	2	0
		Cunctochrysa albolineata	1	1	0
		Nineta flava	4	7	0
Orthoptera	Acrididae	*Oedipoda caerulescens*	1	2	1
	Gryllidae	*Gryllus campestris*	1	1	0
	Tettigoniidae	*Platycleis affinis/albopunctata/intermedia*	4	18	0
		Tessellana tessellata	1	4	0
		Tettigonia viridissima	1	3	0
Trichoptera	Limnephilidae	*Micropterna fissa*	3	8	0
		Stenophylax nycterobius	1	2	0
		Stenophylax vibex	1	4	0
		Stenophylax sp.1	2	4	0
Unknown	Unknown	Insecta 1	3	5	0

Order	Family	Species	No. Bats	No. Pellets	No. pools
		Insecta 2	1	3	0
		Insecta 3	1	1	0
		Insecta 4	1	1	0
		Insecta 5	1	1	0
		Insecta 6	1	2	0
		Insecta 7	2	2	0
		Insecta 8	1	1	0
		Insecta 9	1	3	0
		Insecta 10	1	1	0
Total			20	300	20

Table S2.3 – Summary results of a Beta regression model (Pseudo R-squared = 0.8428) relating the error rates in the frequency of occurrence estimates of prey items in the diet of European free-tailed bats, in relation to the number of pellets analysed (pellets), the frequency of occurrence of each prey item in the sample of bats analysed (FOtot), and the frequency of occurrence of each prey item in pellets of each individual that consumed that item (FOpel).

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	2.5748	0.0354	72.8300	<0.0001
Pellets	-0.2660	0.0048	-55.0570	<0.0001
FOtot	-0.7123	0.2662	-2.6760	0.0075
FOpel	-4.3645	0.1220	-35.7890	<0.0001
Pellets:FOtot	0.2073	0.0384	5.3990	<0.0001
Pellets:FOpel	-1.1104	0.0211	-52.7020	<0.0001
Ftot:FOpel	0.1340	0.5052	0.2650	0.7908
Pellets:FOtot:FOpel	1.0019	0.0783	12.8030	<0.0001

Table S2.4 – Summary results of a Beta regression model (Pseudo R-squared = 0.2691) relating the error rates in the frequency of occurrence estimates of prey items in the diet of European free-tailed bats, estimated through the analysis of pools of 15 pellets per individual, in relation to the frequency of occurrence of each prey item in the sample of bats analysed (FOtot), and the frequency of occurrence of each prey item in pellets of each individual that consumed that item (FOpel).

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.7906	0.2558	6.999	<0.001
FOtot	-0.9427	1.8910	-0.498	0.618
FOpel	-2.3303	0.7259	-3.210	0.001
Ftot:FOpel	-0.1420	3.1473	-0.045	0.964

Chapter 3

Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists

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Abstract

The application of DNA metabarcoding to dietary analysis of trophic generalists requires using multiple markers in order to overcome problems of primer specificity and bias. However, limited attention has been given to the integration of information from multiple markers, particularly when they partly overlap in the taxa amplified, and vary in taxonomic resolution and biases. Here we test the use of a mix of universal and specific markers, provide criteria to integrate multi-marker metabarcoding data and a python script to implement such criteria and produce a single list of taxa ingested per sample. We then compare the results of dietary analysis based on morphological methods, single markers, and the proposed combination of multiple markers. The study was based on the analysis of 115 faeces from a small passerine, the Black Wheatears (*Oenanthe leucura*). Morphological analysis detected far fewer plant taxa (12) than either a universal 18S marker (57) or the plant trnL marker (124). This may partly reflect the detection of secondary ingestion by molecular methods. Morphological identification also detected far fewer taxa (23) than when using 18S (91) or the arthropod markers IN16STK (244) and ZBJ (231), though each method missed or underestimated some prey items. Integration of multi-marker data provided far more detailed dietary information than any single marker and estimated higher frequencies of occurrence of all taxa. Overall, our results show the value of integrating data from multiple, taxonomically overlapping markers in an example dietary dataset.

3.1 Introduction

Studies on trophic interactions using next generation sequencing (NGS) approaches have had an increasing impact on ecological research (Taberlet et al., 2012, 2018; Bohmann et al., 2014; Deiner et al., 2017), revolutionizing the breadth and depth of dietary studies, making it possible to process hundreds or even thousands of samples in a relatively short time (Pompanon et al., 2012; Galan et al., 2018; Nielsen et al., 2018). Furthermore, metabarcoding makes it possible to identify virtually all species consumed by a predator or herbivore, including rare food items (Soininen et al., 2009; Razgour et al., 2011; Hope et al., 2014; Nielsen et al., 2018), though this is conditional on DNA quality and the availability of DNA reference databases (Deagle et al., 2006; Elbrecht et al., 2016; Gerwing et al., 2016). Due to its strengths and cost-effectiveness, this approach has been increasingly used to describe the diet of many animals (Soininen et al., 2009; Kaunisto et al., 2017; Macías-Hernández et al., 2018; Deagle et al., 2019) and even carnivorous plants (Littlefair et al., 2019). However, there are still significant uncertainties regarding potential biases and pitfalls of metabarcoding, and how best to address them, which may significantly impact on the results of dietary analysis (Nielsen et al., 2018).

One problem that has attracted much attention is the selection of molecular markers, because primer specificity and biases can greatly affect the results of dietary studies (Taberlet et al., 2018; Alberdi et al., 2019). In general, studies build on previous knowledge of the diet of one or more species of interest, or of ecologically similar species, to select a primer that amplifies DNA from the main food items expected to be consumed (Coghlan et al., 2013; Alberdi et al., 2019). For instance, the studies of Soininen et al. (2009) and Valentini et al. (2009) used primers amplifying a fragment of the chloroplast *trn*L intron to analyse the diet of a number of herbivore species. Likewise, many studies on insectivore diets often used the ZBJ primer amplifying a fragment of the COI mitochondrial gene (Razgour et al., 2011; Zeale et al., 2011). This single marker approach has been widely used in many studies (Gordon et al., 2019; McClenaghan et al., 2019; Moran et al., 2019), but it may produce significant biases due to differential primer affinity for different taxa. For instance, although ZBJ is often used as a “universal” marker for arthropods (Crisol-Martínez et al., 2016; Trevelline et al., 2016, 2018; Jedlicka et al., 2017), it may have strong positive or negative bias depending on the taxa (Clarke et al., 2014; Piñol et al., 2015). The challenge is even worse in the case of omnivorous diets, because the variety of taxonomic clades consumed cannot be analysed using a single marker (De Barba et al., 2014; Taberlet et al., 2018). Therefore, it is increasingly recognised that molecular dietary studies should be based on a mix of markers that adequately amplify

the full complement of prey ingested, which requires integration of data from several markers for each sample (Deagle et al., 2009; Alberdi et al., 2018, 2019; Taberlet et al., 2018).

In multi-marker dietary studies, the most common approach is to divide the expected diet in various components (e.g., vascular plants, cephalopods, arthropods and vertebrates), and then use a primer designed to target each component (Coghlan et al., 2013; Groom et al., 2017; Robeson et al., 2018; Sullins et al., 2018). The integration of this type of multi-marker data is relatively straightforward, as information from each dietary component is retrieved from a single marker, and so a list of taxa detected in each sample can be inferred simply by adding taxa lists across markers. However, in some cases it may be necessary to use a mix of primers overlapping in the range of taxa amplified, making data integration more difficult. For instance, in dietary analysis of trophic generalists it may be useful to combine a universal marker with more specific markers, to account for the consumption of unexpected taxa that are not adequately detected by any of the specific primers used (De Barba et al., 2014; Deagle et al., 2009; Taberlet et al., 2018). Also, in dietary analysis involving highly diverse prey groups such as arthropods it may be necessary to avoid biases by combining primers that vary in affinity for different orders or even families, but that may overlap considerably in the range of taxa amplified (De Barba et al., 2014; Kaunisto et al., 2017; Aizpurua et al., 2018). Integration of such data cannot be made simply by adding the taxa lists retrieved across markers, because the same individual prey may be detected at different taxonomic levels by different markers, due to differences in taxonomic resolution or in the availability of reference databases (Elbrecht et al., 2016). To combine such data, it is necessary to identify duplications across markers, and to retain in each case the most taxonomically resolved taxa. Although these approaches based on taxonomically overlapping markers may advance dietary studies of trophic generalist species by maximising the diversity of species detected, they remain underutilised, there are no well-established criteria for integrating data across markers, and there is no simple computation procedure to implement such criteria.

Here we test the use of multiple overlapping markers, the criteria for integrating data from them in dietary analysis of a trophic generalist bird, and provide a python script to implement our data integration scheme. Prey remains retrieved from Black Wheatear (*Oenanthe leucura*) faeces were identified morphologically and using DNA metabarcoding with 4 molecular markers. Molecular data was integrated by the means of a python script to provide a single list of taxa detected per sample, controlling for duplications by collapsing less resolved taxa detected by one marker (e.g., order and family level) with higher resolved taxa detected using a different marker (e.g., genus and species). We then evaluated differences between morphological, single marker and multi-marker approaches in the estimates of dietary descriptors, in terms of (i) taxonomic resolution, (ii) diet diversity, (iii) the identity of

taxa recorded, and (iv) the composition of diet considering the taxa recorded and their representation in the samples.

3.2 Materials and Methods

3.2.1 Study species and sample collection

The Black Wheatear is a small (~ 35 g) black and white passerine that occurs in cliffs and rocky slopes of arid areas in western North Africa and Iberia. Although the species is not globally threatened, European populations are steadily declining, and the species is now regionally vulnerable (BirdLife International, 2017). Black Wheatears have a very diverse diet, feeding on freshly fruits, insects, arachnids, centipedes and sometimes even lizards (Richardson, 1965; Prodon, 1985; Hodar, 1995). The wheatear is a good study system to test our methodology due to its large feeding spectrum, including both plants and animals, and thus allowing us to test many food items simultaneously, and serving as model for other generalist terrestrial vertebrates. We collected 115 faecal samples from 143 Black Wheatears captured with spring-traps baited with Mealworms (*Tenebrio molitor*) throughout their known distribution in the Douro Valley in Portugal (Supplementary Figure S3.1), during spring and summer of 2014-2016. All birds were ringed to allow for individual recognition, which indicated that only four samples resulted from re-trapped individuals, two from birds collected three months apart, and two from birds collected in different years. Faecal samples were collected from clean cotton bags (soaked in 10% bleach for 1 hour and then washed between each use) or directly from stones used to disguise the bottom of the spring-traps (Oehm et al., 2011; McInnes et al., 2017). Samples were stored in 98% ethanol and refrigerated at 4ºC until processed in the laboratory.

3.2.2 Molecular analysis

DNA was extracted from each faecal sample using the Stool DNA Isolation Kit (Norgen Biotek Corporation) following the manufacturer's protocol. Samples were extracted in batches of 23 plus a negative control in which no faecal material was added. After DNA extraction the remaining faecal fragments used for DNA extraction were preserved for morphological identification. This was possible because morphological identification was based on hard faecal fragments such as chitinous body parts of invertebrates, vertebrate bones, and plant seeds and epidermis, which were not destroyed by the extraction method, as assessed through visual comparison of extracted and non-extracted samples.

Four different marker sets were used to analyse the diet. A universal eukaryote 18S marker Jarman et al., 2013; two arthropod markers: a modified version of IN16STK Kartzinel & Pringle, 2015 for the 16S region in which some degenerate bases were added to increase the affinity of the primers (IN16STK-1F_mod: 5' – TRAACTCARATCAYGTAA – 3', IN16STK-1R_mod: 5' – TTAGGGATAACAGCRTWA – 3') and ZBJ (Zeale et al., 2011) for COI region; and finally the gh plant specific marker for the *trn*L intron (Taberlet et al., 2007). All primers were modified to contain Illumina adaptors at the 5' end of the sequence (forward primers: 5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – 3', reverse primers: 5' – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – 3'). PCR reactions were carried-out in volumes of 10 µl, comprising 5 µl of QIAGEN Multiplex PCR Master Mix, 0.3 µl of each 10mM primer, 3.4 µl of ultra-pure water, and 1 µl of DNA extract. Cycling conditions used initial denaturing at 95 °C for 15 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Each marker was amplified in an independent PCR reaction, without any multiplexing. A very low PCR amplification temperature was used for all markers in order to reduce as much as possible the level of primer bias, this way allowing primers to anneal with less matching templates. This has been tested for some COI markers with positive results (Clarke et al., 2014). We also did not do any PCR replicates because recent studies have shown that, for faecal samples, variation in prey species composition among PCR replicates is much smaller than variation among samples (Mata et al., 2019). Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% gel stained agarose (GelRed Biotium, USA). PCR products were subjected to a second round of PCR with P5 and P7 indexes, after an initial dilution of 1:4 in order to reduce the amount of initial template and guarantee the complete incorporation of indexes in the library. Each index contained a unique 7bp long barcode that differed at least 3bp from any other index, allowing for the multiplex of several hundred samples in a single run (P5: 5' – AATGATACGGCGACCACCGAGATCTACACxxxxxxxTCGTCGGCAGCGTC – 3', P7: 5' – CAAGCAGAAGACGGCATACGAGATxxxxxxxGTCTCGTGGGCTCGG – 3'). PCR reactions and cycling conditions were similar to the ones of the first PCR except that only 8 cycles of denaturing, annealing and extension were done, with annealing at 50ºC. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter), and subsequently quantified using Nanodrop and diluted to 15nM. Purified and normalized PCR products were pooled per marker. These 4 libraries were then individually quantified using qPCR (KAPA Library Quant Kit qPCR Mix, Bio-Rad iCycler) and diluted to 4 nM. Finally, libraries were pooled equimolarly and sequenced using approximately half a lane of a 500 cycles v2 MiSeq run (Illumina) for an expected average of 24,000 paired-end reads per sample-marker combination.

3.2.3 Bioinformatic analysis

Bioinformatic processing of sequencing reads was done using OBITools (Boyer et al., 2016), with a separate analysis for each molecular marker. First, paired-end reads were aligned using the command 'illuminapairedend' and discarded if overlapping quality was less than 40 Taberlet et al., 2018. Second, reads were assigned to samples and primer sequences were removed using 'ngsfilter', allowing a total of 4 mismatches to the expected primer sequence. Finally, reads were collapsed into exact sequence variants (ESVs) and singletons were removed. ESV diversity and read count per fragment length, as well as bibliographic information of each marker was used to discard ESVs shorter and/or longer than expected. This way, we kept fragments with 94-153bp for 18S, 72-119bp for IN16STK, 155-159bp for ZBJ, and 30-93bp for *trnL*. The command 'obiclean' was then used to denoise the data by removing potentially spurious sequences with an 'r' level of one. This means that any 'A' ESV differing one base-pair from a 'B' ESV, with an absolute read count lower than 'B', and that was not found without the presence of 'B' in any PCR product, was removed as it was most likely a PCR or sequencing error. The PCR products that exhibited less than 100 reads in total after this step were considered to have failed and excluded from further analyses. This only happened for negative controls and taxa specific markers (IN16STK, ZBJ, and *trn*L), meaning that all samples contained amplifiable DNA. For the remaining ones, we removed from each PCR product all ESVs that had a read count <1% of the total number of reads of that PCR (Mata et al., 2016). This should allow the removal of most PCR and sequencing errors that still passed the 'obiclean' denoising step.

For each marker, prey items were identified by comparing the ESVs retained against online databases (BOLD and NCBI) using BLAST algorithm, as well as unpublished sequences of 1846 species of arthropods collected in northern Portugal in the case of COI (for further details see Ferreira et al., 2018). Whenever an ESV matched several species, genus, or families at similar identity levels, we selected the most inclusive taxonomic rank. For example, if a given 16S ESV matched with 99% similarity to two species of different genus belonging to the same family, we identified that ESV only to family level. For ESVs not identified to species level, we built a neighbour-joining tree in Geneious (Biomatters), visually inspected the corresponding alignment, and checked for patterns of co-occurrence of similar ESVs in order to cluster (~98%) them into distinct taxa (e.g., Carabidae 1, Carabidae 2, and so on), also referred as molecular operational taxonomic units (MOTUs). After this step, we removed every taxa not belonging to either the Plantae or Animal kingdoms, as well as all non-vascular plants, birds (mostly ESVs matching Black Wheatear), mammals (human and pig), internal parasites (phylum Nematoda), as well as mealworms (and the only

Tenebrionidae MOTU found with 18S and assumed to be mealworm) due to the high probability of being bait contamination. In the end, for each marker we counted the total number of taxa identified in each sample at the highest possible taxonomic resolution, thereby summing the number of taxa identified at species level with other MOTUs identified at higher taxonomic categories.

To build a consensus diet incorporating all molecular markers, we developed a python 3.0 script that merges the dietary information derived from the four markers into a single taxa list per sample. The script functions by merging in each individual sample the different taxa obtained with the different markers, considering the differences in taxonomic resolution yielded by different markers. This merging assumed that a given item recovered at higher taxonomic resolution (e.g., order or family) by a given marker was the same as items of the same taxonomic group recovered at lower resolution by other markers (e.g., genus or species). For example, if in a given sample the 18S marker detected a Coleoptera, the IN16STK a Chrysomelidae, and ZBJ a species belonging to the Chrysomelidae family, we assumed that all the markers were detecting the same taxa and merged them all into the most taxonomically resolved taxa. In contrast, we assumed the presence of different items when taxonomy at different levels of resolution was inconsistent across markers. For instance, if the 18S detected a Coleoptera, IN16STK a Carabidae, and ZBJ a species belonging to the Chrysomelidae family, we assumed there were 2 distinct taxa: the Carabidae and the Chrysomelidae species. This was expected to enhance complementarities and avoid redundancies across markers. However, since for many MOTUs it is impossible to establish a clear taxonomic relationship between the different markers, due to different taxonomic resolutions and lack of clear co-occurrences, we opted to merge MOTUs only on a sample by sample basis. For instance, MOTU-1 from 18S identified as undetermined Coleoptera1 could be merged in one sample with MOTU-2 from IN16STK identified as undetermined Carabidae1, but in a different sample could be merged with MOTU-3 identified as undetermined Chrysomelidae1. This could happen because the different families of beetles could share the same 18S MOTU, but also because different taxa are being detected with each marker. However, since there is no way to distinguish both situations, we believe our merging approach to be conservative and to avoid overestimating dietary diversity. Taxa richness per sample was computed as for the individual markers, by counting the total number of taxa identified at the highest possible taxonomic resolution. The python code is provided in supporting information (merge_script.rar) with a “readme” explanatory file containing an example of data input, and in github at https://github.com/PJADPereira/merge_markers.

3.2.4 Morphological identification

Plant and animal remains from faecal samples were analysed under a dissecting microscope, except plant epitheliums that were seen under a compound microscope, after DNA extraction. Plant remains like seeds and epidermis were identified by comparison with plants collected at capture sites. Animal parts were identified to the order or family level whenever possible, using specialized bibliography (Barrientos, 2004). In each sample, we also identified the total number of animal morphospecies of each order, thereby producing an approximation to the total number of taxa per sample. We did not, however, compare morphospecies across all samples in order to estimate the total diversity, because they were rarely comparable due to differences in the fragments recorded in each sample. This should not affect the analysis as all comparisons with molecular data were done on a sample by sample basis.

3.2.5 Data analysis

Statistical analysis was conducted to detect significant variation in estimates of dietary descriptors (i.e., diet diversity and composition) between different molecular markers, and to compare estimates obtained with each individual marker and the multi-marker approach. As multi-marker data combines information from all individual markers, it was used as the benchmark against which the performance of each individual marker was compared. This allowed, for instance, to assess what taxa are consistently missed or underestimated by the single markers. Plant and animal components of the diet were always analysed separately due to the different taxonomic range of the primers. Statistical significance was considered for p-values ≤ 0.05. All analysis were carried in R v3.3.0 (R Core Team, 2015).

To evaluate whether there were differences between methods in diet diversity estimates, for both plant and animal components, we compared among methods (i) the numbers of taxa per sample, and (ii) the number of orders per sample, using generalized linear mixed models (GLMM) with a Poisson distribution and a log link, specifying the sample as random effect. GLMMs were performed using the packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017). We then used multi-comparisons with Bonferroni corrections to identify in which pairs the observed differences occurred, using the package multcomp (Hothorn et al., 2008). To evaluate differences between methods in the estimates of diet composition, we used Multivariate Generalized Linear Models, assuming negative binomial errors, with the package mvabund (Wang et al., 2012). Analysis were carried out using numbers of taxa of each order detected per sample as response variable. To detect

which orders contributed to differences among methods we used univariate tests with adjusted p-values for multiple testing. Finally, we used Czekanowski's overlap index (Nielsen et al., 2018) to estimate the pairwise overlap in diet composition estimated by different methods, using the R package EcoSimR (Gotelli et al., 2015). This index ranges from 0 (no overlap) to 1 (complete overlap) and compares pairwise similarities based on frequency of occurrence data.

3.3 Results

3.3.1 Plant component

Morphological examination detected plants in 73 out of 115 faecal samples, yielding 12 taxa from 5 orders, of which 5 taxa were identified to genus or species levels (Figure 3.1). The most frequent taxon was *Solanum nigrum*, order Solanales (Figure 3.2). Metabarcoding detected plants in more faecal samples and yielded more taxa than morphology using either 18S (100 samples; 57 taxa from 16 orders; 2479±220 reads/sample) or *trn*L (110 samples; 124 taxa from 27 orders; 7462±387 reads/sample) (Figure 3.2). Besides detecting almost twice as many taxa, the taxonomic resolution was much higher for *trn*L (54% of taxa identified to genus or species) than 18S (19% to genus or species; Figure 3.1). The taxa recorded most frequently using either 18S or *trn*L was an unidentified plant of the family Vitaceae, most likely *Vitis vinifera* (Figure 3.2). There was significant variation among methods in the number of orders (χ^2 = 200.77, df = 2, p < 0.001) and taxa (χ^2 = 289.58, df = 2, p < 0.001) detected, with much lower values for morphology than metabarcoding with either 18S or *trn*L (Table 3.1**;** Supplementary Table S3.1). There were also significant differences in plant composition between methods (Wald value = 11.21, p < 0.001), with univariate tests indicating that 15 plant orders, particularly Vitales and Asterales, significantly contributed to such differences (Figure 3.1; Supplementary Table S3.2). Overlap was high between the results of 18S and *trn*L (0.757), but each had low overlap with morphology (<0.350) (Figure 3.3).

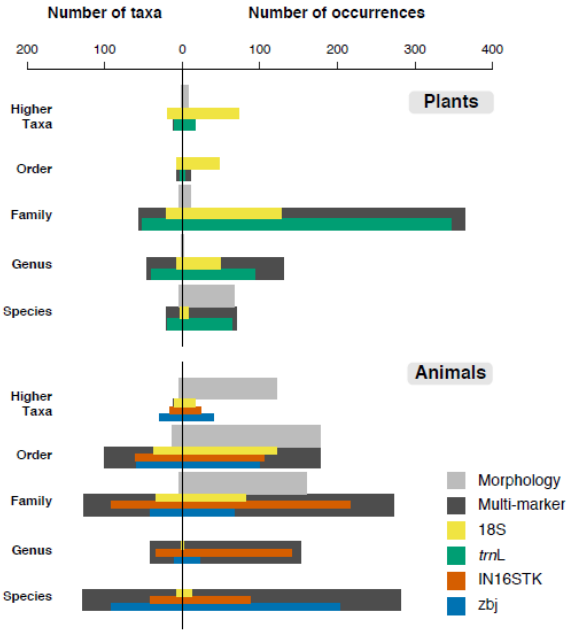

Figure 3.1 – Number of consumed taxa observed at different taxonomic levels (left) and number of occurrences observed at each taxonomic level (right), during the morphological identification (Morphology), with 4 individual molecular markers (18S, universal marker; *trn*L, plant marker; IN16STK and ZBJ, arthropod specific markers) and with the multi-marker approach, for plants and animals. Note that for the morphological identification, animal fragments were not compared across samples, and therefore the total number of taxa corresponds to the sum of the maximum number of morphotypes detected per family and order.

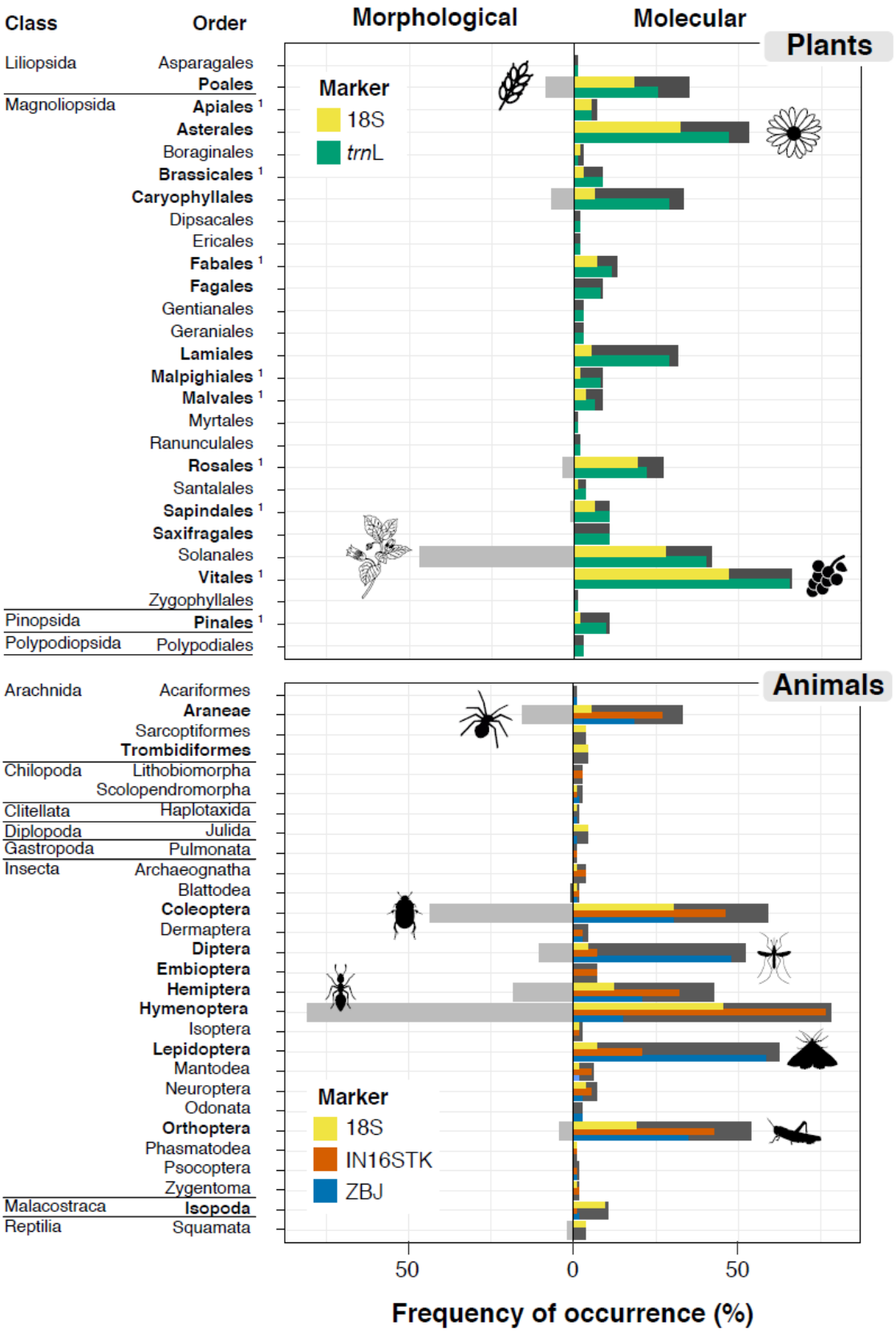

Figure 3.2 – Frequencies of occurrence of each order of plants and animals in the diet of Black Wheatears obtained through morphological and molecular analysis (multi-marker, dark grey bar, and for each set of primers). The orders highlighted in bold indicate significant differences at univariate tests of Multivariate Generalized Linear Models. 1 indicates orders that only showed significant differences among the molecular markers and morphological identification.

3.3.2 Animal component

Morphological examination detected animal prey in 112 samples, yielding 23 taxa from 8 orders, all of which were identified at best to family level (Figure 3.1; Figure 3.2). The most frequent order was Hymenoptera (81%), mainly due to the family Formicidae (70%) (Figure 3.2). Metabarcoding using 18S detected animals in 94 samples (3008±299 reads/sample), yielding 91 taxa from 21 orders, of which 10% were assigned to a genus or a species (Figure 3.1; Figure 3.2). The most frequent order was also Hymenoptera (45%). The two arthropod specific markers, IN16STK and ZBJ detected animals in 113 (6765±342 reads/sample) and 108 (2829±202 reads/sample) samples, yielding 244 and 231 taxa from 21 and 18 orders, respectively. From the taxa identified, 31% and 42%, IN16STK and ZBJ respectively, were identified to genus or species (Figure 3.1). The most frequent order detected by IN16STK was Hymenoptera (77%), mainly due to Formicidae (71%) as in the morphological analysis, while ZBJ detected most frequently Lepidoptera (59%) and only detected Hymenoptera in 15% of the samples, failing to detect the family Formicidae (Figure 3.2).

The mean number of taxa per sample varied significantly among the morphological identification and the markers ($\chi^2 = 148.78$, df = 3, $p < 0.001$), with all differing significantly from each other, except morphology from ZBJ (Table 3.1; Supplementary Table S3.1). Likewise, there was significant variation in the mean number of orders per sample across methods ($\chi^2 = 54.78$, df = 3, $p < 0.001$), with all differing significantly from each other, except morphology from 18S, and IN16STK from ZBJ (Table 3.1; Supplementary Table S3.1). Finally, there were significant differences in animal composition among morphological and molecular methods (Wald value = 21.29, $p < 0.001$), with univariate tests indicating that 10 orders, particularly Hymenoptera, Lepidoptera and Orthoptera, significantly contributed to such differences (Table 3.1; Supplementary Table S3.3). Overlap between morphology and each molecular marker (0.435-0.673) was only slightly lower than the pairwise overlap between markers (0.525-0.781; Figure 3.3).

Table 3.1 – Average ± standard error of the number of orders and taxa detected per sample. The number of taxa combines the number of species identified and the number of MOTUs identified at higher taxonomic levels.

	Method	Order	Taxa
Plant	Morphological	1.05 ± 0.05	1.12 ± 0.04
	18S	2.14 ± 0.11	3.03 ± 0.17
	*trn*L	3.70 ± 0.18	4.76 ± 0.31
	Multi-marker	4.09 ± 0.19	5.30 ± 0.31
Animal	Morphological	1.80 ± 0.10	4.09 ± 0.20
	18S	1.91 ± 0.12	2.38 ± 0.16
	IN16STK	2.93 ± 0.13	5.07 ± 0.27
	ZBJ	2.58 ± 0.12	4.00 ± 0.20
	Multi-marker	4.56 ± 0.17	7.92 ± 0.32

3.3.3 Multi-marker approach

When integrating information from the four molecular markers used, the initial 2064 occurrences (828 plant and 1236 animal) were reduced to 1492 (591 plant and 901 animal), indicating that only approximately one quarter of the information provided by the individual markers was redundant. The multi-marker approach detected a total of 27 plant and 28 animal orders, in 112 and 115 samples, respectively. The most detected plant order was Vitales, and the most detected animal order was Hymenoptera (Figure 3.2). As expected, individual markers differed significantly from each other and from the multi-marker approach in terms of taxa detected for both plants ($\chi 2 = 142.43$, df = 2, $p < 0.001$) and animals ($\chi 2 = 444.93$, df = 3, $p < 0.001$). The multi-marker approach provided more occurrences with high taxonomic resolution, i.e. genus or species, (Figure 3.1) and also detected a higher number of taxa and orders per sample than any individual marker, except for *trn*L that contributed to most of the plant taxa present in the multi-marker approach (Table 3.1; Supplementary Table S3.1). The overlap of the multi-marker data for the plant component was very high in relation to *trn*L (0.959) and very low in relation to morphology (0.342), while regarding the animal component the overlap was lowest with morphology (0.563) and had similarly high values with each individual marker (0.711-0.777; Figure 3.3).

Finally, plant and animal composition differed among the multi-marker approach and individual markers (plants: Wald value = 10.53, $p < 0.001$; animals: Wald value = 22.58, $p < 0.001$). For plants, univariate tests, adjusted for multiple testing, indicated that these

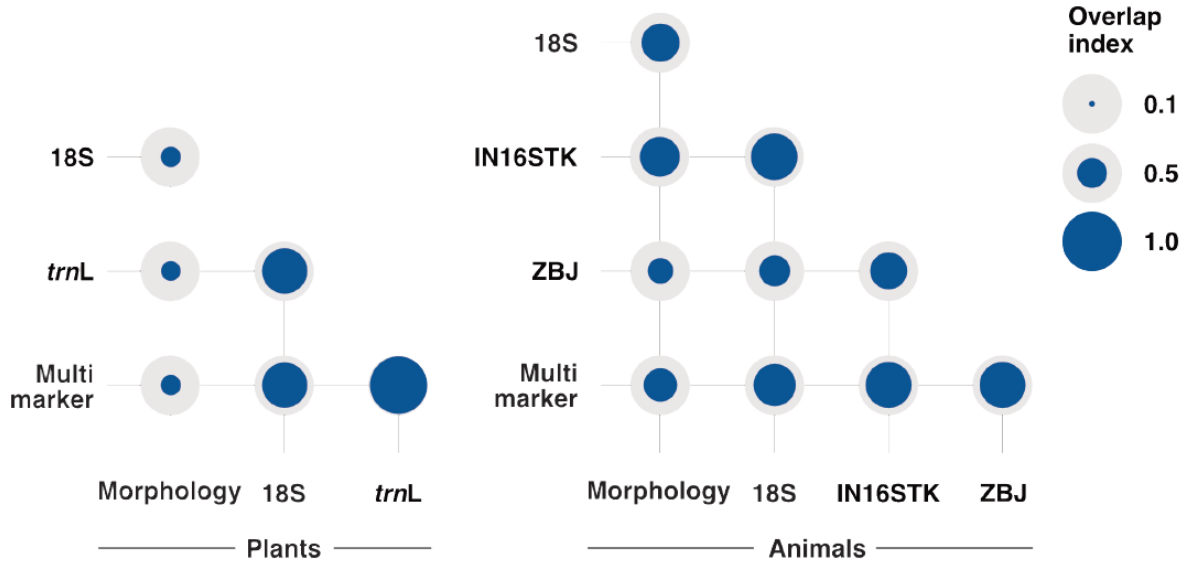

Figure 3.3 – Czekanowski's overlap index for plants and animals, between the morphological identification, the several molecular markers and the multi-marker approach used in Black Wheatear diet analysis.

differences were caused by 6 orders, mainly Caryophyllales, Lamiales, and Saxifragales (Supplementary Table S3.2). For animals, these differences were caused by 10 orders, mainly Diptera, Lepidoptera and Hymenoptera (Supplementary Table S3.3).

3.4 Discussion

Our study highlights the challenges involved in the description of the diet of trophic generalist animals, showing that results greatly vary depending on the method used. As expected, there were major differences in estimates of diet diversity, prey taxonomic identity, and composition between morphological and molecular methods, but there were also large variations in the results produced using different molecular markers. In particular, we found that widely used markers consistently underrepresented or missed some heavily consumed taxa, including taxa that were easily detected using the morphological analysis. The multi-marker approach appeared to largely overcome the problems of underestimate biodiversity that single marker dietary or non-molecular analysis produce, though it shares problems such as the detection of secondary ingestion. Overall, we suggest that using a mix of universal eukaryote and more taxon-specific markers can advance the description of trophic generalist diets and underline the importance of adequately integrating data to overcome problems associated with different taxonomic resolution across markers.

3.4.1 Biases and pitfalls in morphological and molecular dietary data

Most plant material recovered visually from Black Wheatear faeces were seeds of berry-producing plants, mainly *S. nigrum* and, to a much lesser extent, *P. americana*. These results suggest that wheatears regularly consumed berries in our study area, more so than suggested by previous studies (Richardson, 1965; Prodon, 1985; Hodar, 1995). Surprisingly, metabarcoding showed an even greater consumption of plants, with 18S and particularly *trn*L detecting a very large diversity of taxa, most of which produce dry seeds rather than berries. Reasons for this are unknown, but it may be a consequence of several non-exclusive factors. One possibility is that metabarcoding detects direct consumption of items for periods longer than the defecation time (Deagle et al., 2010; Oehm et al., 2011) especially if short amplicons are used (Kamenova et al., 2018). This can explain for instance, why *trn*L detected the berry-producing *Pistacia terebinthus* in 11 samples, while the seeds of this plant were detected in a single faecal sample. Another hypothesis is that the method is detecting plants that left no hard parts, and thus could not be detected visually. Lack of seeds can occur when wheatears only eat the flesh of berries, which might explain the high prevalence of *Vitis vinifera* detected through metabarcoding but not visually. However, this is questionable because grapes at the

time of sampling were unripe and thus unlikely to be eaten by the birds. The typical insectivore morphology and behaviour of Black Wheatears Richardson, 1965 also question the hypothesis of direct consumption to explain the detection of DNA from species with small and dry seeds such as Asterales, Lamiales and Poales, or with large acorns such as oaks *Quercus* spp.. It is also highly implausible that wheatears are feeding on other parts of these plants such as buds, flowers, or pollen. A more likely explanation may thus be indirect consumption through the stomach contents of animal prey, which may be recovered by molecular markers amplifying small DNA fragments (<200bp), such as the 18S and *trn*L markers used in our study (Sheppard et al., 2005; Kamenova et al., 2018). Detection of secondary consumption is well documented even through traditional methods (Johnson et al., 1997) but it is usually considered as having little importance (Barrett et al., 2007). In metabarcoding diet studies the effect of secondary consumption is not often explored in detail, and depending on the studied species it is considered to have low impact (Gerwing et al., 2016) or considerable influence on the range of the species detected in the diet (Bowser et al., 2013). If we consider only plants likely to be directly eaten by the wheatear, i.e. with fleshy fruits ripe during the sampling period, we will only retain 8.7% and 8.0% of the plants identified by the 18S and *trn*L, respectively. This shows that secondary detection can cause a strong bias on inferring the diet of generalist vertebrates if other sources of information such as morphological analysis and behavioural studies are not used to differentiate between primary and secondary consumption. Wheatears may also accidentally ingest some plant material when capturing small prey, as suggested by the small Poales seeds found in the morphological analysis. Also, it cannot be ruled out the possibility that some of the plant DNA recovered from faeces reflects environmental contamination, including for instance contamination with pollen spread through the air. We believe, however, that these problems should have had limited impact in our results, because most samples were collected from clean bags in which environmental contamination should be minimum, and we have followed the established protocols to minimize direct contamination (McInnes et al., 2017). Finally, it is possible that the high detection of secondary ingestion was particularly high in a largely carnivore species such as wheatears, because in many faecal samples there were no remains of plant material ingested directly, and so the primers amplified the only available plant DNA, i.e. meals of herbivorous insect prey. Whatever the reasons, our results suggest that dietary metabarcoding may record DNA of many plants that are not directly ingested by the target species.

The animal prey detected visually in Black Wheatear faeces was in line with previous studies (Richardson, 1965; Prodon, 1985; Hodar, 1995), showing a prevalence for Hymenoptera, mainly Formicidae, and Coleoptera, Hemiptera, Araneae and Diptera. As expected, these groups were largely recovered through molecular analysis, though

metabarcoding yielded a much larger diversity of prey and higher taxonomic resolution, particularly in the case of the COI marker ZBJ (Razgour et al., 2011; Hope et al., 2014; Krüger et al., 2014a, 2014b). Furthermore, some taxa were far more often detected through metabarcoding than by visual examination, including orders that seemed to be important in the diet such as Lepidoptera and Orthoptera. This may be a consequence of the ingestion of soft-bodied animals leaving few or no hard parts (Sutherland, 2004; Nielsen et al., 2018), as it was probably the case of caterpillars (Lepidoptera). Lack of Orthoptera remains are more difficult to explain because they have a heavy chitinous exoskeleton, but this may be a consequence of wheatears eating only the soft parts of the abdomen and leaving the head, thorax and legs, thereby ingesting fewer hard parts with morphological taxonomic value.

Although we cannot rule out the possibility of some animal prey detected through metabarcoding but not morphology, such as orthoptera and other taxa, being the result of secondary predation, this seems highly unlikely as it is congruent with what is known of the wheatears feeding behaviour. However, where no other sources of dietary information are available, it might be impossible to distinguish primary from secondary ingestion. On the contrary, some taxa are easily recognized as non-dietary items, due to their very small size and parasitic nature. For example, mites of the orders Acariformes, Trombidiformes, and Sarcoptiformes, detected through metabarcoding but not through visual examination, were probably not directly preyed by wheatears. These may have been ingested indirectly through the stomach contents of arthropod predators (Sheppard et al., 2005), or as parasites occurring in the body of arthropod prey or the birds themselves (Di Prisco et al., 2016; Gerwing et al., 2016; Martinho et al., 2017). Nonetheless, detection caused by secondary predation of animal prey appeared to be lower than that detected for plants. The reasons for this are not totally clear but may at least partly be explained by the very small size of the amplicon used for plants, which might have detected very small fragments of DNA originating from arthropod stomach contents.

Some animal preys were easily detected visually but not by some molecular markers. Formicidae, in particular, were often detected in faeces, while they were missed altogether by ZBJ. This was probably a consequence of the well-known positive bias of ZBJ towards Diptera and Lepidoptera, at the expenses of other arthropod orders Clarke et al., 2014. Although the failure to detect Formicidae was solved when using 18S and IN16STK, these tended to provide a lower taxonomic resolution of prey items, particularly in the case of Lepidoptera for which there was a very comprehensive reference database of COI barcodes. Therefore, only the combination of the three markers provided a detailed description of the animal component of Black Wheatear's diet.

3.4.2 Implications to describing diets with multi-marker approaches

Overall, the combination of visual and molecular approaches used in this study highlighted two important sources of potential errors in the analysis of trophic generalist diets and provided some clues on how to address these problems. First, our study suggests that morphological examination and/or previous ecological information may be important in order to detect unexpected biases and pitfalls of molecular methods, providing a basis to interpret and eventually correct results. This is highlighted by the detection of a range of animal and plant taxa that likely resulted from secondary ingestion or contamination, which may be a widespread problem in molecular analysis of trophic generalists, particularly when using small amplicons such as gh for plant *trn*L (Groom et al., 2017; Liu et al., 2018; Sullins et al., 2018) or generalist molecular markers (Bowser et al., 2013). This problem might be important, for instance, in conservation studies aiming to assess key trophic resources for a given species (Groom et al., 2017; Liu et al., 2018), in behavioural ecology research (Quéméré et al., 2013; Aizpurua et al., 2018), and even when reconstructing trophic networks from molecular data (Evans et al., 2016). To address this problem, visual analysis of a subset of samples would be desirable (Haarsma et al., 2016), providing information on the range of taxa that are eaten, which could then be compared against the results of metabarcoding. As this may often be impractical, researchers should at least check their metabarcoding results against the literature on conventional dietary studies of the target or closely related species (Gerwing et al., 2016), as well as ancillary information on morphology, behaviour and ecology, which may provide a basis to assess the plausibility of direct ingestion of unexpected taxa detected in samples. Another potential way to identify secondary consumption could be to look at the proportion of reads of each taxon and try to understand if it always occurs at a low proportion or not (Deagle et al., 2019). By filtering all taxa with less than 1% of the total number of reads of the corresponding PCR, one could expect that secondary consumption would disappear. However, in our study we observed that this is not always the case, with high number of reads obtained for some taxa that probably resulted from secondary ingestion. Nevertheless, detection resulting from secondary ingestion may not always be a problem, e.g. if the study aim is to know the entire intake of a given species, irrespective of whether it was ingested directly or indirectly (Pompanon et al., 2012).

Second, our study confirmed the value of using multiple markers, but suggests that previous studies based on a mix of non-overlapping specific markers each targeting a particular dietary component (e.g., Coghlan et al., 2013; Groom et al., 2017; Robeson et al., 2018; Sullins et al., 2018) may not be sufficient to overcome marker biases and thus provide a reliable diet composition. This is because markers considered universal for a given

taxonomic clade may still have considerable variations in affinity across taxa within that clade, and thus may not amplify some important items in the diet (Bowser et al., 2013; Clarke et al., 2014; Piñol et al., 2015; Kaunisto et al., 2017; Aizpurua et al., 2018; Alberdi et al., 2018, 2019). The problem was clearly illustrated by the high level of bias detected for ZBJ, which is sometimes regarded as universal for arthropods and is still the only marker used in many studies (Gordon et al., 2019; McClenaghan et al., 2019; Moran et al., 2019). In our study ZBJ completely missed Formicidae and other Hymenoptera, which was a key component of the diet identified through other methods, and probably overestimated the dietary importance of Lepidoptera and Diptera. The 16S marker used appeared less biased and thus may provide an alternative to ZBJ (Clarke et al., 2014; Deagle et al., 2014), but it still underestimated some important dietary components, which may be partly due to the less comprehensive reference databases available when compared to COI (Elbrecht et al., 2016).

To overcome the problems of marker bias and taxonomic resolution, a mix of taxonomic data from different markers needs to be integrated, by eliminating the duplicates resulting from the same individual prey being detected at different taxonomic resolutions. This should be a relatively easy task using the criteria and the python script provided in our study. Notwithstanding, newer and better molecular markers have been developed and are now available, and these may reduce the need for a multi-marker approach, e.g. UniPlant for plants (Moorhouse-Gann et al., 2018) and fwh for insects (Vamos et al., 2017). Unfortunately, there are no perfect markers, thus multiple primer sets should most of the times detect more taxa, mainly in highly diverse groups such as invertebrates (Corse et al., 2019). Even though untested in this study, our script should also prove useful in any broadscale biodiversity assessment, using either eDNA or bulk samples, allowing the integration of taxa detected using any combination of molecular markers, as well as of taxa detected through other methods like morphological identification.

Acknowledgments

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Supporting Information

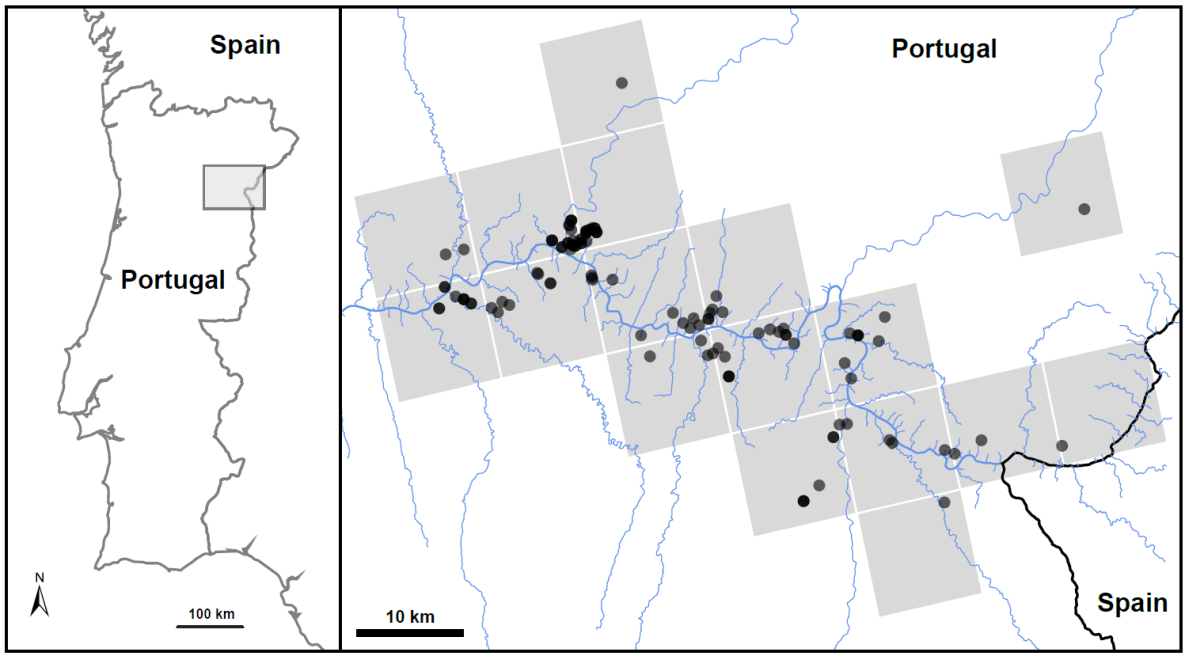

Figure S3.1 – Black Wheatear distribution along the Douro valley in Portugal (10 km^2 grey squares in ETRS89 projection) and the sample collection points.

Table S3.1 – Multiple comparison of taxa and order diversity among different diet assessment methods and molecular markers. Significant values are highlighted in bold.

	Group	Comparison	Estimate	Std. error	z-value	p-value
No. Taxa	Plant	morph – 18S	-1.0986	0.1308	-8.397	**<0.001**
		morph – *trn*L	1.8867	0.1216	15.517	**<0.001**
		18S – *trn*L	0.7881	0.0789	9.988	**<0.001**
		multi – 18S	0.8979	0.0772	11.631	**<0.001**
		multi – *trn*L	-0.1099	0.0604	-1.818	0.207
	Animal	morph – 18S	0.4494	0.0869	5.171	**<0.001**
		morph – IN16STK	-0.4880	0.0689	-7.082	**<0.001**
		morph – ZBJ	0.1537	0.0739	2.079	0.225
		18S – IN16STK	0.9375	0.0801	11.701	**<0.001**
		18S – ZBJ	0.6032	0.0845	7.141	**<0.001**
		IN16STK – ZBJ	-0.3343	0.0658	-5.081	**<0.001**
		multi – 18S	1.4240	0.0755	18.867	**<0.001**
		multi – IN16STK	0.4865	0.0539	9.029	**<0.001**
		multi – ZBJ	-0.8208	0.0601	-13.647	**<0.001**
No. Orders	Plant	morph – 18S	-1.0222	0.1329	-7.693	**<0.001**
		morph – *trn*L	1.6650	0.1243	13.400	**<0.001**
		18S – *trn*L	0.6428	0.0844	7.614	**<0.001**
		multi – 18S	0.7521	0.0823	9.137	**<0.001**
		multi – *trn*L	-0.1093	0.0678	-1.613	0.320
	Animal	morph – 18S	0.0879	0.1018	0.864	1.000
		morph – IN16STK	-0.4939	0.0893	-5.531	**<0.001**
		morph – ZBJ	0.3229	0.0924	3.496	**0.003**
		18S – IN16STK	0.5818	0.0918	6.338	**<0.001**
		18S – ZBJ	0.4109	0.0948	4.333	**<0.001**
		IN16STK – ZBJ	-0.1709	0.0813	-2.103	0.212
		multi – 18S	1.0411	0.0853	12.210	**<0.001**
		multi – IN16STK	0.4594	0.0700	6.562	**<0.001**
		multi – ZBJ	-0.6303	0.0739	-8.530	**<0.001**

Table S3.2 – Multivariate Generalized Linear Models univariate comparisons among plant orders. Significant values are highlighted in bold.

	morphological vs markers		markers vs multi-marker	
Order	Deviance	p-value	Deviance	p-value
Asparagales	2.206	0.603	1.631	0.985
Poales	19.934	**0.001**	12.749	**0.027**
Apiales	9.731	**0.040**	0.388	0.985
Asterales	101.287	**0.001**	18.261	**0.003**
Boraginales	2.449	0.603	0.553	0.985
Brassicales	14.518	**0.004**	4.999	0.740
Caryophyllales	34.946	**0.001**	34.999	**0.001**
Dipsacales	4.394	0.418	3.243	0.940
Ericales	4.394	0.418	3.243	0.940
Fabales	20.673	**0.001**	4.637	0.740
Fagales	20.334	**0.001**	16.171	**0.006**
Gentianales	6.655	0.149	4.93	0.740
Geraniales	6.591	0.159	4.865	0.740
Lamiales	57.754	**0.001**	31.347	**0.001**
Malpighiales	14.461	**0.004**	7.305	0.324
Malvales	9.749	**0.040**	2.657	0.940
Myrtales	2.197	0.603	1.622	0.985
Ranunculales	4.394	0.418	3.243	0.940
Rosales	23.359	**0.001**	2.894	0.940
Santalales	5.982	0.192	2.717	0.940
Sapindales	10.996	**0.024**	1.725	0.985
Saxifragales	27.293	**0.001**	20.401	**0.001**
Solanales	6.239	0.192	7.089	0.327
Vitales	108.044	**0.001**	4.702	0.740
Zygophyllales	2.197	0.603	1.622	0.985
Pinales	17.401	**0.002**	9.151	0.137
Polypodiales	6.591	0.149	4.865	0.740

Table S3.3 – Multivariate Generalized Linear Models univariate comparisons among animal orders. Significant values are highlighted in bold.

	morphological vs markers		markers vs multi-marker	
Order	Deviance	p-value	Deviance	p-value
Acariformes	2.772	0.910	2.772	0.979
Araneae	23.783	**0.002**	41.289	**0.001**
Sarcoptiformes	11.090	0.062	11.090	0.143
Trombidiformes	13.862	**0.024**	13.862	**0.030**
Lithobiomorpha	8.380	0.239	8.401	0.413
Scolopendromorpha	2.772	0.896	1.530	0.979
Haplotaxida	2.772	0.910	2.772	0.979
Julida	11.228	0.054	9.933	0.229
Pulmonata	2.772	0.910	2.772	0.979
Archaeognatha	8.858	0.228	7.584	0.519
Blattodea	0.541	0.950	0.680	0.979
Coleoptera	12.934	**0.040**	37.884	**0.001**
Dermaptera	8.317	0.239	7.022	0.592
Diptera	97.800	**0.001**	130.311	**0.001**
Embioptera	22.180	**0.002**	22.180	**0.001**
Hemiptera	18.272	**0.003**	34.360	**0.001**
Hymenoptera	190.467	**0.001**	180.358	**0.001**
Isoptera	5.545	0.557	5.623	0.787
Lepidoptera	160.152	**0.001**	138.303	**0.001**
Mantodea	8.720	0.239	5.094	0.864
Neuroptera	8.538	0.239	4.232	0.977
Odonata	8.317	0.239	8.317	0.413
Orthoptera	78.267	**0.001**	52.062	**0.001**
Phasmatodea	2.772	0.910	1.726	0.979
Psocoptera	2.772	0.910	2.772	0.979
Zygentoma	4.498	0.851	3.314	0.979
Isopoda	21.577	**0.003**	19.208	**0.001**
Squamata	9.445	0.151	11.255	0.125

Chapter 4

Female dietary bias towards large migratory moths in the European free-tailed bat

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Abstract

In bats, sexual segregation has been described in relation to differential use of roosting and foraging habitats. It is possible that variation may also exist between genders in the use of different prey types. However, until recently this idea was difficult to test due to poorly resolved taxonomy of dietary studies. Here we use high throughput sequencing to describe gender-related variation in diet composition of the European Free-tailed bat (*Tadarida teniotis*), while controlling for effects of age and season. We analysed guano pellets collected from 143 individuals mist-netted from April to October 2012 and 2013, in north-east Portugal. Moths (Lepidoptera; mainly Noctuidae and Geometridae) were by far the most frequently recorded prey, occurring in nearly all samples and accounting for 96 out of 115 prey taxa. There were significant dietary differences between males and females, irrespective of age and season. Compared to males, females tended to consume larger moths and more moths of migratory behaviour (e.g. *Autographa gamma*). Our study provides the first example of gender-related dietary variation in bats, illustrating the value of novel molecular tools for revealing intraspecific variation in food resource use in bats and other insectivores.

4.1 Introduction

Sexual segregation in resource use is common in vertebrates (Ruckstuhl & Neuhaus, 2005). Segregation is often associated with morphological and behavioural differences between sexes, which in turn affect a number of ecological and life-history traits such as home range, habitat selection, diet, foraging behaviour, and survival rates (Ruckstuhl & Neuhaus, 2005). Therefore, research on sexual segregation and its underlying causes is important to understand vertebrate ecology, demography and evolution, with implications in wildlife management and conservation.

In bats, most species do not exhibit obvious sexual dimorphism, but segregation between sexes has been described in relation to roosting and foraging habitat use, particularly during the maternity season (Ruckstuhl & Neuhaus, 2005). In temperate bats, females tend to use warmer roosts for maximizing fetal growth rate and milk production (Racey & Entwistle, 2000), while males tend to choose colder roosts to make use of torpor and maximize energy saving (Hamilton & Barclay, 1994). In some species, females also tend to forage closer to roosts (Entwistle et al., 1996; Wilkinson & Barclay, 1997; Encarnação, 2012), as this seems to be more cost-efficient and can lead to lower infant mortality (Tuttle, 1979), while males seem to be forced to feed away from breeding areas, thereby reducing potential competition with females (Senior et al., 2005). As a result, it is possible that segregation may also occur in the use of different prey types (Husar, 1976). Testing this hypothesis, however, has been hindered by poor taxonomic resolution of most bat dietary studies, though the recent development of molecular tools for dietary analysis provide the opportunity to examine this issue in great detail Zeale et al., 2011.

We used high throughput DNA metabarcoding to examine dietary sexual segregation in the European free-tailed bat (*Tadarida teniotis*). This is a medium-sized bat without obvious sexual dimorphism, which hunts at high altitude and has a large foraging range (Marques et al., 2004), and forms mixed colonies composed mainly of females (Amorim et al., 2015). The species has a highly specialized diet composed predominantly of nocturnal moths, but no dietary variation between sexes has been described (Rydell & Arlettaz, 1994). Yet, it might be expected that due to their high energetic requirements during breeding, females should feed more than males on large prey and on prey with high energetic value such as migratory moths (Angelo & Slansky Jr., 1984). To test this idea, we provide a detailed description of the diet of European free-tailed bats. Additionally, while controlling for potentially confounding factors related to age and sampling season, we assess differences between males and females in

relation to *(i)* prey species composition, *(ii)* prey species richness; *(iii)* prey size; and *(iv)* prevalence of migratory moth species.

4.2 Methods

4.2.1 Study area

The study was carried out in north-east Portugal (N41º09'– 42º00', W7º15'– 6º15'), within the watershed of the River Sabor (Supplementary Figure S4.1). Climate is transitional between meso- and supra-mediterranean, with cold winters (average temperature of the coldest month < 6ºC) and dry hot summers (total annual precipitation <600 mm, of which < 5% in July-August, average temperature of the warmest month > 24ºC), which are particularly hot in some valleys where monthly average temperatures exceed 21ºC (Monteiro-Henriques, 2010). Topography is characterized by plateaus with average altitudes of 700-800 m above sea level. River valleys are deep and narrow, and watercourses can have steep slopes and highly variable hydrological regimes, with many of them drying out seasonally while others persist year-round. Native vegetation comprises a complex mosaic including patches of evergreen oak (*Quercus suber*, *Q. rotundifolia*) woodlands and large expanses of shrublands dominated mainly by *Cytisus multiflorus, Lavandula pedunculata* and *Cistus ladanifer* (Hoelzer, 2003). Human occupation is sparse, and agricultural land is mainly covered by almond and olive groves, extensive pastureland, and some fields cultivated with cereals and other annual crops (Hoelzer, 2003).

4.2.2 Bat sampling

In April-October 2012 and 2013, we mist-netted bats at their roost in five bridges (Supplementary Figure S4.1), under an on-going monitoring program for this species (e.g. Amorim et al., 2015). The roosts were constituted by several hundred individuals, arranged horizontally in harems along the lateral crevices of the bridges. Although females far outnumber males at sampling roosts (Amorim et al., 2015), both genders were collected in every roost. The sampling was done monthly, to obtain a sufficiently large sample of different individuals throughout the breeding season. The close proximity of all roost sites and the large nightly flight distances of these bats (at least ~30km from roosts during foraging bouts Marques et al., 2004) suggest that individuals from the five sampling bridges are likely to use similar foraging areas. Therefore, individual bats were used as independent sampling units throughout our study.

After capture, bats were placed in clean individual cotton bags, whence guano pellets were subsequently collected. We recorded gender, maturity (juveniles versus adults), and sampling date (1st of April = day 1) of each individual. Juveniles were separated from adults based on the criteria described by Amorim et al. (2015), including the presence of unfused epiphyses, combined with the presence of a small non-secreting gular gland and the smaller size of testes in young males, and small nipples and smaller size in young females. All individuals captured before September were considered adults, because young of the year were only observed from that month onwards, and individuals born in the previous year (sub-adults) were no longer distinguishable. Pellets were stored in tubes containing silica-gel and refrigerated at 4ºC until DNA extraction.

4.2.3 Laboratory procedures

We extracted DNA from one random pellet per individual (n = 143, of which 55 were adult females, 47 adult males, 14 juvenile females, and 27 juvenile males) using the QIAamp DNA Stool Kit (Qiagen) following the standard protocol with adjustments suggested by Zeale et al. (2011). We used only one pellet per individual to standardise the sample across individuals, because during capture each bat could produce from as few as two or three pellets to more than 30. Also, the use of a single pellet was justified because each one weights the maximum recommended per extraction (50-100mg), and because we wanted to minimize the amount of inhibitors in the extracted DNA.

We amplified DNA using arthropod general COI primers ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale et al., 2011), modified to contain Illumina adaptors and a small identification barcode. The PCR comprised 10 µl QIAGEN Multiplex PCR Kit reactions, with 0.4 µl of each 10mM primer and 1 µl of DNA. Cycling conditions used initial denaturing at 95 °C for 15 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel. Library preparation followed the manufacturer's protocol for metagenomic sequencing (Illumina). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter) and subsequently quantified using PicoGreen (Promega) and diluted to similar concentrations. Illumina indexes were added to the cleaned PCR products using the Nextera XT Kit (Illumina), allowing individual identification of each amplified product. Indexed samples were pooled and sequenced using a whole run of a MiSeq desktop sequencer (Illumina).

4.2.4 Prey identification

We used OBITools (https://git.metabarcoding.org/obitools/obitools) for general sequence processing. Primers and barcodes were first trimmed, and reads smaller than 150bp removed. The obiuniq function was then used to collapse reads into unique haplotypes. Obiclean was used to remove potentially erroneous haplotypes resultant from PCR errors. This function eliminates haplotypes that differ by 1bp from more abundant haplotypes. At this step, a total of 22,901 haplotypes were left. From each pellet we then removed haplotypes representing less than 1% of the total number of reads and those containing stop codons. In the end, 315 haplotypes were left distributed in an average of 34,084±14,511 (SD) reads per pellet. The final set of haplotypes was manually compared against known reference sequences within the BOLD database (www.boldsystems.org). Haplotypes that best matched the same species were collapsed into a single taxon unit. All these units (n=115) except eight contained at least one sequence whose similarity between known species was higher than 98.5%, and were identified to species level. When the same haplotype matched more than one species, we only considered species known to occur in the Iberian Peninsula, or classified them into a species group.

Because the European free-tailed bats is considered a specialist in nocturnal moths [6] and most haplotypes recovered were indeed from Lepidoptera, particular attention was taken to guarantee the accurate identification of this taxonomic group. Therefore, identifications based on DNA barcoding were checked against a recent and thorough checklist of Lepidoptera from Portugal (Corley, 2015), assessing whether each taxa had been recorded in the study region or not. In addition, records obtained from bat pellets were screened against the known flight times of each moth species in Portugal, based on unpublished information from one of the co-authors (M.F.V. Corley). We discarded from statistical analysis all species identifications that conflicted with known geographic distribution or flight time data.

To assess whether predation by male and female European free-tailed bats was associated with moth size, we estimated from the literature the wingspan (in mm) of each species (Supplementary Table S4.1). Wingspan was used as a proxy of moth size instead of body mass, because the later was unavailable for many species and it may vary over time due for instance to the energetic costs incurred during migration (e.g. Casey, 1976; Rankin & Burchsted, 1992). To assess whether predation was associated with migratory behaviour, we used literature sources to classify each species according to whether it shows or not seasonal migratory movements (Supplementary Table S4.1). Although our dietary data could not show whether predation involved migrating or resident individuals from migratory species, we assumed that the presence of migratory species in pellets could be taken to indicate at least

some predation on migrants. This was because migrating moths are known to move in large numbers at high altitude during the night (e.g. Chapman et al., 2008, 2010), and the European free-tailed bat is a high-flying species.

4.2.5 Data analysis

To compare the diet composition of individual bats in relation to gender, age class, and sampling day, we used a PerMANOVA analysis implemented in the function 'adonis' of the vegan package (http://cran.r-project.org/package=vegan) for R (www.r-project.org). A jaccard distance matrix was used based on presence-absence data of each prey per pellet. Prey contribution to differentiation between groups was assessed with a similarity percentage analysis, implemented in the function 'simper' also available in vegan.

Generalized linear models (GLM) were used to estimate the effect of gender, age and sampling date on diet species richness (negative binomial errors; log link) and proportion of migratory species (binomial; logit) per individual. Generalized Linear Mixed Models (GLMMs) were used to examine the effect of these variables on the size of moth species found in pellets

Table 4.1 – Prey items recorded in the diet of female (n=69) and male (n=74) Tadarida teniotis. N.I. = Not identified.

Order-Family	Species (common name)	No. Prey items	% Samples	
			F	M
Coleoptera		2	1.4	1.4
Diptera		6	10.1	12.2
Tipulidae	*Tipula oleracea* (Crane fly)	1	5.8	9.5
	other Tipulidae	3	1.4	2.7
other Diptera		1	1.4	1.4
Diptera N.I.		1	1.4	0.0
Hemiptera		1	2.9	8.1
Lepidoptera		96	98.6	100.0
Crambidae	*Nomophila noctuella* (Rush veneer)	1	7.2	14.9
	other Crambidae	6	5.8	9.5
Gelechiidae	*Mirificarma mulinella*	1	7.2	14.9
	other Gelechiidae	1	0.0	2.7
Geometridae	*Aspitates ochrearia* (Yellow belle)	1	4.3	10.8
	Rhodometra sacraria (Vestal)	1	14.5	36.5
	other Geometridae	12	20.3	13.5
Noctuidae	*Agrotis ipsilon* (Dark sword-grass)	1	21.7	4.1
	Agrotis puta (Shuttle-shaped dart)	1	13.0	18.9
	Agrotis segetum/trux (Turnip/Crescent dart)	1	37.7	36.5
	Autographa gamma (Silver Y)	1	43.5	23.0
	Hoplodrina ambigua (Vine's rustic)	1	18.8	35.1
	Mythimna albipuncta (White-point)	1	5.8	9.5
	Mythimna vitellina (Delicate)	1	24.6	28.4
	Noctua pronuba/janthe (Large/Lesser broad-bordered yellow underwing)	1	14.5	13.5
	Peridroma saucia (Pearly underwing)	1	24.6	12.2
	Phlogophora meticulosa (Angle shades)	1	18.8	8.1
	other Noctuidae	37	31.9	36.5
Tortricidae	*Tortrix viridana* (European oak leafroller)	1	5.8	13.5
	other Tortricidae	4	2.9	5.4
other Lepidoptera		14	17.4	21.6
Lepidoptera N.I		7	13.0	23.0
Neuroptera		7	7.2	12.2
Insecta N.I.		2	1.4	1.4
Arthropoda N.I.		1	7.2	5.4

(Gaussian, identity), using individuals as random factors. Inference was based on the information-theoretic approach Burnham & Anderson, 2002, using as candidates all model subsets built with three predictors and their possible interactions. For each dependent variable we then computed an average model based on the 95% confidence set of candidate models (minimum number of models whose Akaike weight sum up to 0.95), and estimated the selection probability (w+) of each explanatory variable as a measure of its relative importance in the model. Uncertainty in parameter estimates were assessed through 95% confidence intervals, considering as equivocal the meaning of coefficients with intervals overlapping zero. GLM analysis were conducted with the MuMIn package (http://CRAN.R-project.org/package=MuMIn).

4.3 Results

In the diet of *T. teniotis* we identified 115 prey items within five insect orders (Table 4.1). Lepidoptera accounted for 83.5% of prey items and occurred in 99% of pellets. Most Lepidoptera were Noctuidae (47 prey items) and Geometridae (14). Diptera and Neuroptera each occurred in about 10% of pellets, while the occurrence of Coleoptera and Hemiptera was much lower. Each pellet contained on average 4.1±2.2 prey items of which 56.9%±36.7 were migratory moth species.

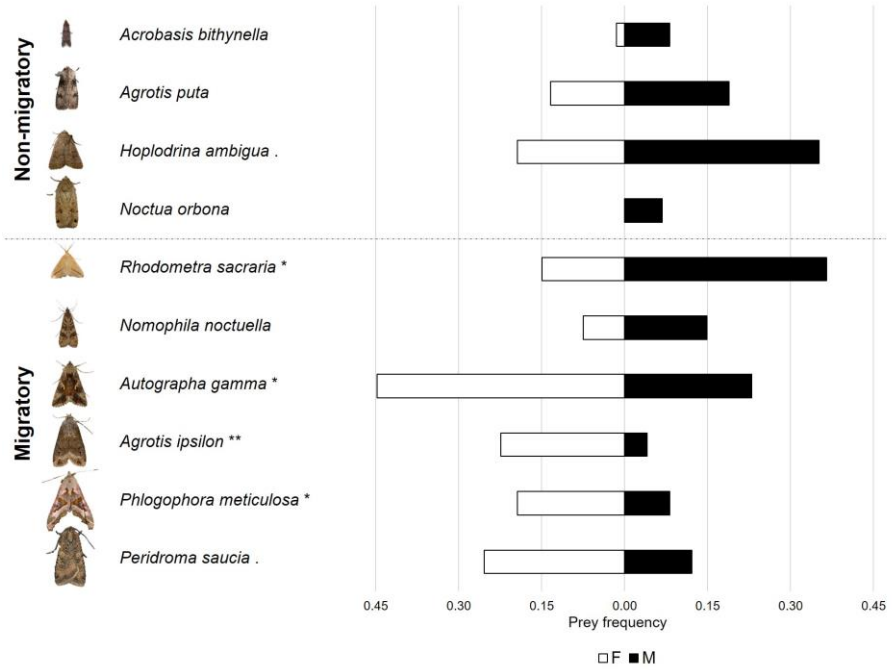

Figure 4.1 – Prey species with the highest contributions to dietary differences between female (F) and male (M) Tadarida teniotis according to the analysis of similarity percentages. Species whose frequencies of occurrence are significantly different between genders are marked as follows: . = P < 0.1; * = P < 0.05; ** = P < 0.01.

PerMANOVA revealed significant variation in diet composition related to gender, but not in relation to age or sampling day (Supplementary Table S4.2). Gender-related differences were primarily due to a higher frequency of *Rhodometra sacraria* and *Hoplodrina ambigua* in males, and of *Agrotis ipsilon* and *Autographa gamma* in females (Figure 4.1). GLM and GLMM models (Supplementary Table S4.3 and Table S4.4) provided strong support ($w+>0.90$) for females consuming larger prey and a higher proportion of migratory moth species than males (Figure 4.2). There was moderate support ($w+=0.82$) for juveniles consuming smaller moths than adults, but the confidence interval overlapped zero and the effect size was small (Figure 4.2).

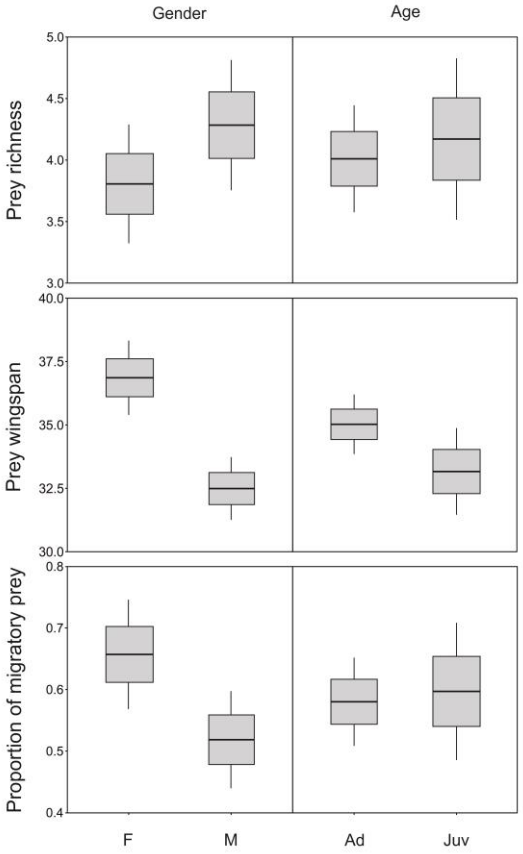

Figure 4.2 – Boxplots representing the average, standard error, and 95% confidence interval of prey richness, prey wingspan (in mm) and proportion of migratory prey in diets of female (F) and male (M), and adult (Ad) and juvenile (Juv) *Tadarida teniotis*.

4.4 Discussion

Our results showed for the first time the occurrence of gender-related dietary differences in an insectivorous bat species. Although there was a substantial overlap in diet composition, the average size (wingspan) and the frequency of migratory moth species was much higher in females than in males. Together with previous studies on the use of roost and foraging habitats, these results suggest that gender-related ecological segregation may be more frequent in bats than previously recognised (Hamilton & Barclay, 1994; Encarnação, 2012).

The higher consumption of large and migratory moths by females may be a consequence of their high energy demands during pregnancy and lactation (Racey & Entwistle, 2000). Feeding on large moths may be particularly rewarding because they provide a large energy intake per individual captured, though this should be weighed against the effort needed to catch each prey and its digestibility, which are unknown in *T. teniotis*. Likewise, migratory moths may be rewarding because they tend to be large and to build energetic reserves to sustain migration (Angelo & Slansky Jr., 1984). Finally, migratory moths may provide attractive foraging patches, as heavily eaten species such as *Autographa gamma*

migrate during the night in very large swarms (Chapman et al., 2010). *T. teniotis* may be particularly adapted to explore this valuable resource, because it is a fast and high-flying species with low manoeuvrability, which feeds in open areas, mostly above canopy level. Studies on the congeneric *Tadarida brasiliensis*, have shown that large numbers of individuals track migratory moth swarms at 400-500 meters above ground level (McCracken et al., 2008).

If large and migratory moths are particularly rewarding prey, it might be expected that males should use them heavily as well, instead of resorting to relatively smaller and sedentary species. As suggested in previous studies on bat sexual segregation in foraging habitats (Wilkinson & Barclay, 1997; Senior et al., 2005; Encarnação, 2012), this may be driven to some extent by intraspecific competition, with males avoiding to forage close to putatively dominant females. Irrespective of current competition, however, dietary segregation may also be a consequence of gender-related differences in morphology (Lisón et al., 2014), echolocation (Grilliot et al., 2009; Schuchmann et al., 2012), or social and physiological needs (Levin et al., 2013), resulting in distinct foraging habitats or prey types. Although the single radio-tracking study on *T. teniotis* did not find any evidence of gender-related differences in habitat use (Marques et al., 2004), this could be a consequence of small sample sizes and short tracking periods.

The gender-related differences documented here may also be a consequence of vertical segregation in space use, with females foraging more frequently at the high altitudes used by large migrating moths (McCracken et al., 2008; Chapman et al., 2010), and males foraging closer to the ground where encounters with sedentary species should be more frequent. Interestingly, the only migratory moth that males fed on more often was *Rhodometra sacraria*, a small species known to migrate at relatively low altitudes (Hausmann, 2004). The pattern of altitudinal segregation suggested here for *T. teniotis* is inverse from that described in other species (Grindal et al., 1999; Encarnação, 2012), where females were found in lower elevations, associated with resource abundant riparian habitats. However, vertical segregation of genders over the same foraging grounds has never been analysed or found before. Clarifying the occurrence of vertical segregation between male and female *T. teniotis*, and how this affects predation on high-flying migratory moths, should be the subject of further research, using for instance altimeter tags to estimate the altitude of foraging individuals.

Overall, our study points out the importance of understanding gender-related ecological segregation in bats, and the unique opportunities raised by DNA metabarcoding (Zeale et al., 2011). For instance, our results suggest that female *T. teniotis* may be more susceptible to large scale climate changes driving moth migrations (Sparks et al., 2005), whereas males may be more vulnerable to local moth declines arising for example due to land use changes. Conservation actions targeting this species should thus consider the different gender requirements. These conservation implications would have gone unnoticed using

conventional diet analysis, because low taxonomic resolution would have blurred dietary differences between sexes. Future metabarcoding studies are needed to describe gender-related dietary variation in other bats and insectivores, which should contribute to our understanding of species ecology and evolution, and aid in the design of conservation strategies.

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Supporting Information

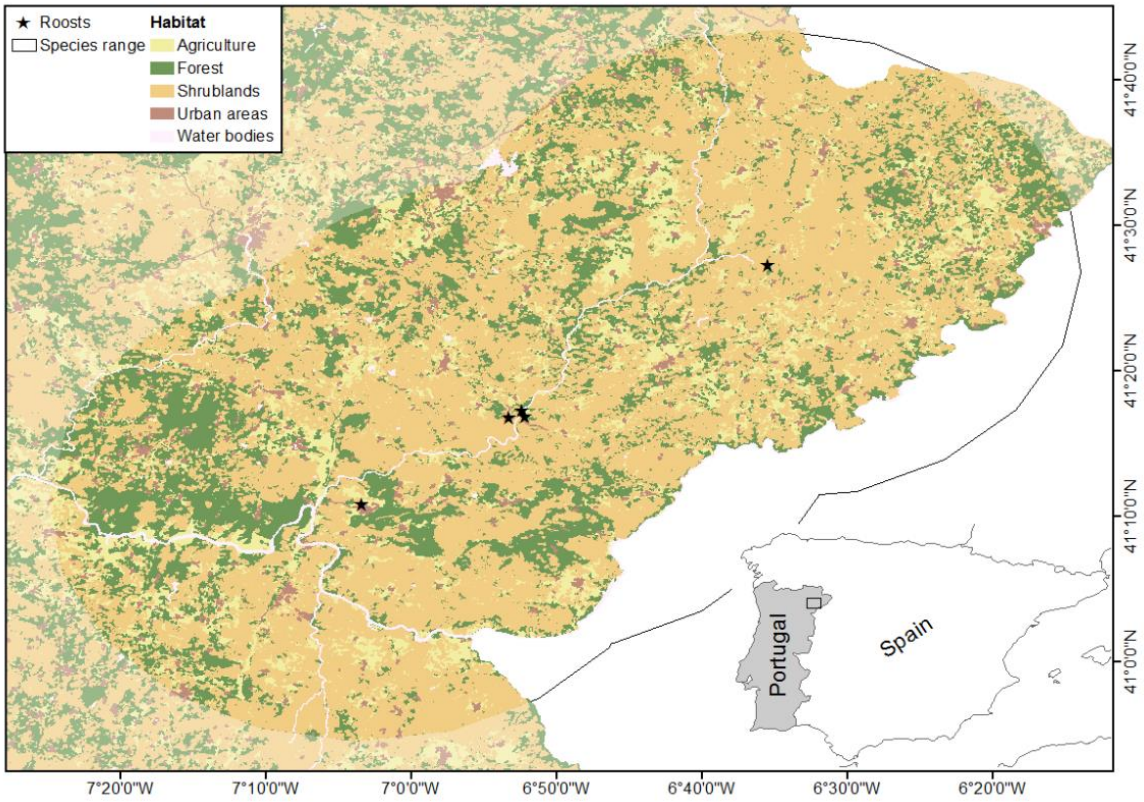

Figure S4.1 – Roost sampling locations and surrounding habitat. Buffer represents a minimal estimate of foraging range of the sampled colonies (~30km). White area refers to Spain for which we have no habitat data.

Table S4.1 – Prey species detected in this study. Wingspan (in mm) and migratory behaviour (yes or no) of each prey item used in statistical analysis. *, ª - Species that are probably misidentifications. Either because their known distribution does not include Portugal, or because they do not occur at the recorded time of the year. The ones marked with * were included in the composition and richness analysis, but not on ecological analysis (prey size and migratory behaviour).

Order	Family	Name	Match	Wingspan	Migratory	Reference
Coleoptera	Carabidae	*Bradycellus verbasci*	99.4	NA	NA	
	Curculionidae	*Curculio elephas*	100	NA	NA	
Diptera	Culicidae	*Culiseta annulata/ subochrea*	100	NA	NA	
	Tipulidae	*Tipula invenusta*	98.7	NA	NA	
		Tipula oleracea	100	NA	NA	
		Tipula paludosa	99.3	NA	NA	
		Tipula subcunctans	99.4	NA	NA	
	Unclassified Diptera	Diptera sp. 1	94.1	NA	NA	
Hemiptera	Lygaeidae	*Nysius graminicola*	100	NA	NA	
Lepidoptera	Crambidae	*Agriphila geniculea*	100	23	no	
		*Angustalius malacellus**	100	NA	NA	
		Prays oleae	98.7	30	no	Leraut, 2012
		Chrysoteuchia culmella	100	21	no	Leraut, 2012
		Nomophila noctuella	100	29	yes	Leraut, 2012
		Pyrausta aurata	99.4	16	no	Leraut, 2012
		Uresiphita gilvata	100	32	yes	Leraut, 2012
	Depressariidae	*Agonopterix pupillana**	99.2	NA	NA	
		Agonopterix scopariella	100	19.5	no	Emmet & Langmaid 2002a
		Agonopterix thapsiella	98.1	21	no	
		Ethmia bipunctella	100	23.5	no	Emmet & Langmaid 2002a
	Erebidae	Lepidoptera sp. 1	98.0	NA	NA	
		Autophila dilucida	99.4	42	no	Goater et al., 2003
		Eublemma ostrina	100	21.5	yes	Skinner, 2009
	Gelechiidae	Lepidoptera sp. 2	96.8	NA	NA	
		Mirificarma mulinella	100	13	no	Emmet & Langmaid 2002b
	Gelechiidae/ Geometridae	*Teleiopsis diffinis/bagriotella*ª/ *Eupithecia sp.*	100	NA	NA	
	Geometridae	*Aspitates ochrearia*	100	29.5	no	Redondo et al., 2009
		Camptogramma bilineata	100	28.5	no	Redondo et al., 2009
		*Compsoptera opacaria**	100	NA	NA	
		Crocallis dardoinaria	100	49	no	Redondo et al., 2009
		Cyclophora puppillaria	100	25.5	yes	Redondo et al., 2009
		*Ennomos quercinaria**	100	NA	NA	
		Eupithecia laquaearia	100	18	no	Redondo et al., 2009
		Gymnoscelis rufifasciata	100	15	no	Redondo et al., 2009
		Idaea degeneraria	100	23.5	no	Redondo et al., 2009
		*Idaea laevigata**	100	NA	NA	
		Idaea sardoniata	100	19	no	Redondo et al., 2009
		Onychora agaritharia	99.4	36.5	no	Redondo et al., 2009
		Pachycnemia hippocastanaria	100	28	no	Redondo et al., 2009
		Rhodometra sacraria	100	25	yes	Redondo et al., 2009
	Lycaenidae	*Plebejus argus**	99.0	NA	NA	
	Noctuidae	*Agrochola lunosa*	100	35	no	Skinner, 2009
		Agrotis ipsilon	100	47.5	yes	Skinner, 2009
		*Agrotis lata**	99.4	NA	NA	
		Agrotis puta	100	31	no	Skinner, 2009
		Agrotis segetum/trux	100	NA	NA	
		*Aporophyla lueneburgensis**	100	NA	NA	
		Aporophyla nigra	100	43	no	Skinner, 2009
		Autographa gamma	100	40	yes	Skinner, 2009
		Calophasia platyptera	100	29	no	Skinner, 2009
		Caradrina clavipalpis/selini	100	30	no	Skinner, 2009
		*Caradrina morpheus**	100	NA	NA	
		Chloantha hyperici/ Helicoverpa armigera	100	NA	NA	
		*Cryphia algae/pallida**	100	NA	NA	
	Noctuidae/ Geometridae	*Coenobia rufa/ Cyclophora suppunctaria*	100	25	no	Redondo et al., 2009
	Noctuidae	*Dryobotodes eremita**	100	NA	NA	
		*Eucoptocnemis optabilis**	100	NA	NA	
		*Eugnorisma arenoflavida**	100	NA	NA	
		Eugnorisma glareosa	100	35	no	Fibiger, 1990

Order	Family	Name	Match	Wingspan	Migratory	Reference
		Euxoa tritici/obelisca	100	34	no	Fibiger, 1990
		*Euxoa temera**	100	NA	NA	
		Heliothis peltigera	100	38	yes	Skinner, 2009
		Hoplodrina ambigua/superstes[a]	100	33	no	Skinner, 2009
		Leucania loreyi	100	39	yes	Skinner, 2009
		Leucochlaena oditis	99.4	34	no	Skinner, 2009
		Mythimna albipuncta	100	34.5	yes	Skinner, 2009
		Mythimna riparia	100	32.5	no	Ronkay et al., 2001
		Mythimna unipuncta/ Leucania zeae	100	44.5	yes	Skinner, 2009
		Mythimna vitellina	100	39.5	yes	Skinner, 2009
		Noctua comes	100	43	no	Fibiger, 1993
		Noctua orbona	100	41.5	no	Fibiger, 1993
		Noctua pronuba/janthe	100	NA	NA	
		Noctua tirrenica	100	54	no	Fibiger, 1993
		Nyctobrya muralis	100	30.5	no	Skinner, 2009
		*Papaipema nebris**	99.4	NA	NA	
		Peridroma saucia	100	50.5	yes	Skinner, 2009
		Phlogophora meticulosa	100	48.5	yes	Skinner, 2009
		*Polymixis argillaceago**	100	NA	NA	
		Pseudenargia ulicis	100	40	no	
	Geometridae	*Rhoptria asperaria*	100	22.5	no	Redondo et al., 2009
		Rhyacia simulans	100	48	yes	Fibiger, 1993
		Spodoptera exigua	100	29	yes	Skinner, 2009
		Thalpophila vitalba	100	42	no	Fibiger & Hacker, 2007
		Trigonophora haasi	100	37	no	Fibiger & Hacker, 2007
		*Trigonophora jodea**	100	NA	NA	
		Xestia agathina	100	32	no	Skinner, 2009
		Xestia c-nigrum	98.9	40	yes	Skinner, 2009
		*Xestia sexstrigata**	98.9	NA	NA	
	Noctuidae/ Geometridae/ Tortricidae	*Anarta trifolii/ Plemyria rubiginata*[a]*/ Choristoneura hebenstreitella*	100	NA	NA	
	Noctuidae/ Crambidae	*Chloantha hyperici/ Helicoverpa armigera/ Palpita vitrealis*	100	NA	NA	
	Noctuidae/ Gelechiidae/ Noctuidae	*Caradrina morpheus/ Carpatolechia sp./ Lacanobia contigua*[a]	100	NA	NA	
	Nolidae	*Nycteola revayana*	100	24	no	Skinner, 2009
	Pyralidae	*Acrobasis bithynella*	99.4	20	no	Leraut, 2014
		Acrobasis obliqua	100	20.5	no	Leraut, 2014
		*Psorosa nucleolella**	100	NA	NA	
		Pyralis farinalis	100	24	no	Leraut, 2014
		Synaphe punctalis	100	21.5	no	Leraut, 2014
	Tortricidae	*Cnephasia alfacarana**	98.7	NA	NA	
		Crocidosema plebejana	100	14	no	Razowski, 2003
		Cydia fagiglandana	100	14	no	Razowski, 2003
		Cydia pomonella	97.9	18	no	Razowski, 2003
	Tortricidae/ Crambidae	*Tortrix viridana/ Udea ferrugalis*	100	20.5	NA	Razowski, 2001
	unclassified Lepidoptera	*Caradrina morpheus/ Hypena sp./ Eupithecia sp.*	100	NA	NA	
		Eupithecia oxycedrata/ Cucullia sp.	100	NA	NA	
		Hoplodrina octogenaria/ Caradrina flavirena/aspersa	100	NA	NA	
Neuroptera	Chrysopidae	*Chrysopa viridana*	100	NA	NA	
		Chrysoperla lucasina/carnea	100	NA	NA	
		Chrysopidae sp. 1	98.7	NA	NA	
		Chrysopidae sp. 2	99.4	NA	NA	
		Cunctochrysa albolineata	99.4	NA	NA	
	Hemerobiidae	*Hemerobius stigma*	100	NA	NA	
		Wesmaelius subnebulosus	100	NA	NA	
unclassified Arthropoda	unclassified Arthropoda	Arthropoda sp.1	93.2	NA	NA	

Order	Family	Name	Match	Wingspan	Migratory	Reference
Unclassified	Unclassified	Insecta sp. 2	97.6	NA	NA	
Insecta	Insecta	Insecta sp. 1	92.7	NA	NA	

Table S4.2 – Adonis (PerMANOVA) results of season, gender and age effect on diet composition of *Tadarida teniotis*. Significant P-values (≤0.05) are highlighted in bold.

	d.f.	SS	MS	FModel	R^2	P
Age	1	0.337	0.337	0.812	0.0057	0.7012
Day	1	0.562	0.562	1.351	0.0095	0.1174
Gender	1	1.116	1.116	2.684	0.0189	**0.0002**
Residuals	137	56.947	0.416		0.9658	
Total	140	58.962			1.0000	

d.f. = degrees of freedom; SS = sum of squares; MS = mean of squares

Table S4.3 – Summary results of information-theoretic model selection for the effects of explanatory variables (and all their interactions) on prey richness, prey size, and proportion of migratory prey in the diet of *Tadarida teniotis*. For each dependent variable we show the 95% confidence set of best-ranked regression models, and for each one we provide: k, number of variables included in the model; logLik, maximized log-likelihood value; i , delta Akaike information criteria (AIC); wi , Akaike weight; w+, cumulative sum of Akaike weights.

	k	logLik	AIC	i	w_i	*w*+
Prey Richness						
~ Null	2	-303.897	611.8	0.00	0.221	0.221
~ Gender	3	-303.049	612.1	0.30	0.190	0.411
~ Day	3	-303.595	613.2	1.39	0.110	0.521
~ Age	3	-303.819	613.6	1.84	0.088	0.609
~ Day + Gender	4	-302.822	613.6	1.85	0.088	0.697
~ Age + Gender	4	-303.034	614.1	2.27	0.071	0.768
~ Age + Day	4	-303.593	615.2	3.39	0.040	0.808
~ Age + Gender + Age*Gender	5	-302.771	615.5	3.75	0.034	0.842
~ Age + Day + Gender	5	-302.812	615.6	3.83	0.033	0.875
~ Day + Gender + Day*Gender	5	-302.822	615.6	3.85	0.032	0.907
~ Age + Day + Age*Day	5	-303.179	616.4	4.56	0.023	0.930
~ Age + Day + Gender + Age*Day	6	-302.418	616.8	5.04	0.018	0.948
~ Age + Day + Gender + Age*Gender	6	-302.543	617.1	5.29	0.016	0.964
Prey Size						
~ Age + Gender + Age*Gender	6	-1466.886	2945.8	0.00	0.592	0.592
~ Age + Gender	5	-1468.855	2947.7	1.94	0.225	0.817
~ Gender	4	-1470.177	2948.4	2.58	0.163	0.980
Proportion of Migratory Prey						
~ Gender	2	-166.860	337.7	0.00	0.329	0.329
~ Day + Gender	3	-166.619	339.2	1.52	0.154	0.483
~ Age + Gender	3	-166.860	339.7	2.00	0.121	0.604
~ Day + Gender + Day*Gender	4	-166.443	340.9	3.16	0.068	0.672
~ Age + Day + Gender + Age*Day	5	-165.568	341.1	3.42	0.060	0.732
~ Age + Day + Gender	4	-166.579	341.2	3.44	0.059	0.791
~ Age + Gender + Age*Gender	4	-166.849	341.7	3.98	0.045	0.836
~ Age + Day + Gender + Age*Day + Day*Gender	6	-165.380	342.8	5.04	0.026	0.862
~ Age + Day + Gender + Day*Gender	5	-166.413	342.8	5.11	0.026	0.888
~ Age + Day + Gender + Age*Day + Age*Gender	6	-165.532	343.1	5.34	0.023	0.911
~ Age + Day + Gender + Age*Gender	5	-166.571	343.1	5.42	0.022	0.933
~ Null	1	-170.801	343.6	5.88	0.017	0.950

Table S4.4 – Summary statistics of average models regarding prey richness, prey size and prey migratory behaviour in *Tadarida teniotis*. For each variable and model, we indicate the coefficient estimate (Estimate), the standard error (Std. Error), the 95% confidence intervals (95% CI) and variable relative importance (w+). Confidence intervals that do not overlap 0 are highlighted in bold. Ad=Adults; Juv=Juveniles; M=Males; F=Females.

	Estimate	Std. Error	95% CI	*w+*
Prey richness				
(Intercept)	1.342	0.096	**[1.153, 1.531]**	
Age (Ad=0, Juv=1)	0.166	0.697	[-1.207, 1.539]	0.33
Day	0.001	0.001	[-0.001, 0.002]	0.37
Gender (M=1;F=0)	0.111	0.106	[-0.099, 0.322]	0.50
Age*Day	-0.008	0.009	[-0.025, 0.009]	0.04
Age*Gender	0.152	0.210	[-0.263, 0.567]	0.05
Day*Gender	0.000	0.001	[-0.003, 0.003]	0.03
Prey size				
(Intercept)	37.195	0.965	**[35.298, 39.092]**	
Age (Ad=0, Juv=1)	-0.794	2.053	[-4.829, 3.241]	0.83
Gender (M=1;F=0)	-4.558	1.374	**[-7.259, -1.858]**	1.00
Age*Gender	0.416	2.831	[-5.150, 5.982]	0.60
Prey migratory behavior				
(Intercept)	0.690	0.259	**[0.179, 1.201]**	
Age (Ad=0, Juv=1)	-1.402	2.942	[-7.192, 4.388]	0.40
Day	-0.002	0.002	[-0.006, 0.003]	0.46
Gender (M=1;F=0)	-0.5098	0.277	**[-1.145, -0.050]**	0.98
Age*Day	0.029	0.021	[-0.012, 0.070]	0.11
Age*Gender	0.083	0.486	[-0.879, 1.045]	0.09
Day*Gender	0.002	0.003	[-0.005, 0.009]	0.13

Chapter 5

DNA metabarcoding illuminates pest control services in complex multifunctional landscapes

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Keywords: pest control; ecological networks; bats; metabarcoding; predator-prey interactions; community;

Abstract

In multifunctional landscapes, diverse communities of small vertebrates can provide vital ecosystem services such as pest control. However, understanding the role of individual species within such communities is difficult, though this knowledge would be important to promote ecological intensification for food and fibre production. Here we provide a framework to identify small vertebrate species potentially important to biocontrol services, by combining dietary screening using DNA metabarcoding with ecological network analysis. In a heterogeneous mosaic landscape, our approach revealed a complex interaction network involving 19 bat species feeding across habitats on 132 insect pests. Just six generalist bats potentially regulated over three quarters of the pests, though functional redundancy within the community was high. Some pests were potentially regulated only by a few trophic specialists with high niche differentiation. Our approach underlines the functional importance of conserving diverse vertebrate communities in multifunctional landscapes, while identifying candidate species that could be favoured to intensify the control of pests.

5.1 Introduction

Multifunctional landscapes are key to combine food security and biodiversity conservation worldwide, as they integrate human activities with the preservation of ecosystem structure and function (Lovell & Johnston, 2009; Kremen & Merenlender, 2018; Manning et al., 2018). In such landscapes, biodiversity contributes to the production of food and fibre, and to reduce their negative environmental externalities, by providing critical services such as pollination and pest control (Mace et al., 2012; Maas et al., 2016; Kaiser-Bunbury et al., 2017; Heath & Long, 2019). To enhance the flow, stability and resilience of ecosystem services supporting production (i.e., ecological intensification *sensu* Doré et al., 2011), the management of multifunctional landscapes should target at benefiting service-providing organisms (Bommarco et al., 2013; Tittonell, 2014). Designing management strategies towards ecological intensification thus requires a detailed understanding of what species or groups of species are key to service delivery (Birkhofer et al., 2018).

Natural pest control is one of the services required to sustain production while minimising artificial inputs (Kleijn et al., 2019), with predation by small insectivorous vertebrates such as birds and bats strongly contributing to pest suppression (Mooney et al., 2010). The importance of these organisms has been repeatedly demonstrated through a variety of enclosure experiments, with studies on corn and cotton plantations in the USA, and coffee and cocoa in the tropics, showing that their biocontrol services can sum up to billions of dollars annually (Maine & Boyles, 2015; Maas et al., 2016). These studies have shown that predator identity is important by analysing the relative contribution of birds versus bats to pest suppression, but otherwise have largely attributed biocontrol to diverse predator communities rather than to any particular species. However, some individual species may have a disproportionate contribution to crop productivity, particularly those that are abundant and feed heavily on pests, as shown in the case of cotton and the Mexican free-tailed bat in the USA (*Tadarida brasiliensis*; Cleveland et al., 2006; Federico et al., 2008; McCracken et al., 2012), and rice and the Wrinkle-Lipped Bat (*Tadarida plicata*) in Thailand (Wanger et al., 2014; Srilopan et al., 2018; Nguyen et al., 2019). Management benefiting these species may thus intensify pest control services.

In multifunctional landscapes, multiple land uses coexist across space and over time (e.g., crop rotation), with each land use associated with multiple pests, and each pest predated by multiple vertebrate predators (Bianchi et al., 2006; Chaplin-Kramer et al., 2011). In these circumstances, suppression of all pests represented in a multifunctional landscape may require diverse predator communities rather than any particular predator species, and generic

management prescriptions such as maintaining landscape heterogeneity and/or maximising coverage by natural habitats may be sufficient to enhance pest biocontrol (Bianchi et al., 2006; Rusch et al., 2016; Karp et al., 2018). Even in these landscapes, however, management to benefit individual species can be important to ensure that each pest species has at least a natural enemy and that there is functional redundancy across predators to ensure the stability of service delivery over space and time (Peralta et al., 2014). For instance, specific management prescriptions may be required to benefit endangered species and or habitat specialists, which otherwise may decline and release specific pests from effective biocontrol.

Species' roles in systems involving multiple predators and pests are challenging to evaluate, requiring for instance estimates of changes in pest density, biomass or damages when selectively excluding individual predator species from multiple crops. A practical alternative to address this problem is provided by the analysis of species interaction networks, which can identify candidate species necessary for community functioning (Harvey et al., 2017). In bipartite networks such as those representing predator-pest interactions, a species having many interactions (i.e., high degree centrality) can be considered particularly important, especially if the network has a nested structure (i.e., the interactions of species with a lower degree are a subset of those with a higher degree; Bascompte et al., 2003). If the network is modular (i.e., with groups of species interacting strongly with other species within but not across modules), however, species acting as 'network hubs' (i.e., interacting with many species across modules) may be the most important, though relevance should also be given to 'module hubs' (i.e., interacting with many species within a module; Delmas et al., 2019). Irrespective of network structure, attention by managers should be given to species that have low trophic niche overlap with others and thus complement their functional role, though species that are functionally redundant may also be important to ensure stability in the delivery of pest control services.

Network analysis has been widely used to understand species roles in mutualistic (e.g., pollination, seed dispersal) and antagonistic (e.g., parasitoid-host) networks, but much less attention has been given to predator-pest interaction networks, due to difficulties in assessing the diet of natural predator communities (but see Roubinet et al., 2018; Feit et al., 2019; Sint et al., 2019). Molecular diet analysis through DNA metabarcoding has recently overcome this problem (Pompanon et al., 2012; Nielsen et al., 2018), by simultaneously providing species level identification of hundreds of prey and the capacity to processing several hundred samples in a short period, and thus allowing the reconstruction of entire insectivore interaction networks with relative ease (Galan et al., 2018; Gordon et al., 2019). Moreover, reduced costs, coupled with technical refinements such as multi-marker approaches (da Silva et al., 2019) and the availability of ever more comprehensive barcode databases (e.g. Dincă et al., 2015;

Hendrich et al., 2015; Wirta et al., 2016; Morinière et al., 2017), are making this method increasingly powerful and readily available for practical applications.

This study provides a framework combining DNA metabarcoding and ecological network analysis to identify the functional role of individual species of small vertebrate insectivores in multifunctional landscapes. We illustrate its practical application with a case study that focuses on a diverse bat community inhabiting a Mediterranean landscape comprising multiple crops and forest production systems, which can be attacked by over one hundred insect pests. We focused on bats because they play important roles as arthropod pest suppressors (Boyles et al., 2011), while the focus on Mediterranean mosaic landscapes is justified because they are often considered the epitome of multifunctionality (Pinto-Correia & Vos, 2004; Bugalho et al., 2011). Specifically, the study aimed at: (i) describing predation on arthropod pests (i.e., frequency of occurrence and interaction) by all bat species; (ii) to use this information to produce a predator-pest network and characterize its properties; and to estimate species' roles in the network by assessing (iii) species' centrality and (iv) the presence of species that can act as 'network hubs' or 'modular hubs', (v) the functional complementarity and redundancy across species. We found that although all bats predated on several pest species to a variable extent, there were a few particularly important species in the network and that should thus be the focus of management attention, because of their frequency of interaction with pests, key role and high redundancy levels in network interactions, and finally for their distinctiveness in pest species predation.

5.2 Methods and materials

5.2.1 Study area and field sampling

Bats were sampled in north-eastern Portugal around 'Vale da Vilariça', one of the most fertile valleys of the country (41°20'15.9"N 7°03'24.4"W). The valley is about 22km long and 2-8km wide, with a small alluvial plain of 5000 hectares, and is located over the Bragança-Manteigas fault, within the Demarcated Douro Region. The fertility of the valley derives from the periodic floods that hit the area whenever the neighbouring Sabor river refluxes due to the high flow of the Douro river. Climate is transitional between meso- and supra-mediterranean, with cold winters (average temperature of the coldest month < 6ºC) and dry and hot summers (total annual precipitation <600 mm, of which < 5% in July-August, average temperature of the warmest month > 24ºC). Native vegetation comprises a complex mosaic including patches of evergreen oak woodlands (*Quercus suber, Q. rotundifolia*) and large expanses of shrublands dominated mainly by *Cytisus multiflorus*, *Lavandula pedunculata* and *Cistus*

ladanifer. Forest plantations of pine trees (*Pinus spp*.) and other coniferous trees are also common in the region, usually at hilltops. Human occupation is stronger in the alluvial plain where a variety of crops are explored, mostly vineyards, and olive and almond groves, but also a diversity of fruits and greens.

Bats were captured using mist nets placed either at the entrance of roosts, feeding perches, or foraging areas, between May and July of 2016 and 2017. A total of 443 individual bats belonging to 19 different species were captured and its faeces collected. Additionally, 148 fresh faecal pellets were collected from known roosts, whose bats were not possible to catch. Faecal pellets were stored in 2mL tubes containing silica beads and stored at -20ºC until further processing.

5.2.2 Laboratory procedures

From each sampled bat individual up to three faecal pellets were individually processed and its DNA extracted. For pellets collected in roosts we also extracted DNA from each individual pellet. This corresponded to a total of 1282 individual guano pellets extractions. We used a custom protocol that consisted of an initial incubation period using a lysis buffer (0.1 mTris–HCl, 0.1 mEDTA, 0.01 mNaCl, 1% N-lauroylsarcosine, pH 7.5-8), followed by inhibitor removal using Inhibitex tablets (QIAGEN), and cell lysis, DNA precipitation and washing using E.Z.N.A. Tissue Kits (Omega). The extraction protocol started by adding one faecal pellet to 800 µl of lysis buffer. Samples were homogenized with a spatula, vortexed and left in a dry bath at 70ºC for 30 min. Afterwards, samples were short-spinned and up to 700 µl of supernatant was transferred to a new tube containing one quarter of an inhibitex tablet. Samples were then vortexed for 1 min and centrifuged at 8,000 rpm for 30 sec. Up to 500 µl of supernatant was transferred to a new tube and 25 µl of OB Protease was added. The remaining steps followed the kit recommendations, except that DNA was eluted two times in 50 µl into different extracts. DNA was extracted in batches of 23 samples plus one negative control in which no faecal pellet was added. Extracted DNA was distributed in 96-well plates where the last well was left empty for PCR negative control. Prey DNA was independently amplified using 2 different COI primer sets, the ZBJ-ArtF1c-R2c and FwhF2-R2n (Zeale et al., 2011; Vamos et al., 2017; respectively), modified to contain Illumina adaptors. The reaction consisted in 5 µl of Qiagen Multiplex Master Mix, 0.3 µl of each 10 nM primer, 3.4 µl of water and 1 µl of DNA extract. Cycling conditions consisted in a 15 min period at 95ºC, 35 cycles of 30 sec denaturation at 95 ºC, 30 sec annealing at 45 ºC for ZBJ and at 52 ºC for Fwh2, and 30 sec extension at 72 ºC, and a final extension period of 10 min at 72 ºC. Bat identification was double checked by amplifying a small COI fragment using the bat specific primers SFF_145f-351r (Walker et al., 2016), also adapted to be sequenced with an Illumina machine.

PCR reactions and cycling conditions were similar to that of prey, except that MyTaq Mix (Bioline) was used, and annealing was done at 56 ºC. All PCR products were diluted 1:4 with water and further subjected to a second PCR reaction in order to incorporate 7bp long identification tags and Illumina P5 and P7 adaptors. PCR reactions and cycling conditions were similar to the first PCR except that KAPA HiFi HotStart ReadyMix (Rocher) was used and only 8 cycles of denaturing, annealing and extension were done, with annealing at 55 ºC. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter), and subsequently quantified using Nanodrop and diluted to 15 nM. Purified and normalized PCR products were pooled per marker. These 3 libraries were then individually quantified using qPCR (KAPA Library Quant Kit qPCR Mix, Rocher) and diluted to 4 nM. Finally, libraries were pooled by mixing 51uL of Fwh2 library with 41 µl of ZBJ library and 8 µl of SFF library. This final library was sequenced using ~30% of a lane of a HiSeq Rapid SBS Kit v2 (500 cycles) for a target of 38k, 25k and 5k reads/pellet for Fwh2, ZBJ, and SFF amplicons respectively. The aimed coverages were selected based on the differences in taxa amplified by the different markers. Fwh2 not only amplifies insects, but also some fungi and vertebrates like bats, which leads to the loss of some reads to non-dietary items. ZBJ amplifies mostly insects, in particular Lepidoptera and Diptera, and does not amplify bat DNA. Finally, SFF is expected to amplify only the bat origin of the pellet as it was designed to not amplify the insects contained in the diet.

5.2.3 Bioinformatic analysis

Bioinformatic processing was done using Obitools (Boyer et al., 2016) and followed Silva et al. (2019). Shortly, paired-end reads were aligned, primer sequences removed, and reads collapsed into exact sequence variants (ESVs). ESVs that did not have a total read count in the entire dataset of 50 were removed, as well as if their length was outside the expected range for the targeted taxa (202-208bp for Fwh2 amplicons, 154-160bp for ZBJ, and 202bp for SFF). Finally, the ESV data was denoised using the command ‘obiclean’ with an ‘r’ level of one. ESVs were then compared to online databases (BOLD and NCBI) and identified to the lowest taxonomic rank possible. Whenever an ESV matched several species, genus, or families at similar identity levels, we selected the most inclusive taxonomic rank. Each ESV was also categorized as either being “diet” or “not diet” depending on its taxonomic identification. In general, fungi, internal and external parasites were categorized as “not diet”. Samples that did not have at least 100 reads belonging to dietary items were considered to have failed and were discarded. Samples collected from roosts whose bat identity was not possible to assess using any of the primer pairs were also discarded. From each sample we further removed all taxa representing less than 1% of the total number of dietary reads of that

sample. Finally, for bat individuals where more than 1 pellet was analysed, the taxa found in the different pellets was merged.

5.2.4 Insect pest assessment

In order to categorize each prey item as either a pest or not, we reviewed over 20 entomology books, as well as agronomy bulletins from Iberia and online databases dedicated to the listing of insect pests. We then created a list of all the taxa mentioned as pest and cross-checked which of our diet items were considered pests. We further reviewed those taxa and kept only the ones mentioned either as forest or agricultural pests.

5.2.5 Statistical analysis

Insect pest predation

To describe the overall predation patterns of insect pests by bats, we calculated both the frequency of occurrence of pests (FO), as well as the frequency of interaction (FI). Frequency of occurrence was calculated as the number of individuals and guano pellets collected in roosts in which a pest was detected, divided by the total number of individuals and roost samples. Frequency of interaction was calculated as the number of interactions with pest insects, divided by the total number of prey interactions. We considered an interaction whenever a prey species was detected in a bat/roost sample.

Ecological network characterization

We built a bipartite bat-pest interaction network using the R package ‘bipartite’ and characterized this network both in terms of i) modularity, to assess if bats were structured in terms of preyed pests, ii) nestedness, to see if the services provided by specialist bats was contained within that of generalist bats, and iii) specialization, to assess if the network was dominated by generalist or specialist species. We also built cumulative curves to assess the overall sample completeness of the network.

For modularity, we calculated the maximum modularity (Q) in bat-pest interactions using the function ‘metaComputeModules’ in the package ‘bipartite’, using the ‘Beckett’ method and 10,000 replicates. Modularity level was compared to 1000 null models built with the function ‘nullmodel’, and the method ‘vaznull’ that randomizes matrices with the same dimensions and connectivity as the initial web. High modularity values translate into strong modules, where bats interact mostly with pest species of the same module.

Network nestedness was calculated using the function ‘networklevel’ and the index ‘weighted NODF’. The statistical significance of the value was assessed by comparing it to the ones obtained in 1000 ‘vaznull’ null models, calculated as before. High values indicate that specialist species are feeding on common resources also used by generalists, while low values support a niche differentiation between generalist and specialist species.
Network specialization level, as well as its significance, were calculated as network nestedness, but this time using the index ‘H2’. With this index, high values reflect the dominance of specialist species and low values of generalist ones.
Finally, cumulative curves were built using the function ‘iNEXT’ from the package ‘iNEXT’ with 1000 bootstraps for both richness and sample coverage of bats, insect pests, and bat-pest interactions, per sample. Observed richness levels were then compared to Hills numbers estimates of richness (q=0) in order to assess the percentage of species sampled Hsieh et al., 2016b.

Bat species role in network

We assessed the role each species played in pest suppression patterns by calculating their level of centrality in the interaction network, as well as their contribution to network structure. For species centrality we considered 3 complemental centrality measures: degree, closeness and betweenness. Centrality degree is a simple measure that evaluates the number of pest species that each bat preys on, or in other words pest species diversity in diet. Centrality degree evaluates the proximity of a bat species to all the other bat species in the network. The more a bat shares at least one pest species with another bat, the higher its closeness value will be. Finally, betweenness measures how often a certain species is able to link two other species that do not share resources by sharing resources with them and thus acting like a “bridge” or “connector” in the network. These values were calculated using the function ‘specieslevel’ that computes these and other indexes for each species in the network. We used the weighted version of these last two metrics in order to take into account the number of interactions and not only their pattern of presence-absence (Opsahl et al., 2010).

Regarding the species role in network structure, we calculated both the participation coefficient (PC or just c) and intra module connection (z-score) to evaluate whether bat species behaved as peripherals, connectors, module hubs of network hubs. For this we used the function ‘czvalues’ on the most modular network configuration calculated previously, that computes those values for each species in the network. To assess if c and z values were statistically high or not, we also calculated the c and z values for each bat species based on 1000 ‘vaznull’ models and defined critical c and z values based on the 95% quantiles across species (Dormann & Strauss, 2014).

Functional redundancy and complementarity among bats

To evaluate the levels of complementarity and redundancy of pest regulating services offered by the bat community we calculated two indexes i) standardized specialization d', and ii) diet distinctiveness of each bat species, and conducted extinction simulations to evaluate how that affected overall pest suppression services.

The standardized specialization index d' evaluates how specialized a bat species is by taking into account the abundance of the prey it interacts with. This way, species with high d' values interact with few prey species and that no other bat species interact with, while species with low d' values interact with prey species that are very common in the network and with whom many other bat species also interact. Index d' was calculated using the function 'specieslevel'.

Diet distinctiveness is a new index proposed by us and essentially consists on the inverse of the average Pianka niche overlap of each bat species against the remaining bats in the community ($1 - \frac{\sum_i^{n-1} Pianka\ Niche\ overlap}{n-1}$).This index reflects how distinct the diet of each bat species is in comparison to the other bat species in the community, thus reflecting the uniqueness of the pest regulation service offered by bats. Higher values reflect a higher distinctness, this way indicating species that contribute to the complementarity of the system, while lower values reflect less distinct diets, and therefore species that contribute more to the redundancy of the services. We calculated the Pianka niche overlap for each pair of species using the function 'niche.overlap' of the package 'spaa' with the method 'pianka'. The diet distinctiveness of each species was then calculated by summing all the pairwise overlaps in which that species was involved, divided by the total number of partner species, and subtracting that to one, so that higher values could reflect a higher distinctness. Since Pianka niche overlap is calculated based on the frequency of occurrence of the different prey items in the diet of each species, we calculated the diet distinctiveness only for species whose sample size was above 10, in order to avoid interpreting averages based on poorly estimated index values.

Finally, to further explore the role each bat species plays in sustaining pest regulatory services, we conducted extinction simulations to see how the regulation of pest services would decay with the disappearance of bats. We extinguished bat species in three different ways. In the optimistic scenario, bats were removed according to the number of interactions they displayed with pest species. Bats with less interactions were first removed, while bats with more interactions were the last to disappear. We considered this the optimistic scenario in the sense that it allows the least number of bat species to maintain the regulation of the highest number of pest species. In the second scenario, bats were eliminated according to their

conservation status. We assumed that bats with less favourable conservation status are more likely to disappear first in the future and tested whether this would lead to an accelerating decline in pest regulation services compared to a random scenario. Bats were thus randomly removed according to their conservation status in Portugal (Cabral et al., 2005), with critically endangered (CR) species being eliminated first and least concern (LC) species last. Finally, we extinguished bats randomly and compared the outcome with the other two methods. For both the second and third method, we performed 10,000 randomizations and calculated the average and 95% confidence interval number of regulated pest species for each extinction step. Extinction curves were calculated using the function 'second.extinct' of the package 'bipartite'.

5.3 Results

Overall, a total of 19 bat species fed on 132 different agricultural and forest insect pests (Supplementary Table S1). For most species, over 50% of the individuals fed at least once on a pest species (frequency of occurrence; Figure 5.1), while relative interaction with pests was

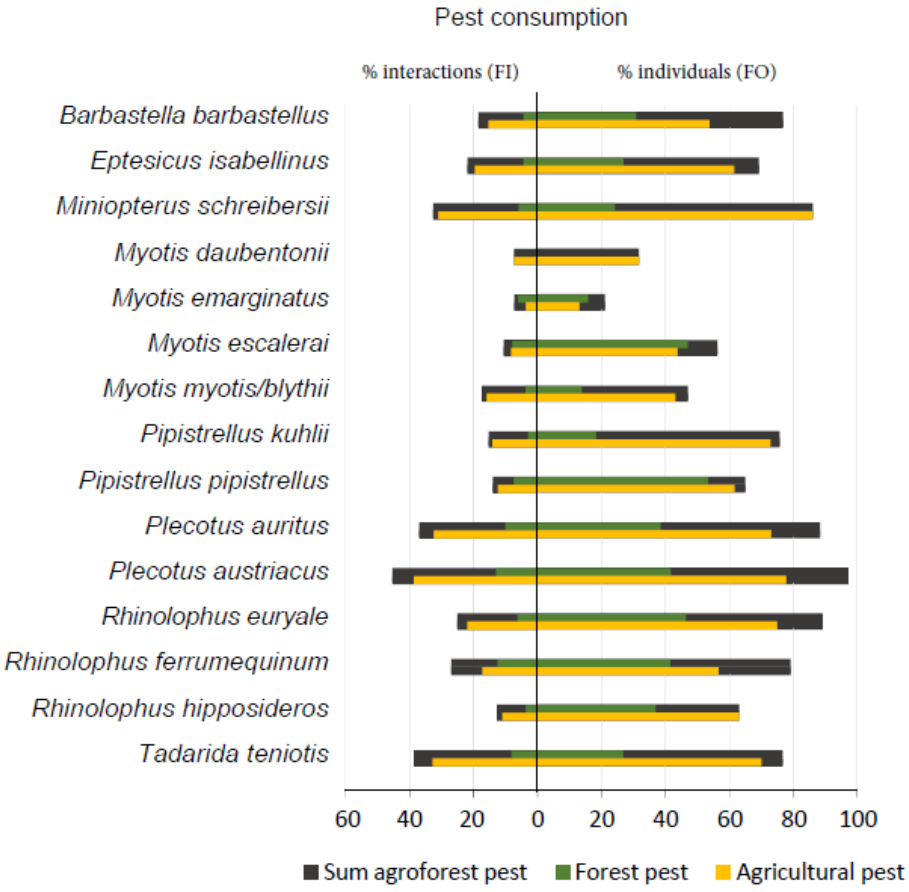

Figure 5.1 – Frequency of occurrence and of interaction with forest and agricultural pests per bat species. Species whose sample size was below 10 are not shown.

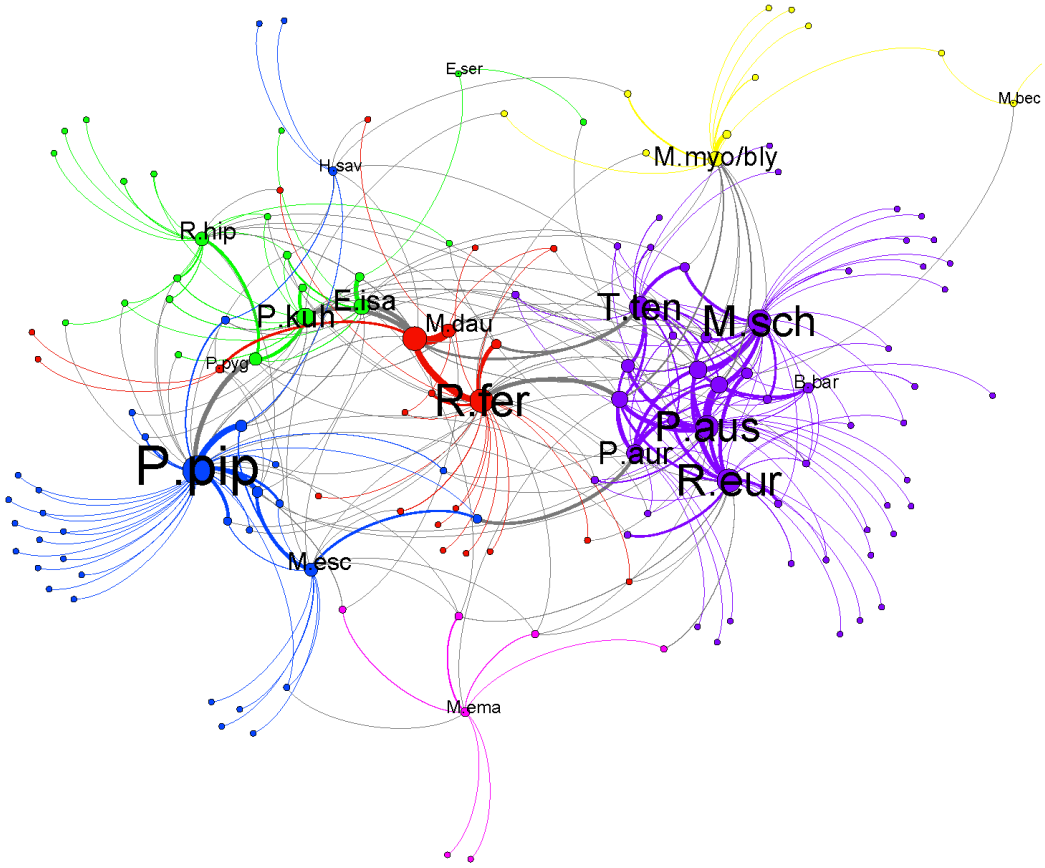

Figure 5.2 – Bat and pest species interaction network. Nodes (species) and edges (interaction links) are colored according to the modules to which they were assigned. Grey edges represent interactions outside the species respective module. Node, edge and label sizes are proportional to the number of observed interactions.

much lower (frequency of interaction), with only four species having at least one third of its diet composed by pests. The frequency of occurrence of pests in bat diet was as high as 97% and constituted up to 45% of prey interactions in grey long eared bats (*Plecotus austriacus*; Figure 5.1), while for other species like the Geoffroy's bat (Myotis emarginatus) these values were much lower, with only 21% of individuals feeding on pests and pest species representing only 7% of prey interactions.

The bat-pest interaction network was modular (Q = 0.48, p-value < 0.0001), with bats being clustered in 6 groups, each composed by 1-6 bat species (Figure 5.2). Each module was associated to pests belonging to different orders of insects, whose frequency of interaction varied according to module (Figure 5.3). Two modules composed by *M. emarginatus*, Savi's pipistrelle (*Hypsugo savii*), Escalera's bats (*Myotis escalerai*), and common pipistrelle (*Pipistrellus pipistrellus*), were more associated to forestry pests, with 44-88% of predated pest species causing damage on trees (Table S5.1).

Network nestedness was lower than expected (wNODF = 8.9, pvalue < 0.0001), which indicates that the pests predated by specialist bats are not preyed by the generalist bats. Network specialization (H_2) was higher than expected (H_2 = 0.36, pvalue < 0.0001), but still

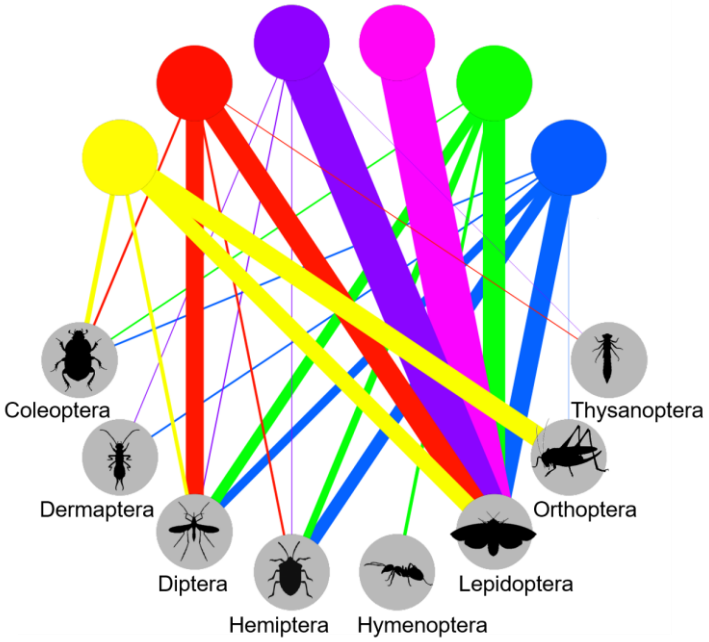

Figure 5.3 – Interaction network between bat modules and major insect orders. Edges (interaction links) are weighted according to the observed percentage of occurrence frequency.

relatively low, thus indicating the dominance of generalist species with the existence of few relatively common more specialized species.

Overall richness and sample completeness of the bat-pest interaction network was not perfect, with bat's species richness having reached an asymptote, but not insect pests (Figure S5.1). A sample coverage of about 90% was obtained for insect pests and 70% for bat-pest interactions, which means that 10% of the bat-pest interactions in the community occur with currently unsampled pest species, while the other 20% occur between bat and pest species already sampled, but whose interaction was not observed.

The species with highest scores in the different components of network centrality (degree, closeness and betweenness) were almost always the same three (Table S5.2). The common pipistrelle was the one found feeding on a higher number of pest species – highest degree, followed by the greater horseshoe bat (*Rhinolophus ferrumequinum*) and the common bent-wing bat (*Miniopterus schreibersii*). Regarding closeness, which measures the proximity of a species to all the other species in the network, *R. ferrumequinum* showed the highest values, followed by *P. austriacus* and *M. schreibersii*. Finally, the highest values of betweenness, which essentially reflects species that better connect different modules of bat-pest interactions, were observed in *R. ferrumequinum*, *M. shreibersii* and *P.pipistrellus*.

The profile of species interactions was mostly characterized by low c (<0.65) and z values (<1.28), thus meaning that most species were considered peripherals, i.e. not well connected within or between network modules. No species could be classified as either connector, i.e. well-connected between modules but not within modules, or network hub, i.e

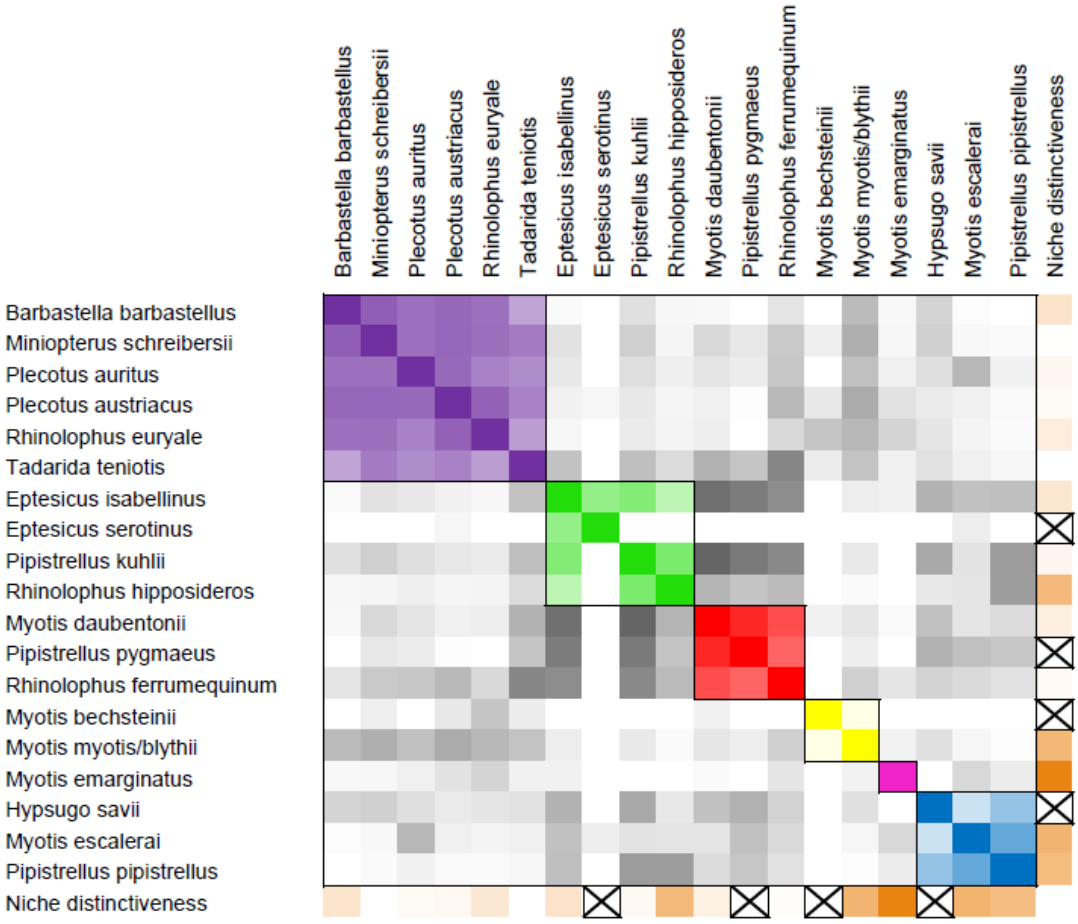

Figure 5.4 – Matrix of pairwise Pianka niche overlap between studied species. Bats are grouped according to network modules. Niche distinctiveness is also represented per species except for species with sample size below ten. Colour strength represents lower and higher values.

connecting the entire community both within and across modules. Nevertheless, two bats, *M. schreibersii* and the little horseshoe bat (*Rhinolophus hipposideros*), were identified as module hubs, thus well connected within their modules, but not between modules.

In terms of diet distinctiveness, *M. emarginatus* showed the most non-overlapping services of pest control, most likely caused by the overall low number of interactions with pest species, many of which were never or rarely predated by other bats (highest d' in Table S5.2, or niche distinctiveness in Figure 5.4).

Extinction simulations revealed that 3 bat species (*P. pipistrellus*, *R. ferrumequinum* and *R. euryale*) are enough to maintain the regulation of ~60% of the pest species in our area, while 6 bat species can regulate about three quarters of the pests (Figure 5.5). However, 3 of these 6 species are either vulnerable (VU) or critically endangered (CR) at the national level. If bat species were extinct according to their conservation status, a significant reduction of regulated pest species was observed, when compared to a random scenario.

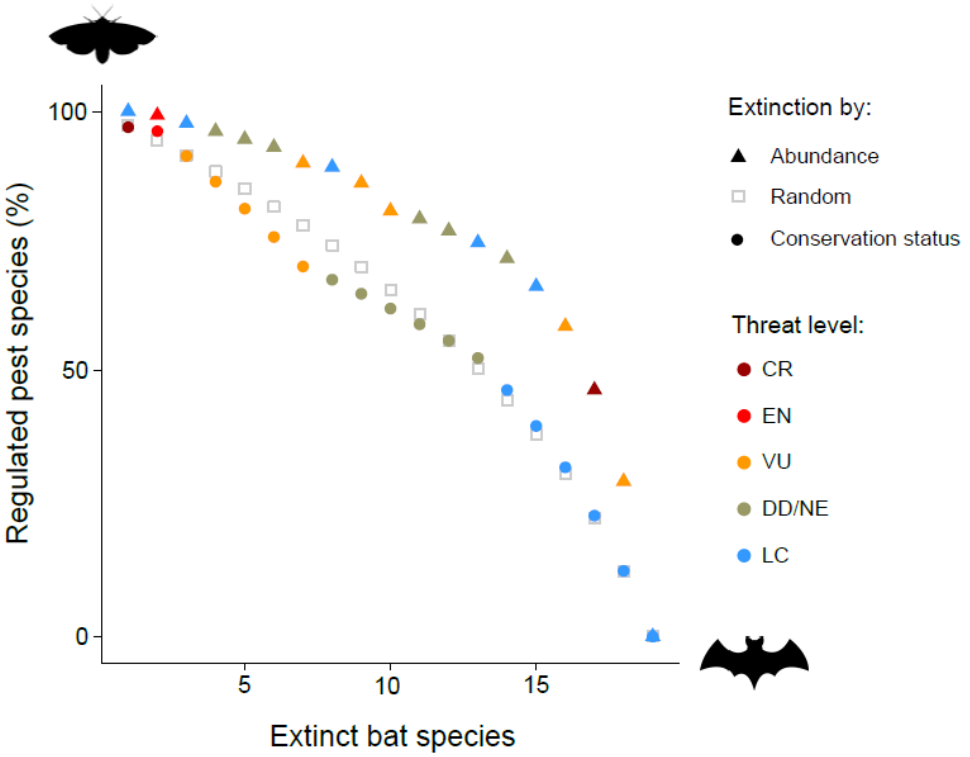

Figure 5.5 – Effect of bat species extinction on the percentage of regulated pest species. Curves were built assuming three different scenarios based on the number of pest interactions (abundance), randomly (random) and by decreasing level of conservation status (conservation status).

5.4 Discussion

We described an insect pest suppression network by bats in a highly heterogeneous landscape with multiple forest and agricultural land uses. Pest predation levels by bat species varied greatly, both in terms of frequency of occurrence and interaction, suggesting that bats do play different roles in biocontrol functions. Although, we found a high redundancy in the bat-pest system, with multiple bats feeding on the same species of pests and thus performing similar regulatory functions, we did observe some levels of niche differentiation, with bats organized in 6 different modules, and species like *Myotis emarginatus* showing a very distinct pattern of pest predation. To our knowledge this is the first time a bat-insect pest predation system involving an entire bat community has been described, with other studies either focusing on just one or very few species (Brown et al., 2015; Aizpurua et al., 2018; Krauel et al., 2018; Baroja et al., 2019; Kemp et al., 2019; Weier et al., 2019), or on other components of the diet (Galan et al., 2018; Gordon et al., 2019). We believe that our ecological network analysis framework coupled with DNA metabarcoding is an important tool to better understand the role insectivorous vertebrates play in the provision of ecosystem services, as pest

regulation and suppression, providing valuable knowledge for the design of more efficient landscapes.

Overall, insect pest predation by bats varied between species, with a few species standing out for both their frequency of occurrence and interaction with pests. Species like *Plecotus spp.*, *Tadarida teniotis* and *Miniopterus schreibersii* showed high levels of predation and interaction with insect pests. Unlike other species that often fed on at least one pest, but also fed on many other non-pest insects (like *Pipistrellus spp.* and *Rhinolophus hipposideros*), these bats had a high proportion of their diet composed by pests and are probably the highest consumers of those insects in their community, but not necessarily the ones mostly contributing to pest suppression in the landscape. *Plecotus* species in particular, although widespread and relatively common, have relatively small home ranges, breed in small colonies usually under 20 individuals, and are not believed to have large population sizes (Dietz et al., 2009). *T. teniotis* and M. *schreibersii* on the other hand, show an elevated potential of pest suppression as both species have large home ranges, are habitat generalists that feed on high altitudes over almost any type of land cover type, can concentrate in large colonies composed by several hundreds to thousands of individuals, and are thought to have large population sizes (Dietz et al., 2009).

Our bat-pest network was characterized as being modular, with bats organized in 6 modules, not nested and with low specialization levels. Unfortunately, we could not find any other insectivorous vertebrate pest control networks to which we could compare our network structure, so it remains to be seen whether these characteristics are common across other communities and organisms or not. The bats found in each module seem to be of very different natures, exhibiting differences in foraging strategies, echolocation calls, habitat preferences, morphological characters, and so on.

One interesting pest suppression module of bats was that composed by *Barbastella barbastellus*, *Plecotus spp., R. euryale*, *T. teniotis* and *M. schreibersii*, in which the only thing shared is probably their fondness for big moths (Goerlitz et al., 2010; Razgour et al., 2011; Arrizabalaga-Escudero et al., 2015; Mata et al., 2016; Aizpurua et al., 2018). Some of these bats forage in open areas way above the canopy, other close to the ground, some are habitat specialists, while others are completely generalists (Dietz et al., 2009). So although all these bats are offering the same regulatory service, and could in theory be inter-replaceable, in reality they are also complimentary in the sense that they are performing the same function at different places, this way increasing the magnitude and stability of the provided biocontrol service. It is also interesting, that forest associated species like *B. barbastellus*, *P. auritus*, *Rhinolophus spp.* and *M. bechsteinii,* did not regulate the highest diversity of forest pests, although some of them did show the highest predation levels of forest pests in terms of frequency of occurrence and interaction. The highest diversity of forest pests was observed in

two other modules composed by *M. emarginatus*, *Hypsugo savii*, *M. escalerai* and *P. pipistrellus*. Unlike the first module, these two contain mostly small Lepidoptera, Hemiptera and Diptera, with a weaker flight capacity, probably reflecting the different dietary niche of those bats. As before, these modules also contain bats with very different echolocation calls and foraging behavior.

The most central species in our network were not the ones more frequently interacting with pest species. In fact, *P. pipistrellus*, one of the top scorers in centrality measures had one of the lowest values of interaction frequency with pests. This way, although pests constitute a small percentage of its diet, it does suppress an overall high number of pests (degree centrality), as well as pests belonging to different modules (betweenness centrality). Moreover, this species showed some moderately high levels of niche distinctiveness, preying on pests not predated by other bats, thus emphasizing its key role in pest control. The other two highly central species in the network were *R. ferrumequinum* and *M. schreibersii*. These two do not only share the high capacity of *P. pipistrellus* to consume a high number of different pests and of different modules, but also feed regularly on pests that are being predated by all other bats in the community (closeness centrality). This last pattern was further confirmed by the lowest niche distinctiveness values observed in these two species, that are this way highly redundant with the rest of the bats, and thus good representatives of bat pest control services. Together, these 3 central bat species are able to regulate about 60% of the pests found in the study area and are thus major candidates for ecosystem intensification.

Finally, the most distinct species in our community, *M. emarginatus*, not only predated less often on pests, the ones they did prey on were either not preyed by other bats or at low frequencies. This uniqueness in pest suppression services was also translated into its role in the network, being isolated in a single species module, as well as in its niche distinctiveness and specialization index. This species is known to be a spider specialist that can forage in highly cluttered vegetation, both gleaning insects from the substrate and hovering in front of foliage, as well as by aerial pursuit (Krull et al., 1991; Goiti et al., 2011). The uniqueness of its pest control service is probably a reflection of its distinct diet and foraging strategy among our bat community.

Regarding the extinction simulations, we found that only a reduced number of bat species are needed to regulate a high proportion of the pests. Surprisingly, many of those species are of conservation concern, and thus management practices aiming to improve biocontrol by bats could potentially benefit vulnerable and endangered species, instead of just abundant and generalist ones. Notwithstanding, for some modules of bat pest control, functionally redundant species seem to be performing their role in different habitats across the landscape. This probably means that there are none or few truly redundant bat species in our

community, and that a diverse functionally redundant system with many different species of bats is much more efficient in regulating pests across the landscape than a reduced number of bats, even if the total number of regulated pest species is the same. In fact, some authors have questioned whether true functional redundancy does exist at all in nature if all niche dimensions are considered (Loreau, 2004), while others argue that functional redundancy might be more common in small size and hyper-diverse groups of organisms like insects (Scheffer et al., 2015).

5.4.1 Limitations and potential shortcomings

Our study, although involving a highly laborious sampling scheme, which included countless hours of fieldwork, as faeces were mostly collected by capturing free-ranging bats, did not fully describe the total diversity of insect pests existing in the region, nor therefore the richness of bat-pest interactions. This problem is quite common in network analysis, with several hundreds of samples usually being required in order to fully capture the diversity of communities, and even more so of species interactions in those communities (Chacoff et al., 2012; Jordano, 2016a). Yet, studies evaluating the effect of under sampling in network structure and characteristics have found that metrics like modularity, nestedness and specialization are quite robust to this issue, provided at least 30% of the species have been found, which is by far our case (Blüthgen et al., 2006; Rivera-Hutinel et al., 2012; Costa et al., 2016). The effect of under sampling in species roles is less well understood, although species specialization d' has been shown to be stable across different network sizes, network asymmetries, and number of interactions (Blüthgen et al., 2006). Nevertheless, centrality roles in the network for example, might be expected to be affected by unevenness in bat species sampling, i.e. species with higher sample sizes (due to their catchability) showing higher centrality roles in pest suppression services. Although such a correlation can be expected, as ultimately all these metrics depend on the number of observed interactions which depend on sample size, in our study this relation was not fully linear. For example, our most sampled bat species *M. daubentonii* (n=79) showed relatively low values for both degree centrality and weighted betweenness, while *M. schreibersii*, a mediumly sampled bat, showed top scores in all centrality measures. This way, we believe that the overall species role in pest suppression of our bat community is fairly well represented, with two of the less sampled bats (*M. bechsteinii* and *E. serotinus*, n<10) being indeed rare in the region, while the other two (*P. pygmaeus* and *H. savii*) being uncommon and perhaps slightly under sampled in comparison to other also uncommon species like B. barbastellus, but whose role should not be that different even with more samples.

One other evident limitation of our study is that it is based on one single network. Indeed, we would be happy to see our approach replicated both in time and space. Pollinator network studies for example, often generate several networks, whose characteristics are then compared to community and landscape traits (e.g. Redhead et al., 2018; Jauker et al., 2019). This allows a deeper understanding of the factors affecting the plant-pollinator interactions, and how these interactions can be maximized in order to increase natural pollination. In insectivorous vertebrate predator studies however, such networks are much harder to generate, as sufficient sample size required to obtain robust and meaningful results are considerably much more difficult to obtain. Also, contrary to predatory invertebrates, the community of flying vertebrates cannot be studied at the patch level, but rather at the landscape level, as the home range of both groups is quite distinct. This further inflates the potential pest diversity each species might find when foraging and the required sample size, especially in highly heterogeneous landscapes like the one studied, where small patches of different vegetables, crops and fruit trees intertwine with patches of shrubs, grasses and forest. Replicates of these types of networks should help getting a better understanding whether the role of each bat species plays in pest regulation changes according to climatic, landscape or other environmental variables. Nevertheless, for disentangling the role each species plays in their community, one single network can provide crucial information about the services each species is providing in the assessed landscape, that can probably be extrapolated to other landscapes with similar bat assemblies and landscape structure (da Silva et al., 2017; Delmas et al., 2019).

5.4.2 Conclusion

We recovered a complex interaction network of bats and insect pests in a multifunctional landscape. Through the combined use of metabarcoding and ecological networks we were able to identify possible key species in pest suppression of Mediterranean agricultural landscapes, as well as describe the overall role each species plays in service provision. We found that pest regulation services were structured and non-nested, but still relatively non-specialized, and that different bats seem to be performing similar functions across different habitats in the landscape.

An important point to include in future analysis, would be to incorporate estimates of bat abundance and energetic requirements. This would allow more realistic estimates of pest suppression levels attainable by each bat species. These aspects come nonetheless with additional methodological challenges. In particular, bat population sizes are extremely difficult to estimate. Unlike birds, where decades of observation have translated into well standardized census methodologies, small insectivorous bats cannot be counted with perhaps the

exception of roosts. Locating all possible roosts of every bat species across a landscape is however an almost impossible task. Acoustic monitoring with ultrasound detectors is often used to assess bat relative abundance, but even this does not happen without its bias. Not all species can be distinguished by their echolocating calls and some species are rarely recorded due to their echolocation characteristics (call intensity and frequency), making population estimates per species difficult. This way, we believe that our approach combining ecological networks and DNA metabarcoding is the most straightforward way to unravel the role each species plays in insect pest control.

Acknowledgments

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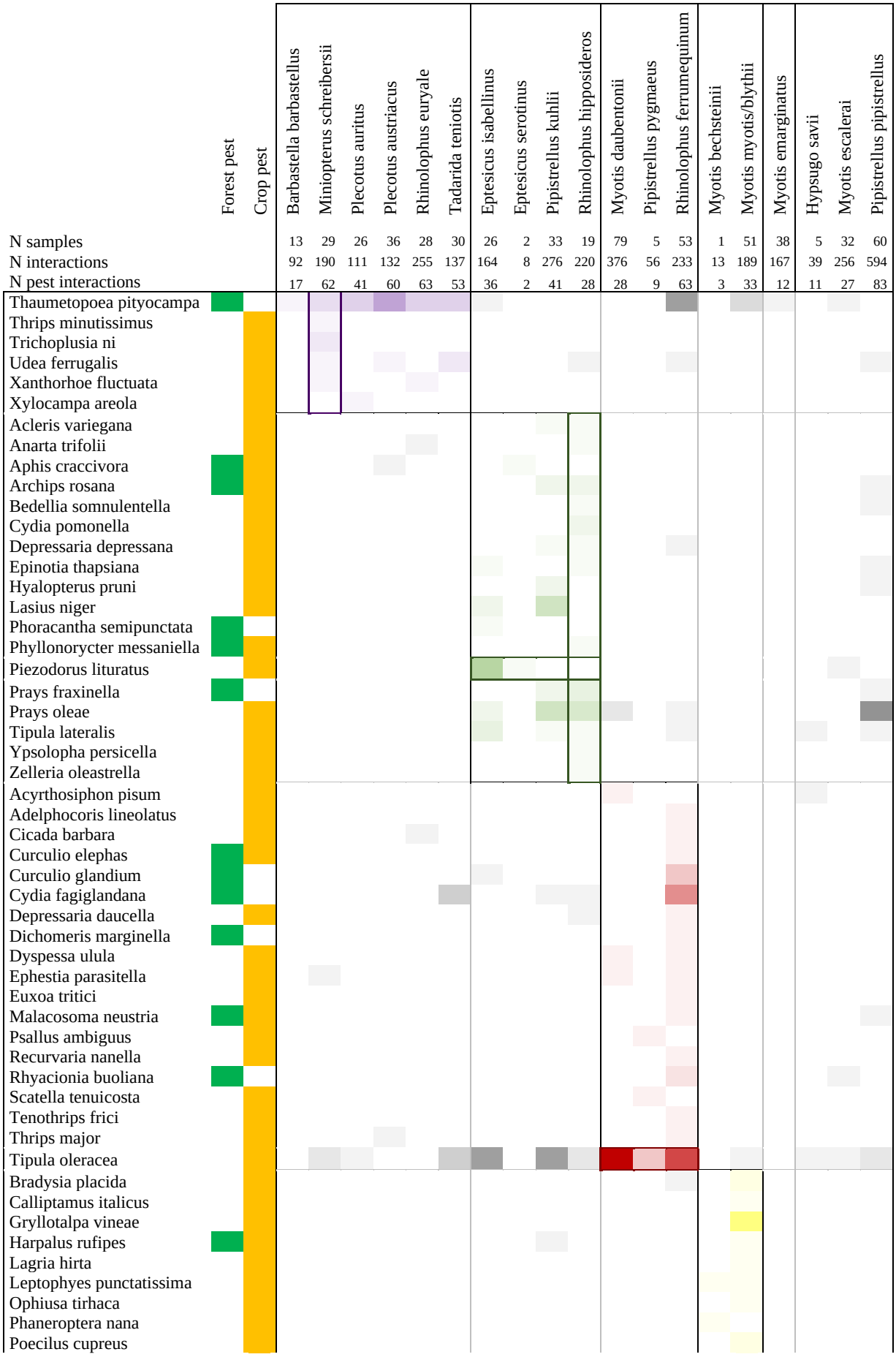
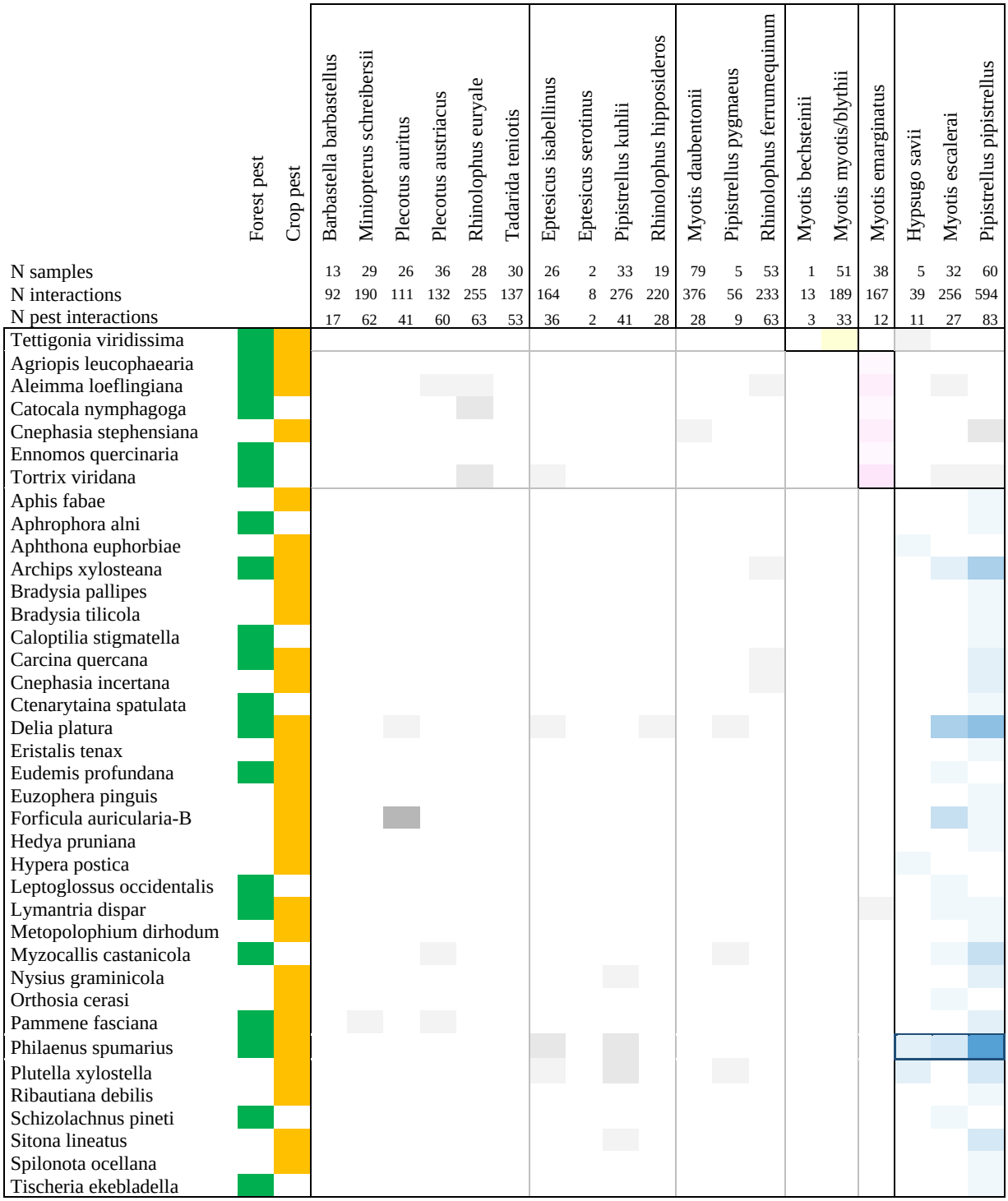
Supporting Information

Table S5.1 – Bat and insect pests interaction matrix. Interactions are coloured according to the network module they were assigned to. Grey cells represent interactions outside the species respective module. Module hub species are also highlighted within their modules. Colour strength reflects the observed number of interactions.

	Forest pest	Crop pest	Barbastella barbastellus	Miniopterus schreibersii	Plecotus auritus	Plecotus austriacus	Rhinolophus euryale	Tadarida teniotis	Eptesicus isabellinus	Eptesicus serotinus	Pipistrellus kuhlii	Rhinolophus hipposideros	Myotis daubentonii	Pipistrellus pygmaeus	Rhinolophus ferrumequinum	Myotis bechsteinii	Myotis myotis/blythii	Myotis emarginatus	Hypsugo savii	Myotis escalerai	Pipistrellus pipistrellus
N samples			13	29	26	36	28	30	26	2	33	19	79	5	53	1	51	38	5	32	60
N interactions			92	190	111	132	255	137	164	8	276	220	376	56	233	13	189	167	39	256	594
N pest interactions			17	62	41	60	63	53	36	2	41	28	28	9	63	3	33	12	11	27	83
Acronicta auricoma	■	■																			
Acronicta rumicis		■																			
Agrotis exclamationis		■																			
Agrotis ipsilon		■																			
Agrotis puta		■																			
Agrotis segetum		■																			
Alcis repandata	■	■																			
Amphipyra pyramidea	■	■																			
Aphis spp.		■																			
Autographa gamma		■																			
Biston betularia	■	■																			
Campaea margaritaria	■	■																			
Chrysodeixis chalcites		■																			
Cosmia trapezina	■	■																			
Cossus cossus		■																			
Dysgonia algira		■																			
Etiella zinckenella		■																			
Euproctis chrysorrhoea	■	■																			
Evergestis limbata		■																			
Gymnoscelis rufifasciata		■																			
Hydriomena furcata	■	■																			
Hypsopygia costalis		■																			
Lacanobia oleracea		■																			
Leucania loreyi		■																			
Liorhyssus hyalinus		■																			
Menophra abruptaria		■																			
Mesapamea secalis		■																			
Mimas tiliae	■	■																			
Noctua comes		■																			
Noctua fimbriata	■	■																			
Noctua pronuba		■																			
Nycteola revayana	■																				
Orthosia incerta	■	■																			
Ourapteryx sambucaria		■																			
Palpita vitrealis		■																			
Panolis flammea	■																				
Peribatodes rhomboidaria		■																			
Peridroma saucia		■																			
Phalera bucephala	■	■																			
Phlogophora meticulosa		■																			
Phragmatobia fuliginosa		■																			
Spodoptera exigua		■																			


Forest pest
Crop pest
Barbastella barbastellus
Miniopterus schreibersii
Plecotus auritus
Plecotus austriacus
Rhinolophus euryale
Tadarida teniotis
Eptesicus isabellinus
Eptesicus serotinus
Pipistrellus kuhlii
Rhinolophus hipposideros
Myotis daubentonii
Pipistrellus pygmaeus
Rhinolophus ferrumequinum
Myotis bechsteinii
Myotis myotis/blythii
Myotis emarginatus
Hypsugo savii
Myotis escalerai
Pipistrellus pipistrellus
N samples 13 29 26 36 28 30 26 2 33 19 79 5 53 1 51 38 5 32 60
N interactions 92 190 111 132 255 137 164 8 276 220 376 56 233 13 189 167 39 256 594
N pest interactions 17 62 41 60 63 53 36 2 41 28 28 9 63 3 33 12 11 27 83
Thaumetopoea pityocampa
Thrips minutissimus
Trichoplusia ni
Udea ferrugalis
Xanthorhoe fluctuata
Xylocampa areola
Acleris variegana
Anarta trifolii
Aphis craccivora
Archips rosana
Bedellia somnulentella
Cydia pomonella
Depressaria depressana
Epinotia thapsiana
Hyalopterus pruni
Lasius niger
Phoracantha semipunctata
Phyllonorycter messaniella
Piezodorus lituratus
Prays fraxinella
Prays oleae
Tipula lateralis
Ypsolopha persicella
Zelleria oleastrella
Acyrthosiphon pisum
Adelphocoris lineolatus
Cicada barbara
Curculio elephas
Curculio glandium
Cydia fagiglandana
Depressaria daucella
Dichomeris marginella
Dyspessa ulula
Ephestia parasitella
Euxoa tritici
Malacosoma neustria
Psallus ambiguus
Recurvaria nanella
Rhyacionia buoliana
Scatella tenuicosta
Tenothrips frici
Thrips major
Tipula oleracea
Bradysia placida
Calliptamus italicus
Gryllotalpa vineae
Harpalus rufipes
Lagria hirta
Leptophyes punctatissima
Ophiusa tirhaca
Phaneroptera nana
Poecilus cupreus


Forest pest
Crop pest
Barbastella barbastellus
Miniopterus schreibersii
Plecotus auritus
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Rhinolophus euryale
Tadarida teniotis
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Rhinolophus hipposideros
Myotis daubentonii
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Rhinolophus ferrumequinum
Myotis bechsteinii
Myotis myotis/blythii
Myotis emarginatus
Hypsugo savii
Myotis escalerai
Pipistrellus pipistrellus
N samples 13 29 26 36 28 30 26 2 33 19 79 5 53 1 51 38 5 32 60
N interactions 92 190 111 132 255 137 164 8 276 220 376 56 233 13 189 167 39 256 594
N pest interactions 17 62 41 60 63 53 36 2 41 28 28 9 63 3 33 12 11 27 83
Tettigonia viridissima
Agriopis leucophaearia
Aleimma loeflingiana
Catocala nymphagoga
Cnephasia stephensiana
Ennomos quercinaria
Tortrix viridana
Aphis fabae
Aphrophora alni
Aphthona euphorbiae
Archips xylosteana
Bradysia pallipes
Bradysia tilicola
Caloptilia stigmatella
Carcina quercana
Cnephasia incertana
Ctenarytaina spatulata
Delia platura
Eristalis tenax
Eudemis profundana
Euzophera pinguis
Forficula auricularia-B
Hedya pruniana
Hypera postica
Leptoglossus occidentalis
Lymantria dispar
Metopolophium dirhodum
Myzocallis castanicola
Nysius graminicola
Orthosia cerasi
Pammene fasciana
Philaenus spumarius
Plutella xylostella
Ribautiana debilis
Schizolachnus pineti
Sitona lineatus
Spilonota ocellana
Tischeria ekebladella

Table S5.2 – Network species' metrics calculated for each bat species. Highest values are highlighted in bold for easier reading.

Species	normalised degree	weighted betweenness	weighted closeness	d'	c	z
Barbastella barbastellus	0.068	0.000	0.036	0.328	0.000	-1.441
Eptesicus isabellinus	0.121	0.084	0.047	0.396	**0.457**	-0.322
Eptesicus serotinus	0.015	0.000	0.004	**0.610**	0.000	-0.999
Hypsugo savii	0.068	0.000	0.020	0.456	**0.453**	-0.752
Miniopterus schreibersii	**0.220**	**0.163**	**0.063**	0.345	0.093	**1.320**
Myotis bechsteinii	0.023	0.000	0.007	**0.691**	0.069	-0.707
Myotis daubentonii	0.068	0.004	0.063	0.424	0.374	-0.548
Myotis emarginatus	0.061	0.000	0.024	**0.673**	0.173	NA
Myotis escalerai	0.121	0.002	0.038	0.487	0.221	-0.383
Myotis myotis/blythii	0.121	0.066	0.032	0.566	0.099	0.707
Pipistrellus kuhlii	0.136	0.054	0.049	0.382	**0.524**	-0.055
Pipistrellus pipistrellus	**0.295**	**0.125**	0.056	0.586	0.278	1.135
Pipistrellus pygmaeus	0.045	0.000	0.021	0.407	0.227	-0.606
Plecotus auritus	0.114	0.039	0.051	0.345	0.203	-0.791
Plecotus austriacus	0.182	0.073	**0.067**	0.367	0.261	0.526
Rhinolophus euryale	0.197	0.112	0.060	0.415	0.322	0.513
Rhinolophus ferrumequinum	**0.242**	**0.228**	**0.070**	0.422	0.363	**1.154**
Rhinolophus hipposideros	0.144	0.000	0.036	0.517	0.189	**1.377**
Tadarida teniotis	0.144	0.051	0.061	0.337	0.091	-0.126

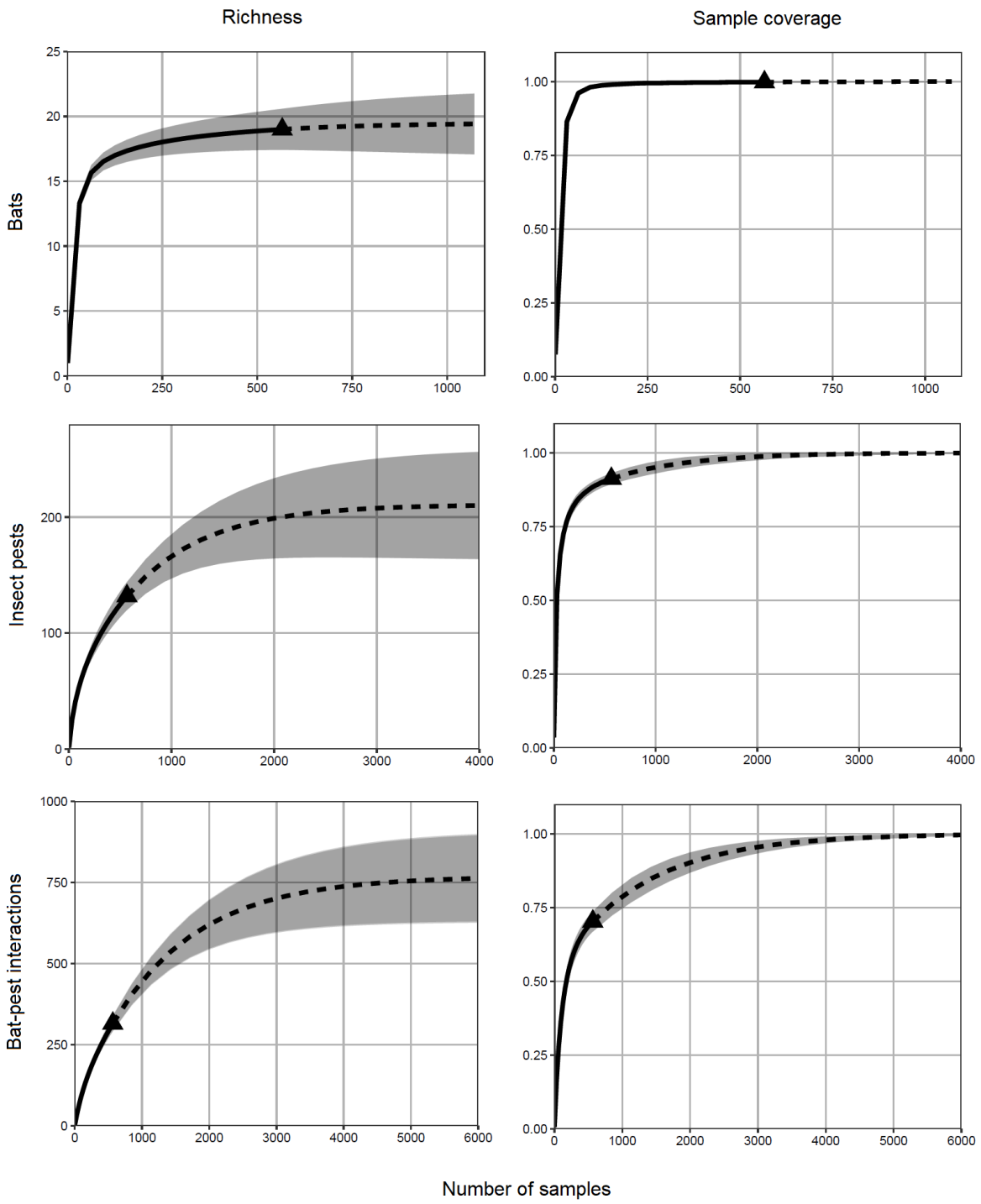

Figure S5.1 – Cumulative curve of bats, insect pests, and bat-pest interactions richness and sample coverage, per number of samples analysed. Triangle symbol represents the observed value and the dashed line the extrapolated values expected with higher sampling effort. Shaded area represents 95% confidence intervals. Observed values were respectively for bats, insect pests, and bat-pest interactions: 100%, 63%, and 41% of estimated richness; 100%, 91%, and 70% of sample coverage.

Chapter 6

General Discussion

6.1 In a nutshell

This thesis presents significant developments in the use of metabarcoding techniques for the study of small vertebrates' diets, thereby contributing to enhance our understanding of species trophic interactions in the context of the management of multifunctional landscapes. We have detailed how biological replication can affect dietary descriptors in comparison to technical replicates, as well as how primer bias and secondary predation can influence the outcome of metabarcoding analysis. At the same time, we have demonstrated the powerfulness of the technique by describing how highly resolved dietary data provided by metabarcoding can illuminate subtle intraspecific variations in predation patterns in a bat species, and the potential for pest control services offered by an entire community of insectivorous bats in a complex multifunctional landscape. Overall, our two technical manuscripts provide important guidance to the design of dietary studies based on metabarcoding, while our two ecological studies highlight its use in answering biological questions and providing vital information for species' conservation and ecosystem management. In this final chapter, I present the main findings of each study in relation to the overall and specific objectives of this thesis, while discussing its implications for the description of trophic species interactions and their importance for species' ecology and the provisioning of ecosystem services.

6.1. Understanding species trophic interactions using metabarcoding

The description of species' trophic interactions is without a doubt an intrinsically complex task. With so many different methods, types of samples, and dietary descriptors available, it is not surprising that trophic ecologists often end up classifying species in broad categories like 'insectivorous' or 'frugivorous'. Many times, species are so plastic and adaptable that their diet changes considerably depending on their life-stage, season, resource availability, sympatric species' identity, and so on (Garvey & Whiles, 2016). Yet, understanding trophic relationships is key to understanding ecosystem functioning and community dynamics, and requires detailed knowledge of species interactions. Metabarcoding has certainly revolutionized this understanding by allowing unprecedented taxonomic resolution, often at species level, and the ability to processing hundreds of samples with relative ease (Taberlet et al., 2018).

Like all methods, though, metabarcoding has its own set of biases and limitations, with many sources of biological and technical biases. The use of metabarcoding for dietary analysis

has often followed the technical steps and recommendations of other areas like molecular biodiversity assessments and species detection from soil and water samples, with a very high focus given to technical replication. In these cases, the nature and quality of the DNA is somewhat different from that in dietary studies, and there are important differences in the biological questions asked. For example, when surveying endangered or invasive species, the presence or absence of particular species in a certain sample originated from a specific site might have important consequences in terms of conservation measures. In those situations, technical limitations and biases of the analysis might have large consequences on the results. However, when analysing the overall diet of a species, particularly of small insectivorous vertebrates who can often feed on several hundreds of different preys, the specific link between a certain sample and a certain prey is not so important. Assuming that the technical errors are randomly distributed among the samples and biological groups under study, then they should not generate important biases that might eventually produce erroneous biological patterns. For instance, although the number of false positives might increase with the lack of stringent technical controls, their random distribution across sampling units may contribute to increased noise in the data but not necessarily to biases distorting the overall biological patterns. Thus, to properly characterize the diet of species, what matters the most is having a good representation of the ecological group under study, which can only be accomplished by biological replication. Although this might sound obvious from a statistical point of view, the prevailing perspective is still quite biased towards the technical lab component. As a reviewer of dietary studies using metabarcoding, I have often come across studies in which a good sample size was obtained, but authors then decided to pool the samples in order to meet their available budget for molecular analysis, this way losing statistical power. The molecular analysis then often involved the use of 3 PCR replicates, along with very deep sequencing coverage, which guarantees the high quality and robustness of their results for each specific sampling unit, but helps them very little in answering their biological questions, which is in the end the main goal of any study. Authors are then ‘forced’ to simply describe the results of their pooled samples, without being able to make statistically supported inferences due to the lack of sufficiently large sample sizes.

In **chapter two** we addressed these issues, showing that biological replication contains much higher variability than technical replication and should thus deserve particular attention by researchers studying species’ diets. Coupled with strict bioinformatic filters, like discarding all haplotypes with a read count below 1% for each sample, achieving high levels of biological replication should be key to consistent and robust results, while PCR replicates should show little or no variation. We also demonstrated that under these circumstances, pools of samples become quite weak in describing the overall diet of a population, as ‘rare’ species become even rarer when multiple samples are combined and thus more likely to be missed during PCR

or discarded during bioinformatic processing. It has been suggested that the poor performance of pools in estimating frequencies of occurrence could be caused by methodological issues during the DNA extraction step (e.g. clogged membrane, Andriollo et al., 2019), but we did not observe such phenomena in our case. In that study, Andriollo et al. (2019) found that pools of samples from bat colonies composed by several individuals and containing guano from several foraging nights, contained higher species richness than single pellets. However, the authors applied very different bioinformatic filters than the ones used in **chapter two**, removing haplotypes with less than 0.01% of the reads per sample, instead of 1%. If such filter was applied in our study, a comparable result would be observed, but it would become impossible to distinguish between 'real' species' haplotypes and spurious ones, resultant from PCR and sequencing errors, tag misassignment events, sequencing errors, intragenomic variability, and so on. Under such scenario, PCR replicates would probably be key in ensuring the robustness of the data. Sequencing pools of samples instead of individual samples could probably be justified if studying ecological gradients, like seasonal changes in diet or the effect of habitat fragmentation on diet composition. In such studies, each data point could be the overall diet of a species in a certain moment of time (evaluated by pools of samples) or across different landscapes. The biological replicates in this case would not be individuals, but different colonies of bats, or landscapes, and should be in a sufficiently high number to be able to answer the biological question, taking into account that only presence/absence data would be obtained per biological replicate.

One of the major limitations of metabarcoding for dietary analysis is the inability to estimating species abundances within samples. Although there are some exceptions, i.e. cases where primers anneal in highly conserved regions or when species specific correction factors were estimated (Thomas et al., 2016; Taberlet et al., 2018; Vasselon et al., 2018), there are always some sort of technical and biological sources of imbalance between what was ingested and what can be observed after sequencing. One of the major causes for these errors are related to primer bias. Although more efficient and highly degenerate primers, i.e., primers that can amplify a wide range of different taxa, have been designed in the last years, uneven amplification still seems to be a problem (Elbrecht et al., 2019; Jusino et al., 2019). In particular, primers with little or no degeneracy targeting highly variable regions like cytochrome oxidase 1 (COI) seem to cause the highest biases, not only in terms of read abundance, but also of taxa detection (Elbrecht et al., 2019; Tournayre et al., 2019). On the other hand, highly degenerate primers can lead to the amplification of non-target taxa like bacteria and fungi, as well as the predator itself, causing a decline in the number of reads assigned to dietary taxa and the increase of haplotype diversity and taxa assignment effort (Taberlet et al., 2018). As we showed in **chapter three**, the use of multiple primers can partly solve the problem of

detection bias, by providing independent descriptions of the species contained in each sample, which can then be combined in a unique dataset for statistical analysis. This multi-marker dataset provides a more thorough description of the diet than any single marker and should thus be the preferred approach when targeting generalist species. Nevertheless, this approach does not solve the issue of secondary predation detection, which can cause a serious distortion of diet composition if no other sources of information are available. This can be particularly troublesome in the case of highly generalist small vertebrates like birds, that can feed on both plants and herbivorous invertebrates, for which secondary detection of plants can be extremely high. It is therefore recommended that in those cases, behavioural studies and visual identification of a subset of samples are combined with molecular data to better assess which dietary links are true or not.

6.2. Further developments in DNA-based analysis of trophic interactions

Overall, I believe that the two technical manuscripts produced in this thesis have contributed to a more thorough understanding of how experimental design can affect the outcome of metabarcoding studies for diet analysis. It certainly has contributed to my own understanding of the sources of error and how they can be mitigated when planning new experiments, but it has also brought many new questions. In particular, bioinformatic procedures and haplotype filtering decisions seem to have significant effects on species occurrence patterns, but its magnitude in comparison to biological and technical replicates is currently unknown. It would thus be interesting to assess if and how different pipelines can impact dietary descriptors and overall biological patterns.

Another interesting issue to explore in the future would be the use of DNA probes for the capture of insect COI fragments, instead of doing PCR amplification or whole genome sequencing. This idea has been essentially developed to avoid the problems of primer bias, while still targeting standard barcode regions and thus allowing species level identification of taxa without the need of deep sequencing and heavy bioinformatic processing associated with metagenomics. Although this approach has never been popularized, probably due to the high costs of generating thousands of different DNA probes and technical difficulties in designing the baits, the few studies that used this technique showed very promising results, with several hundreds of different species being captured and good correlations between initial DNA copy numbers and proportion of sequencing reads. Studies applying DNA capture techniques are nevertheless slowly growing in number, with recent studies trying to assess its ability to monitor African mammals from waterholes (Seeber et al., 2019), description of insect and fish larvae bulk samples (Dowle et al., 2016; Liu et al., 2016; Shokralla et al., 2016; Mariac et al.,

2018; Gauthier et al., 2020), characterization of aquatic environmental DNA (Wilcox et al., 2018), as well as reconstruction of paleo-environments from soil sediments (Murchie et al., 2019). Although never applied to species diets, future developments of the technique could help reducing present technical limitations of metabarcoding. The ever-growing number of species barcodes available in databases could one day lead to the creation of local fauna and flora DNA bait chips, thereby revolutionizing biodiversity monitoring and species interactions studies.

6.3. Using trophic information to understand species' ecology and provision of ecosystem services

Ecological communities are not random groups of 'inert' organisms, but rather complex assemblies of species tied by an intricate network of species interactions (Jordano, 2016b). A high proportion of these interactions are inevitably of trophic nature, as individuals need to consume other organisms to survive, grow, and reproduce. Studying trophic interactions can thus provide critical information on community structure, stability and resilience to change and extinction (Thompson et al., 2012; Heleno et al., 2014). Also, many of the trophic interactions established by organisms translate into well-known provision of services to humans as pollination, seed dispersal and pest suppression. A better understanding of such networks is without doubt a step forward in facilitating the ecological intensification of agricultural landscapes and the design of multifunctional landscapes (Bohan et al., 2013; Gaba et al., 2014).

One of the major challenges in integrating ecological networks with conservation science is the construction of accurate and meaningful interaction networks (Harvey et al., 2017). Complete species networks are rare and often based on non-standard sampling schemes, sometimes combining different sources of information like expert knowledge and co-occurrence data. This can lead to important missing links, overestimation of the importance of some links, and most importantly, to false hypothesized links arising from interactions based in simple species co-occurrence (Morales-Castilla et al., 2015). As described before, the use of molecular techniques like metabarcoding can not only reduce the resources needed to build interaction networks, but also provide species-level interaction links, thereby greatly improving our capacity to generating high quality ecological networks that can then be used to help guiding conservation and management actions. In particular, the effect of species taxonomic resolution on the structure of ecological networks has been poorly assessed, but in a study done by Hemprich-Bennet et al. (2018), the authors found that node resolution (species, genus, family or order) had a great impact on overall network metrics and structure, suggesting

that important ecological patterns might be observed or not depending on the taxonomic scale that we are looking at.

In **chapter four**, we were able to demonstrate using metabarcoding that a trophic specialist species actually exhibits intra-specific variation in prey species' consumption. We found that male and female individuals of European free-tailed bats have slightly different diets, with females predating on average on larger moths, many of which engage in migratory movements. This result would have been otherwise impossible to find with other standard methods like visual identification of faeces fragments or stable isotope analysis, as they lack species level resolution of prey identification. The observed resource partitioning between sexes has given us new insights into the ecology of this bat species and their role in ecosystems, leading us to hypothesize that these bats might be foraging at different altitudes to better meet their energy requirements. If true, males and females could experience different pressures by future climatic and land-use changes, as the scale of the processes governing moth migratory movements is much broader than that of local habitat composition. Future changes in the timing and geographic location of moth migration due to climate change could lead to unpredictable consequences in European free-tailed bats ability to survive. Mismatches between northward migrations of moths in spring and southward in autumn and the bats reproduction period and energy stocking for winter could lead not only to a reduced fit of females and a decrease in reproductive success, but also to, at least temporary, higher survival rate of migratory moths. Many of these migratory moths, like *Autographa gamma*, are major agricultural pests, and as such, their higher survival could lead to major increases in pest damage or need of pesticide use in the future, if such mismatches were to be observed. This scenario would not be too surprising, as changes in species abundances and presence are known to cause cascading effects on ecological systems (Sanders et al., 2018). Sometimes, species do not even need to disappear in order for ecological links to be lost and secondary extinctions to occur (Pearse & Altermatt, 2013a). Also, climate change is expected to have major consequences on overall insect migration, as many species of insects seem to migrate south to the Mediterranean basin only to survive the winter, but it is in northern latitudes where they are able to successfully reproduce and on average increase 4-fold their population levels (Chapman et al., 2012). Recent studies based on stable isotopes suggest that moths arriving in spring at northern latitudes have progressively been originated at higher latitudes (Torniainen & Mikonranta, 2018), probably due to increasing winter temperatures across Europe, so future mismatches might not be that far from happening.

Nevertheless, not all insect pests perform major migratory movements and are thus subject to such broadscale phenomena. As we observed in **chapter five**, different bats can consume a variety of different insect pest species, spanning across different insect orders, many of which do no migrate and could thus be mainly regulated by local variables. Our data

on bat-pest interactions revealed a complex network of species interactions, with bats divided into 6 functional groups feeding on different types of pests. To our knowledge this was the first time an entire pest predation network of a community of vertebrates was described, especially with such high taxonomic resolution. This information is timely, as recent studies have highlighted the need for multi-species assemblages and different foraging strategies when assessing pest suppression services, particularly in agroecosystems (Torrez et al., 2019). By combining metabarcoding with network analysis we were able to identify possible key predator species in pest predation services. The promotion of such key species could help in the process of ecological intensification of food and fibre production by increasing the overall pest suppression services. In the case of bats, insectivorous species seem to benefit from a shared array of general management actions. Management practices like protection and maintenance of roosts, provisioning of permanent drinking water points (either natural or artificial), preservation of riparian habitats, hedges and woodland patches, as well as reduced use of pesticides and of nocturnal light pollution, should help maintaining most landscapes bat-friendly (Dietz et al., 2009; Voigt & Kingston, 2016; Medellin et al., 2017). Still, provisioning of extra artificial roosts like bat boxes or other types of structures mimicking cave conditions (often human built constructions like abandoned houses, bridges, etc) can also help bats increasing their activity levels in certain sites (Flaquer et al., 2006; Amorim et al., 2013; Mering & Chambers, 2014; Puig-Montserrat et al., 2015).

Contrary to what might be expected, i.e. similar bats feeding on similar pest species, our results actually suggest that bats of the same functional group are somewhat complimentary in ecological traits (habitat, foraging strategy, etc) and service provisioning, by delivering their suppression services at different places/habitats. Nevertheless, further analysis correlating bats and pests' ecological traits would be needed to better disentangle what is driving those functional groups. Also, despite the observed modularity in pest predation, we found that bats showed high levels of functional redundancy, being able to predate many different insects and sharing pests with bats of other functional groups. This high redundancy found in our study could be a consequence of the overall good quality of the studied landscape, with the existence of a rich community of bats capable of providing stable and resilient services of pest suppression. In fact, our bat community includes many species of conservation concern, many of which seem to be playing key roles in pest predation services. As seen in our simulations, the disappearance of these endangered species could lead to accelerating declines in pest suppression services.

Although our combined approach of metabarcoding and ecological networks was able to identify possible key species in pest control, it was not able to assess the actual delivery of the service by each bat species. That would require data on predator population sizes and on

the number or biomass of each pest consumed by each predator, which is extremely difficult to obtain. It is also not clear how the provision of those services by bats would translate into actual crop damage and output, fibre production and quality, as well as pesticide reduction, as such knowledge would require other types of experiments based for instance on exclosures. Yet, performing such experiments in complex multifunctional landscapes would require equally complex experimental designs in order to cover all the different crops and forest productions systems at the same time. This is impractical and would probably require an unfeasible amount of resources. Also, such experiments would probably not aid us much in guiding management actions, except perhaps by exerting political pressure by showing exact value 'stamps' of the services offered by rich communities of vertebrates. Previous studies have already shown that insectivorous vertebrates, despite their negative effect in intermediate predators like spiders and other invertebrates, have stronger effects on herbivores, being able to reduce overall plant damage by 40% (Mooney et al., 2010; Maas et al., 2016). This means that having rich communities of vertebrates will in most cases probably help delivering stable ecosystem services.

6.4. Prospects for future research

In this thesis, I provided two case studies showing how metabarcoding can help us gaining new insights into species' ecology and provisioning of ecosystem services by studying their trophic interactions. However, the imaginable applications of metabarcoding and other molecular tools to assess species' diets and interactions are possibly endless. Within the scope of our two biological case studies, many new questions could be further asked and pursued. Given the potential of metabarcoding to process a large number of samples within a rather short time frame, obvious directions to expand our work would be to further increase biological replication over time and across space, thereby gaining the ability to analyse seasonal and landscape effects on species interactions.

In **chapter four**, although we did try to assess whether trophic interactions and their intraspecific variation changed during the breeding season, we failed to find any significant effect. This failure was most likely caused by a lack of a sufficiently large monthly sample sizes, rather than the actual absence of temporal variations. Insects in general are known to have relatively well defined phenological periods on which they are active or flying (Tauber & Tauber, 1976; Wolda, 1988), and thus dietary differences in prey occurrences throughout the year can be expected in any insectivorous predator simply due to availability. Nevertheless, the value of assessing seasonal variations in diet are not related to the differences themselves, but how well they help explaining the ecology of species. For example, it remains to be

understood if males and females of European free-tailed bats have different diets throughout the year, or only when females are pregnant or lactating. If the first case is true, then a series of other ecological questions related to learning, behaviour and sociality can be asked about this species, as how and what generates different foraging behaviours in both sexes. On the contrary, if differences in foraging behaviour are only observed during periods of high energetic requirements, it might mean that bats behave differently because it is more profitable to them as individuals with different energetic requirements, or because resources are not enough and this way the intra-specific competition is reduced. This last option could very well be true, considering that female *T. teniotis* in the study area are known to stop reproducing in extremely dry years, probably due to a lack of resources to sustain pregnancy and stock fat for winter survival (Amorim et al., 2015). Either way, if we look at European free-tailed bats as providers of ecosystem services, it might mean that when trying to calculate their overall consumption of insect pests, factors like sex, season, and reproductive status might be important factors, as each variable combination will lead to different consumption levels of each insect species. This intra-specific variation in diet probably remains unnoticed across many other taxa, possibly obscuring or biasing our current understanding of individual species contribution to ecosystem services.

Regarding the spatial factors, they would be important to consider at both species and community levels. For instance, although European free-tailed bats are habitat generalists that have enormous foraging ranges (>30 km; Marques et al., 2004), important differences may still be found regarding their trophic interactions at regional or even continental scales, especially in relation to the routes taken by migratory insects. Nonetheless, spatial effects may be particularly relevant in community studies carried out at the landscape scale, such as those documented in **chapter five**. In this case, it would be particularly interesting to understand how landscape composition and structure affects the network of trophic interactions, and how this in turn affects the overall pest provision services by bats. Future studies could thus try to assess the role of spatial and temporal dynamics of insect pest occurrence in pest suppression services offered by vertebrates, and how that affects, or not, network structure and metrics. In diverse landscapes it could be expected that central species could change dynamically according to environmental conditions and insect availability, reinforcing the need of rich communities to sustain a stable provisioning of ecosystem services. As the resources to describe and compile such a great number of complex networks are probably unfeasible, it would also be beneficial if general governing rules of predator-prey interactions could be found with further sampling (Bartomeus et al., 2016). For example, by correlating predator species' ecological traits with pest species traits, one could try to predict if and when a certain predator could predate on a certain pest species (Eklöf et al., 2013; Peralta, 2016; Pichler et al., 2019),

even if such interaction has never been observed (Pearse & Altermatt, 2013b). These association rules could greatly improve our capacity in building ecological networks (Harvey et al., 2017). However, this would require highly detailed knowledge of both predators and pests' ecology, as the ecological variables that constrain species interactions are not yet very well known (Poisot et al., 2015; Bartomeus et al., 2016). Unfortunately, ecological traits are unavailable for the majority of species and/or highly scattered across the literature (Laigle et al., 2018), thus hindering any major meta-analysis of what governs predator-pest interactions in small insectivorous vertebrates. As more ecological networks are expected to be constructed in the following years, it would be fundamental that such baseline knowledge would be compiled, as high-resolution networks are impractical to obtain for all landscapes. It would also allow us to better predict how climate and land-use can affect those predator-pest interactions and thus better plan and design resilient landscapes.

Although this thesis only focused on trophic interactions, metabarcoding can also be used to explore a myriad of other species interactions, like pollination, seed and fungi dispersal, as well as host-parasite/parasitoid relations. For example, recent studies have assessed which pollinators visited which flower species, simply by amplifying insect DNA from wild flowers (Thomsen & Sigsgaard, 2019). Molecular approaches can thus scale up the speed in which highly resolved ecological networks can be built, without the need of countless hours of field observation. Scaling-up what was done in **chapter five**, it would be interesting to build a highly resolved ecosystem-wide food web. Such network of networks is still extremely rare in the literature, but a few (although "incomplete") examples do exist (Melián et al., 2009; Pocock et al., 2012; Wirta et al., 2015; Clare et al., 2019). The description of multi-layered networks encompassing many different groups of organisms (predatory, herbivorous and omnivorous vertebrate and invertebrate species, along with their parasites and parasitoids, and of course plants and fungi) involved in antagonist and mutualistic interactions can give us new insights into the stability of ecological communities, and how the robustness of different organisms' interaction networks vary or co-vary to species loss. For example, Pocock et al. (2012) found that in an organic farm in the United Kingdom, the different interaction networks did not co-vary in their robustness, with networks containing pollinators being particularly fragile to species loss. The authors were also able to identify key plant species that could be used in restoration programs of intensively managed farms, this way promoting not only pollinators, but also other beneficial organisms. The combined use of metabarcoding with such ecosystem-wide network approaches could thus open the way to improving agricultural and forest production in multifunctional landscapes while safeguarding biodiversity and ecosystem services via better decision-making.

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Appendix A

Paper Proofs

How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis

Mata V.A., Rebelo H., Amorim F., McCracken G.F., Jarman S., & Beja P. (2019) How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, **28**(2), 165–175. https://doi.org/10.1111/mec.14779

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SPECIAL ISSUE: SPECIES INTERACTIONS, ECOLOGICAL NETWORKS AND COMMUNITY DYNAMICS

WILEY MOLECULAR ECOLOGY

How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis

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Abstract

DNA metabarcoding is increasingly used in dietary studies to estimate diversity, composition and frequency of occurrence of prey items. However, few studies have assessed how technical and biological replication affect the accuracy of diet estimates. This study addresses these issues using the European free-tailed bat *Tadarida teniotis*, involving high-throughput sequencing of a small fragment of the COI gene in 15 separate faecal pellets and a 15-pellet pool per each of 20 bats. We investigated how diet descriptors were affected by variability among (a) individuals, (b) pellets of each individual and (c) PCRs of each pellet. In addition, we investigated the impact of (d) analysing separate pellets vs. pellet pools. We found that diet diversity estimates increased steadily with the number of pellets analysed per individual, with seven pellets required to detect ~80% of prey species. Most variation in diet composition was associated with differences among individual bats, followed by pellets per individual and PCRs per pellet. The accuracy of frequency of occurrence estimates increased with the number of pellets analysed per bat, with the highest error rates recorded for prey consumed infrequently by many individuals. Pools provided poor estimates of diet diversity and frequency of occurrence, which were comparable to analysing a single pellet per individual, and consistently missed the less common prey items. Overall, our results stress that maximizing biological replication is critical in dietary metabarcoding studies and emphasize that analysing several samples per individual rather than pooled samples produce more accurate results.

KEYWORDS
bat ecology, metabarcoding, molecular diet analyses, replication, sampling design, trophic ecology

Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists

da Silva L.P., Mata V.A., Lopes P., Pereira P., Jarman S., Lopes R.J., & Beja P. (2019) Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists. *Molecular Ecology Resources* **19**(6), 1420-1432. https://doi.org/10.1111/1755-0998.13060

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RESOURCE ARTICLE

MOLECULAR ECOLOGY RESOURCES WILEY

Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists

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Abstract

The application of DNA metabarcoding to dietary analysis of trophic generalists requires using multiple markers in order to overcome problems of primer specificity and bias. However, limited attention has been given to the integration of information from multiple markers, particularly when they partly overlap in the taxa amplified, and vary in taxonomic resolution and biases. Here, we test the use of a mix of universal and specific markers, provide criteria to integrate multi-marker metabarcoding data and a python script to implement such criteria and produce a single list of taxa ingested per sample. We then compare the results of dietary analysis based on morphological methods, single markers, and the proposed combination of multiple markers. The study was based on the analysis of 115 faeces from a small passerine, the Black Wheatears (*Oenanthe leucura*). Morphological analysis detected far fewer plant taxa (12) than either a universal 18S marker (57) or the plant *trn*L marker (124). This may partly reflect the detection of secondary ingestion by molecular methods. Morphological identification also detected far fewer taxa (23) than when using 18S (91) or the arthropod markers IN16STK (244) and ZBJ (231), though each method missed or underestimated some prey items. Integration of multi-marker data provided far more detailed dietary information than any single marker and estimated higher frequencies of occurrence of all taxa. Overall, our results show the value of integrating data from multiple, taxonomically overlapping markers in an example dietary data set.

KEYWORDS
bird, diet, metabarcoding, morphological identification, overlapping markers, secondary predation

Luis P. da Silva and Vanessa A. Mata are joint first authors.

Female dietary bias towards large migratory moths in the European free-tailed bat (*Tadarida teniotis*)

Mata V.A., Amorim F., Corley M.F. V., McCracken G.F., Rebelo H., & Beja P. (2016) Female dietary bias towards large migratory moths in the European free-tailed bat (*Tadarida teniotis*). *Biology Letters*, **12**(3), 20150988. https://doi.org/10.1098/rsbl.2015.0988

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Research

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Animal behaviour

Female dietary bias towards large migratory moths in the European free-tailed bat (*Tadarida teniotis*)

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In bats, sexual segregation has been described in relation to differential use of roosting and foraging habitats. It is possible that variation may also exist between genders in the use of different prey types. However, until recently this idea was difficult to test owing to poorly resolved taxonomy of dietary studies. Here, we use high-throughput sequencing to describe gender-related variation in diet composition of the European free-tailed bat (*Tadarida teniotis*), while controlling for effects of age and season. We analysed guano pellets collected from 143 individuals mist-netted from April to October 2012 and 2013, in northeast Portugal. Moths (Lepidoptera; mainly Noctuidae and Geometridae) were by far the most frequently recorded prey, occurring in nearly all samples and accounting for 96 out of 115 prey taxa. There were significant dietary differences between males and females, irrespective of age and season. Compared to males, females tended to consume larger moths and more moths of migratory behaviour (e.g. *Autographa gamma*). Our study provides the first example of gender-related dietary variation in bats, illustrating the value of novel molecular tools for revealing intraspecific variation in food resource use in bats and other insectivores.

Appendix B

Other papers published during the PhD

First complete mitochondrial genomes of molossid bats (Chiroptera: Molossidae)

Mata V.A., Amorim F., Guillén-Servent A., Beja P., & Rebelo H. (2017) First complete mitochondrial genomes of molossid bats (Chiroptera: Molossidae). *Mitochondrial DNA Part B*, **2**(1), 152–154. https://doi.org/10.1080/23802359.2017.1298419

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MITO COMMUNICATION

OPEN ACCESS

First complete mitochondrial genomes of molossid bats (Chiroptera: Molossidae)

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ABSTRACT

Bats represent around one-fourth of the world's mammals and their taxonomy is still controversial. Molossids are one of the most diverse bat families with a wide knowledge gap. In this study, we report the first complete mitochondrial genomes of three molossid bats: the European free-tailed bat *Tadarida teniotis*, the La Touche's free-tailed bat *Tadarida latouchei*, and the Wrinkle-lipped free-tailed bat *Chaerephon plicatus*. The mitogenomes are 16,869 and 16,784 bp long for *T. teniotis* and *T. latouchei*, respectively, while in *C. plicatus* it is at least 16,216 bp although the control region was not fully recovered due to its higher divergence from *T. teniotis*. The genomes show conserved synteny with other mammalian mitogenomes, containing 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes, and 1 control region (d-loop). All protein-coding genes start with the ATG start codon, except for ND2, ND3, and ND5 which begin with ATA or ATT. Eleven protein-coding genes terminated in a canonical stop codon, TAA or TAG, two contain incomplete stop codons, T or TA. Cytochrome b terminates in the mitochondria-specific stop codon AGA. These mitogenomes provide a valuable resource for future studies of Molossidae and other bat and mammal species.

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KEYWORDS
Molossidae; mitogenome; *Tadarida teniotis*; *Tadarida latouchei*; *Chaerephon plicatus*

Agriculture shapes the trophic niche of a bat preying on multiple pest arthropods across Europe: evidence from DNA metabarcoding

Aizpurua O., Budinski I., Georgiakakis P., Gopalakrishnan S., Ibañez C., Mata V., Rebelo H., Russo D., Szodoray-Parádi F., Zhelyazkova V., Zrncic V., Gilbert M.T.P., & Alberdi A. (2018) Agriculture shapes the trophic niche of a bat preying on multiple pest arthropods across Europe: Evidence from DNA metabarcoding. *Molecular Ecology*, **27**(3), 815–825. https://doi.org/10.1111/mec.14474

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ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Agriculture shapes the trophic niche of a bat preying on multiple pest arthropods across Europe: Evidence from DNA metabarcoding

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Abstract

The interaction between agricultural production and wildlife can shape, and even condition, the functioning of both systems. In this study, we i) explored the degree to which a widespread European bat, namely the common bent-wing bat *Miniopterus schreibersii*, consumes crop-damaging insects at a continental scale, and ii) tested whether its dietary niche is shaped by the extension and type of agricultural fields. We employed a dual-primer DNA metabarcoding approach to characterize arthropod 16S and COI DNA sequences within bat faecal pellets collected across 16 Southern European localities, to first characterize the bat species' dietary niche, second measure the incidence of agricultural pests across their ranges and third assess whether geographical dietary variation responds to climatic, landscape diversity, agriculture type and vegetation productivity factors. We detected 12 arthropod orders, among which lepidopterans were predominant. We identified >200 species, 44 of which are known to cause agricultural damage. Pest species were detected at all but one sampling site and in 94% of the analysed samples. Furthermore, the dietary diversity of *M. schreibersii* exhibited a negative linear relation with the area of intensive agricultural fields, thus suggesting crops restrict the dietary niche of bats to prey taxa associated with agricultural production within their foraging range. Overall, our results imply that *M. schreibersii* might be a valuable asset for biological pest suppression in a variety of agricultural productions and highlight the dynamic interplay between wildlife and agricultural systems.

New and interesting Portuguese Lepidoptera records from 2016 (Insecta: Lepidoptera)

Corley M.F.V., Rosete J., Gonçalves A.R., Mata V., Nunes J., & Pires P. (2018). New and interesting Portuguese Lepidoptera records from 2016 (Insecta: Lepidoptera). *SHILAP Revista de Lepidopterologia,* **46**(181), 33-56.

SHILAP Revta. lepid., 46 (181) marzo 2018: 35-56 eISSN: 2340-4078 ISSN: 0300-5267

New and interesting Portuguese Lepidoptera records from 2016 (Insecta: Lepidoptera)

M. F. V. Corley, J. Rosete, A. R. Gonçalves, V. Mata, J. Nunes & P. Pires

Abstract

32 species are added to the Portuguese Lepidoptera fauna, mainly as a result of fieldwork undertaken by the authors and others in 2016. In addition, second and third records for the country, new province records and new food-plant data for a number of species are included. A summary of recent papers affecting the Portuguese fauna is included.

KEY WORDS: Insecta, Lepidoptera, distribution, Portugal.

What is the giant wall gecko having for dinner? Conservation genetics for guiding reserve management in Cabo Verde

Pinho C.J., Santos B., Mata V.A., Seguro M., Romeiras M.M., Lopes R.J., & Vasconcelos R. (2018) What Is the Giant Wall Gecko Having for Dinner? Conservation Genetics for Guiding Reserve Management in Cabo Verde. *Genes*, **9**(12), 599. https://doi.org/10.3390/genes9120599

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Article

What Is the Giant Wall Gecko Having for Dinner? Conservation Genetics for Guiding Reserve Management in Cabo Verde

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Abstract: Knowledge on diet composition of a species is an important step to unveil its ecology and guide conservation actions. This is especially important for species that inhabit remote areas within biodiversity hotspots, with little information about their ecological roles. The emblematic giant wall gecko of Cabo Verde, *Tarentola gigas*, is restricted to the uninhabited Branco and Raso islets, and presents two subspecies. It is classified as Endangered, and locally Extinct on Santa Luzia Island; however, little information is known about its diet and behaviour. In this study, we identified the main plant, arthropods, and vertebrates consumed by both gecko subspecies using next generation sequencing (NGS) (metabarcoding of faecal pellets), and compared them with the species known to occur on Santa Luzia. Results showed that plants have a significant role as diet items and identified vertebrate and invertebrate taxa with higher taxonomic resolution than traditional methods. With this study, we now have data on the diet of both subspecies for evaluating the reintroduction of this threatened gecko on Santa Luzia as potentially successful, considering the generalist character of both populations. The information revealed by these ecological networks is important for the development of conservation plans by governmental authorities, and reinforces the essential and commonly neglected role of reptiles on island systems.

Olive harvest at night kills birds

da Silva, L.P., & Mata V.A. (2019) Olive harvest at night kills birds. *Nature*, **569**(192). https://doi.org/10.1038/d41586-019-01456-4

CORRESPONDENCE · 07 MAY 2019 · CORRECTION 24 MAY 2019

Stop harvesting olives at night — it kills millions of songbirds

Luis P. da Silva & Vanessa A. Mata

From October to January, millions of birds from central and northern Europe winter in the Mediterranean basin. Suction olive harvesting at night kills these legally protected birds on a catastrophic scale as they rest in the bushes. This year, Spain's Andalusian government recommended that the practice be stopped; currently, an estimated 2.6 million birds are vacuumed up annually in the country (see go.nature.com/2zkomts). Other big olive-producing countries should follow their lead.

Some 96,000 birds die in Portugal annually as a result of night-time olive harvesting (see, for example, go.nature.com/2zgy7ml). The Portuguese government has so far taken no action; France and Italy remain silent.

The trees are stripped at night because cool temperatures help to preserve the olives' aromatic compounds. Local governments and local, national and international communities urgently need to assess the impact of the practice and take steps to end it.

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Natural woodlands hold more diverse, abundant, and unique biota than novel anthropogenic forests: a multi group assessment

da Silva L.P., Heleno R.H., Costa J.M., Valente M., Mata V.A., Gonçalves S.C., da Silva A.A., Alves J., & Ramos J.A. (2019) Natural woodlands hold more diverse, abundant, and unique biota than novel anthropogenic forests: a multi-group assessment. *European Journal of Forest Research*, **138**(3), 461–472. https://doi.org/10.1007/s10342-019-01183-5

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ORIGINAL PAPER

Natural woodlands hold more diverse, abundant, and unique biota than novel anthropogenic forests: a multi-group assessment

Luís P. da Silva[1,2,3] · Ruben H. Heleno[1] · José M. Costa[1,2] · Mariana Valente[1,2] · Vanessa A. Mata[3] · Susana C. Gonçalves[1] · António Alves da Silva[1] · Joana Alves[1] · Jaime A. Ramos[2]

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Abstract
Biodiversity sustained by natural ecosystems, particularly forests, provides ecosystem services essential to human well-being. However, many forests have been severely transformed, notably via monospecific plantations and the spread of invasive species. Given the extension of these novel anthropogenic forests (plantations and invasive copses), it is critical to know how they can support forest biodiversity, particularly in highly humanized biodiversity hotspots as the southwest Mediterranean Europe. Because the effects likely vary across taxonomic groups, such assessments require an integrative multi-group approach. Here, we evaluated the abundance, richness, and composition of shrubs, herbs, macrofungi, ground and flying arthropods, birds, small mammals, carnivores, and bats across the four most common forest types in Central Portugal, namely: natural oak woodlands (dominated by *Quercus faginea* Lam.) and anthropogenic forests, invasive *Acacia dealbata* Link copses, *Pinus pinaster* Aiton plantations (native), and *Eucalyptus globulus* Labill. plantations (exotic). Oak woodlands sustained higher abundance, diversity, and a unique species composition compared to the other forests, especially those dominated by exotic species. The greatest changes in biodiversity occurred in herbs and birds. Contrary to our expectations, species richness and composition of macrofungi and carnivores in acacia copses were similar to those of oak woodlands, revealing that groups respond differently to forest changes. The large-scale replacement of natural forests by novel anthropogenic forests has significant negative impacts in most, but not all groups, which should be actively considered for integrative conservation strategies.

Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean

Lopes R.J., Pinho C.J., Santos B., Seguro M., Mata V.A., Egeter B., & Vasconcelos R. (2019) Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean. *Ecology and Evolution*, **9**(8), 4994–5002. https://doi.org/10.1002/ece3.5105

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ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean

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Abstract

Trophic networks in small isolated islands are in a fragile balance, and their disturbance can easily contribute toward the extinction vortex of species. Here, we show, in a small Atlantic island (Raso) in the Cabo Verde Archipelago, using DNA metabarcoding, the extent of trophic dependence of the Endangered giant wall gecko *Tarentola gigas* on endemic populations of vertebrates, including one of the rarest bird species of the world, the Critically Endangered Raso lark *Alauda razae*. We found that the Raso lark (27%), Iago sparrow *Passer iagoensis* (12%), Bulwer's petrel *Bulweria bulwerii* (15%), and the Cabo Verde shearwater *Calonectris edwardsii* (10%) are the most frequent vertebrate signatures found in the feces of the giant wall gecko. This work provides the first integrative assessment of their trophic links, an important issue to be considered for the long-term conservation of these small and isolated island ecosystems.

KEYWORDS
birds, Cabo Verde, DNA metabarcoding, endemics, reptiles, trophic networks

Ypsolopha rhinolophi sp. nov. (Lepidoptera: Ypsolophidae), a new species from Portugal and France unveiled by bats

Corley M., Ferreira S., & Mata V.A. (2019) *Ypsolopha rhinolophi* sp. Nov. (Lepidoptera: Ypsolophidae), a new species from Portugal and France unveiled by bats. *Zootaxa*, **4609**(3), 565–573. https://doi.org/10.11646/zootaxa.4609.3.10

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Ypsolopha rhinolophi sp. nov. (Lepidoptera: Ypsolophidae), a new species from Portugal and France unveiled by bats

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Abstract

A new species *Ypsolopha rhinolophi* Corley is described from northern Portugal and south-east France. It resembles *Y. alpella* (Denis & Schiffermüller, 1775) and *Y. lucella* (Fabricius, 1775) but shows clear differences from both species in DNA barcode and in male and female genitalia. Male genitalia of *Y. lucella* are illustrated for the first time. The new species has been collected at light, reared from larvae on *Quercus pyrenaica* Willd. and recognised from DNA barcode fragments obtained from droppings of horseshoe bats.

Trophic interactions between migratory seabirds, predatory fishes and small pelagics in coastal West Africa.

Correia E., Granadeiro J.P., Mata V.A., Regalla A., & Catry P. (2019) Trophic interactions between migratory seabirds, predatory fishes and small pelagics in coastal West Africa. *Marine Ecology – Progress Series* **622**, 177-189. https://doi.org/10.3354/meps13022

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Trophic interactions between migratory seabirds, predatory fishes and small pelagics in coastal West Africa

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ABSTRACT: Competition, predation and facilitation shape community structure. Yet facilitative behaviour is poorly studied, especially in marine ecosystems. We investigated the diet and foraging behaviour of 5 Afro-Palaearctic migratory seabirds during their non-breeding period in West Africa, focussing on their facilitative associations with predatory fishes. We used next-generation sequencing to describe the diet of 5 tern species, employing DNA metabarcoding for the identification of prey from droppings. This is the first time this method has been used for studying the diet of non-breeding migratory seabirds. Our results showed a high diet overlap among all seabirds, mostly due to the dominance of a single prey species, *Sardinella maderensis* (with a mean frequency of occurrence of 90% in tern diets). The subsurface marine predators identified in association with terns were crevalle jack *Caranx hippos* and West African Spanish mackerel *Scomberomorus tritor*, 2 predatory fishes which also rely on *Sardinella maderensis* as their most frequent prey in the study area, the Bijagós Archipelago. There were marked inter-specific differences in the reliance of terns on subsurface marine predators as facilitators, ranging from completely independent (little tern *Sternula albifrons*) to near-obligatory (black tern *Chlidonias niger*). The varied feeding strategies and small-scale spatial segregation may explain the co-existence of the 5 tern species during the non-breeding period, preying mostly on the same clupeids. Declines both in predatory fishes and in *Sardinella maderensis* and other clupeids are likely to impact the long-distance migrant seabirds studied here, calling for integrated management of fisheries in these coastal ecosystems.

KEY WORDS: Predator–prey interaction · Tern · Next-generation sequencing · DNA metabarcoding · Sympatric predators · Facilitated foraging

Evolutionary history of the European free-tailed-bat, a tropical affinity species spanning across the Mediterranean Basin

Amorim F., Razgour O., Mata V.A., Lopes S., Godinho R., Ibañez C., Juste J., Rossiter S.J., Beja P., & Rebelo H. (*in press*) Evolutionary history of the European free-tailed-bat, a tropical affinity species spanning across the Mediterranean Basin. *Journal of Zoological Systematics and Evolutionary Research*.

ORIGINAL ARTICLE

WILEY

Evolutionary history of the European free-tailed bat, a tropical affinity species spanning across the Mediterranean Basin

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Abstract

The Mediterranean Basin is a global biodiversity hotspot, hosting a number of native species belonging to families that are found almost exclusively in tropical climates. Yet, whether or not these taxa were able to survive in the Mediterranean region during the Quaternary climatic oscillations remains unknown. Focusing on the European free-tailed bat (*Tadarida teniotis*), we aimed to (a) identify potential ancient populations and glacial refugia; (b) determine the post-glacial colonization routes across the Mediterranean; and (c) evaluate current population structure and demography. Mitochondrial and nuclear markers were used to understand *T. teniotis* evolutionary and demographic history. We show that *T. teniotis* is likely restricted to the Western Palearctic, with mitochondrial phylogeny suggesting a split between an Anatolian/Middle East clade and a European clade. Nuclear data pointed to three genetic populations, one of which is an isolated and highly differentiated group in the Canary Islands, another distributed across Iberia, Morocco, and France, and a third stretching from Italy to the east, with admixture following a pattern of isolation by distance. Evolutionary and demographic reconstruction supports a pre-Last Glacial Maximum (LGM) colonization of Italy and the Anatolian/Middle East, while the remaining populations were colonized from Italy after the Younger Dryas. We also found support for demographic expansion following the Iberian colonization. The results show that during the LGM *T. teniotis* persisted in Mediterranean refugia and has subsequently expanded to its current circum-Mediterranean range. Our findings raise questions regarding the physiological and ecological traits that enabled species with tropical affinities to survive in colder climates.

KEYWORDS
bat, demographic history, Molossidae, phylogeography, population structure