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Trophic ecology of Sylviid Warblers using DNA metabarcoding: implications for Optimal Foraging Theory and individual specialization

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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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MSc

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Abstract

Dietary analyses are essential for the development of ecological and evolutionary theories, and to inform about the ecosystem functions and dynamics. Niche partitioning has been traditionally used to explain the coexistence of ecologically similar species through a reduction of interspecific competition, and in more recent theories to emphasize the variance in niche use within a population, through resource partition between individuals. This thesis aims to identify through DNA metabarcoding the prey items composing the diet of two omnivorous warblers, the Sardinian Warbler (*Curruca melanocephala*) and the Eurasian Blackcap (*Sylvia atricapilla*), in northern Portugal; and to evaluate the variation in their diet across time and individual traits under the light of two theories of trophic ecology. For this, DNA markers targeting the COI and ITS2 regions were used to identify the arthropods and plants present on bird droppings. Specifically, the Sardinian Warbler diet was used to measure the fluctuation of intraspecific overlap along seasons, and to test for differences in the total niche width across sexes, ages and seasons. The Blackcap diet was used to compare the dietary variation within and between individuals, and to test for differences in individual niche width according to sex, age and body traits (size and condition).

For both species it was registered a highly diverse arthropod diet and a frequent consumption of fruits from summer to winter months. Sardinian Warblers showed highest degree of overlap between individuals during summer months, when arthropods are abundant and during the peak consumption of blackberries (*Rubus ulmifolius*). During winter months, the total niche width increased and intraspecific overlap decreased. In accordance with the Optimal Foraging Theory (OFT), we registered that during summer when resources are abundant, individuals show a narrow niche width with a high dietary overlap among individuals, likely selecting more profitable prey items. During winter, individuals show more distinct diets and increase their niche width, likely due to the need of incorporating more suboptimal prey items. Although the OFT predicts a flexibility in diet to maximize fitness, data on the Blackcap's diet revealed a temporal consistency in the diet of individuals, suggesting some degree of individual specialization. However, we did not find any relation between specialization and body size or condition. Both OFT and individual specialization theory appear to be at work on the trophic ecology of these two warblers, one driving diet flexibility according to extrinsic conditions (i.e., resource availability) and the other constraining the flexibility at the individual level. Furthermore, our results revealed differences in the diet between sexes both at total niche width and the individual niche width. Sardinian Warbler and Blackcap males showed a more diverse diet than females. Taking into account the absence of a significant body size dimorphism, it is probable that the explorative behavior of males is an explanation for the wider niche width.

This thesis shows that DNA metabarcoding provides large and detailed data that allow to examine temporal and intraspecific dietary differences and evaluate several ecological theories as the optimal foraging and individual specialization. This is crucial to understand the extrinsic and intrinsic factors affecting the trophic ecology of species to accurately predict their response to environmental changes, to estimate patterns on the ecological services provided, and to include intraspecific variance in the strategies of resource management.

Keywords

diet; high-throughput sequencing; foraging theories; omnivory; COI; ITS2; bipartite networks; intraspecific variation

Resumo

Os estudos de dieta são essenciais para o desenvolvimento de teorías ecológicas e evolutivas e para conhecer as dinâmicas e funções do ecossistema. A partição do nicho tem sido tradicionalmente usada para explicar a coexistência de espécies ecologicamente semelhantes através da redução da competição interespecífica, e, em teorias mais recentes, para realçar a variação no uso de nicho dentro de uma população, através da divisão de recursos entre indivíduos. Esta tese pretende identificar, através de ADN metabarcoding, as componentes da dieta de duas toutinegras, a Toutinegra-de-cabeça-preta (*Curruca melanocephala*) e a Toutinegra-de-barrete-preto (*Sylvia atricapila*), no norte de Portugal; e avaliar a variação da sua dieta ao longo do tempo e características individuais considerando duas teorias de ecologia trófica. Para este fim, foram utilizados marcadores de ADN das regiões COI e ITS2 para identificar os artrópodes e plantas presentes nos dejetos das aves. A dieta da Toutinegra-de-cabeça, respetivamente, preta foi usada para avaliar a variação da sobreposição intraespecífica da dieta ao longo das estações do ano e para testar as diferenças na amplitude do nicho entre os sexos, idades e estações. A dieta da Toutinegra-de-barrete-preto foi usada para comparar as diferenças da dieta dos indivíduos e entre eles, e para testar diferenças no nicho individual de acordo com o sexo, idade e características corporais (tamanho e condição).

Para ambas espécies foi observada uma dieta de artrópodes bastante diversificada e um consumo frequente de bagas durante os meses de inverno. A Toutinegra-de-cabeça-preta apresentou um maior grau de sobreposição entre os indivíduos durante os meses de verão, quando os artrópodes são abundantes, e durante o pico de consumo de silva (*Rubus ulmifolius*). Durante os meses de inverno, a amplitude do nicho aumentou e a sobreposição intraespecífica diminuiu. De acordo com a Teoria de Alimentação Ótima (TAO), foi registado que durante o verão, quando os recursos são abundantes, os indivíduos apresentam um nicho mais estreito e uma alta sobreposição alimentar entre indivíduos, provavelmente escolhendo os componentes alimentares mais lucrativos. Durante o inverno, os indivíduos apresentam dietas menos distintas e o seu nicho aumenta, provavelmente devido à necessidade de incorporar componentes alimentares sub-ótimos. Embora a TAO preveja uma flexibilidade na dieta para maximizar a condição física, os dados da dieta da Toutinegra-de-barrete-preto demonstraram uma consistência temporal na dieta individual, sugerindo algum grau de especialização individual. No entanto, não foi encontrada nenhuma relação entre especialização e o tamanho corporal. Tanto a TAO como a teoria da especialização individual parecem ocorrer na ecologia trófica destas toutinegras, uma causando flexibilidade de acordo com as condições extrínsecas (i.e., disponibilidade de recursos) e outra restringindo a flexibilidade ao nível individual. Os resultados demostram ainda diferenças na dieta entre os sexos, tanto na amplitude total do nicho quanto na amplitude do nicho individual. Os machos da Toutinegra-de-cabeça-preta e da Toutinegra-de-barrete-preto apresentaram uma dieta mais diversa que as fêmeas. Considerando a ausência significativa de dimorfismo no tamanho corporal, é provável que o comportamento exploratório dos machos seja responsável por estes apresentarem um nicho mais amplo.

Esta tese demostra que o DNA metabarcoding produz muitos dados detalhados que permitem examinar diferenças temporais e intraespecíficas na dieta e avaliar diversas teorías ecológicas como a TAO e a especialização individual. Isto é crucial para entender os fatores extrínsecos e intrínsecos que afetam a ecologia trófica das espécies, de forma a poder prever com precisão a resposta às mudanças ambientais, para estimar padrões nos serviços prestados e incluir a variação intraespecífica nas estratégias de gestão de recursos.

Palavras-chave

dieta; sequenciação de alto-rendimento; teorias de alimentação; omnivoria; COI; ITS2; redes bipartidas; variação intraespecífica

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List of abbreviations

COI - Cytochrome c oxidase I

ITS2 - Internal transcribed spacer II

DNA - Deoxyribonucleic Acid

BOLD - Barcode of Life Data Systems

NGS - Next Generation Sequencing

NCBI - National Center of Biotechnology Information

OTU - Operational Taxonomic Unit

PCR - Polymerase Chain Reaction

PERMANOVA - Permutational Multivariate Analysis of Variance

BLAST - Basic Local Alignment Search Tool

OFT - Optimal Foraging Theory

1y - First year birds

2y+ - Second year or older birds

ZOTU - Zero-radius Operational Taxonomic Unit

CI - Confidence Interval

General Introduction

Dietary studies not only assist the development of theoretical models of evolutionary and community ecology, but enlighten about the natural history of the species, providing information of their ecosystem services and easing the conservation efforts. For example, studies have determined that in agricultural fragmented landscapes insectivorous birds can provide pest-reduction services that improve crop yields (Maas et al., 2016), while frugivorous birds contribute to the maintenance of diverse plant communities through defecating or regurgitating the seeds of fleshy fruits (González- Varo et al., 2021a). In a food web, consumers adjust the abundance and distribution of other species through predation and competition (Blüthgen et al., 2006). Modifications on the trophic configuration impact the ecosystem's functions, and the population and community dynamics and structure (O'Gorman & Emmerson, 2009; Thébault & Loreau, 2005). It is particularly relevant to understand the existing relationship between consumers and resources, taking into account the present alteration of ecosystems, caused by anthropogenic factors and climate change (Schückel et al., 2015; McMahon et l., 2019). However, in the past, ecological studies in natural food webs have been limited by the complexity of determining the diversity of prey items, especially on consumers with broad diets, like omnivores and generalists (Braley et al., 2010; Rosenblatt et al., 2015). Furthermore, the foraging behavior of several species is flexible, varying highly across localities, seasons, years and demographically (Van Heezik, 1990; Amato et al., 2015; da Silva et al., 2020).

Analysis of stable-isotopes have been implemented to understand the patterns of resource consumption. However, the use of stable-isotopes has some limitations, isotopes are not able to differentiate between resources with similar isotopic profiles or when the quantity of resources is greater than the quantity of isotopes utilized (Layman et al., 2012). In contrast, molecular methods provide a high taxonomic resolution of prey items, resulting in a finer discrimination between organisms (Soininen et al., 2014; Moorhouse-Gann et al., 2018; Elbrecht et al., 2019). The development of high-throughput sequencing (HTS) and metabarcoding techniques allow the detection and identification of taxa present in complex samples of multiple species DNA in a cost and time-efficient manner when analyzing many samples (Macgregor et al., 2019). The rapid advancement of HTS and its decreasing costs, has raised the production and precision of many ecological analyses, which includes dietary studies (Carreon- Martinez & Heath, 2010; Monterroso et al., 2019). Nevertheless, this technique also has some limitations to be considered, including taxa-specific biases in the recovery of sequences, or the availability of DNA barcode libraries of the potential taxa of the sample (Collins & Cruickshank, 2013).

The former genus *Sylvia* (now split in *Sylvia* and *Curruca* by most ornithological entities) is composed by warbler species with a wide trophic niche. Their diet during the breeding season consists mainly of insects, but there is a shift to fruits when they become available in later summer and autumn. It has also been reported the consumption of nectar and pollen, mainly during winter and early spring (Calvario et al., 1989; da Silva et al., 2014). The adjustments in foraging across the year (Telleria & Perez-Tris, 2007; Assandri et al., 2017), the generalist trends and plasticity in diet (Rey & Valera, 1999; Catoni et al., 2011), the phylogenetic closeness between the species (Bohning-Gaese et al., 2003) and the previous evidence of competition for territory and microhabitat (Garcia, 1981; Spina et al., 1985) makes the genus a good model to test for intraspecific changes in niche partitioning and diet specialization across the year and during a strong influx of conspecifics. The Sardinian Warbler (*Curruca melanocephala*) and the Eurasian Blackcap (*Sylvia atricapilla*) are two of the most common birds, the latter mainly during winter, in the Mediterranean basin (Cramp & Simmons, 2004), a region considered a biodiversity hotspot (Mittermeier et al., 2011). Both species have a wide distribution, inhabiting almost all habitats around the Mediterranean basin, from maquis to other shrublands, agricultural mosaics, urban areas, and forest clearings (Schaefer & Barkow, 2004; Ieronymidou et al., 2012).

Objectives

In this thesis I will analyze the diet composition of one resident bird species (*C. melanocephala*) and one partially resident and migratory species (*S. atricapilla*), obtained through DNA metabarcoding of droppings. The thesis is organized in two chapters, each focused on the diet of each bird species. These chapters aim to answer different ecological questions, using each bird as a model system. In the first chapter, I aim to understand how dietary richness and niche overlap varies according to the sex and age of individuals, as well as throughout seasons. In the second chapter, my main goal is to assess the role of individual traits (i.e., body condition, size, sex, and age) on individual's diet specialization within their community. In particular, I will test how different network derived specialization and centrality indexes are affected by these individual traits, as well as how variation in diet differs between and within individuals.

Manuscript I

Annual fluctuation in dietary niche width and intraspecific overlap of the Sardinian Warbler: a DNA metabarcoding approach

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Abstract

In temperate regions, animals face strong variations in food availability that potentially constrain their foraging behavior, diet, and role in ecosystem function and stability. Generalist and omnivorous birds in particular, can play key functions in their environment by providing essential ecosystem services like seed dispersal, pollination, and pest control. Optimal foraging theory predicts that species' niche varies according to the seasonal variation in resource availability, contracting in times of food abundance and widening in times of shortage. Still, examples of intra-specific variation in generalist birds' diet are still lacking in the literature. The Sardinian Warbler (*Curruca melanocephala*) is a widespread and omnivorous passerine that occupies most of the warm areas of the Mediterranean Basin. Here, we use primers targeting the COI and ITS2 regions to identify animal and plant taxa composing the diet of the Sardinian Warbler in northeast Portugal. We describe the annual fluctuation of the diet and of the degree of intraspecific diet overlap, and test for differences between sexes, age categories, and months. Younger birds reported a higher richness of families on their diet, while males presented a richer diet of OTUs than females. The prevalence of arthropods on the diet along the year differed between families, with peaks in June-September and December-March. Fruit consumption was registered the entire year except for April and May, with the highest prevalence occurring in August and September. Further, the highest richness of the diet was registered in winter, also when the lowest intraspecific overlap of diet occurred, supporting the optimal foraging theory. Finally, we identified several common agricultural and forestry pests on the diet of the Sardinian Warbler, suggesting that it might provide pest reduction services. The ecological information reported on this study can help the development of conservation efforts, and improves the knowledge on seasonality and ecosystem services in the region.

Keywords

Bird; high-throughput sequencing; trophic ecology; insectivory; ecosystem services; omnivory; Optimal Foraging Theory.

Introduction

Dietary studies are essential for understanding how species interact with each other and the role they play in ecosystems (Şekercioğlu, 2006; Buechley & Şekercioğlu, 2016). Generalist and omnivorous species in particular can have key roles in ecosystem stability by interacting with several species in the community (Brechtel et al., 2019). This behavior flexibility allows species to more easily respond to changes in local resource availability and might help them survive periods of food-shortage (McClenaghan et al., 2019). In temperate regions, insectivorous species face strong variations in food availability, with insect biomass strongly declining in winter (da Silva et al., 2019). Many flying species, like birds, respond by migrating south in search of more favorable conditions, but some species are able to stay all-year round, often switching to a more plant-based diet (Herrera, 1984). Still, seasonal variations in prey consumption are expected to occur in insectivorous species simply due to differences in insect species phenology (e.g., Loureiro et al., 2009; Deblauwe, 2009). Understanding these seasonal changes is extremely important for comprehending how they might affect the species roles in the ecosystem, as well as the ecosystem services that each species is providing (Koch et al., 2009; Vasconcellos et al., 2010). In heterogeneous landscapes with agriculture, for example, insectivorous birds have been associated with pest-reduction services which result in an increase of crop yields (Maas et al., 2016; Barbaro et al., 2017). On the other hand, frugivorous birds are known to regurgitate or defecate seeds, assisting in the establishment of seedlings and supporting diverse plant communities (González-Varo et al., 2021a). Yet, studies describing species diets during an entire annual cycle are not common, most often focusing on just one period or comparing distinct periods (e.g., Gryz & Krauze-Gryz, 2015; Ponce et al., 2018), likely due to practical difficulties in sufficiently sampling a species during a continuous and long period of time. This task becomes even more complex for species that can show intra-specific variability either caused by differences in sexes (Phillips et al., 2011; da Silva et al., 2020), age (Rutz et al., 2006; Riotte-Lambert & Weimerskirch, 2013), or habitat use (Quevedo et al., 2009).

The Optimal Foraging Theory (OFT) has been extensively used to model a population's preference of prey items and foraging area. A fundamental assumption of the OFT is that natural selection will promote the most economically advantageous foraging pattern to maximize the energetic intake. When optimal prey items are abundant, animals should concentrate on these prey items, narrowing their niche. Foragers should become more opportunistic as these optimal prey items decline, incorporating sub-optimal prey items into their diet and expanding their niche width (Pyke, 1984). However, despite the fact that phenotypic variation is a foundation of Darwin's evolutionary theory, most OFT models assume that individuals within a species are ecologically equivalent. Bolnick *et al.,* proposed that niche width is a property of the individual phenotype, and therefore should be defined at the individual level (2010). For instance, a release on resource competition can lead to an increase in the population's niche width due to a growth on individual niche width with a constant niche overlap between individuals. Alternatively, an increment in niche overlap as individual niche width increases could result in no differences in the population's niche width (Bolnick et al., 2010).

The Sardinian Warbler (*Curruca melanocephala*) is one of the most common birds around the Mediterranean basin (Cramp & Simmons, 2004), a region considered a biodiversity hotspot (Mittermeier et al., 2011). Its populations are mainly sedentary, but some can be partially migratory (Cramp & Simmons, 2004). It has a wide distribution, inhabiting almost all habitats around the Mediterranean basin, from maquis to other shrublands, agricultural mosaics, urban areas, and forests (Cramp & Simmons, 2004; Schaefer & Barkow, 2004; Ieronymidou et al., 2012; Aparicio, 2016). Individuals are known to be very tolerant to human activities, dwelling on gardens and close to roads (Bannerman, 1953). In southwest Europe they breed from March to July in low shrubs and are territorial the entire year (Aparicio, 2016; Cramp & Simmons, 2004; Bas et al., 2005). The species is dimorphic in its coloration, but body measurements are almost equal between sexes, with males showing only slightly longer wings than females, but without significant differences in body weight (Cramp & Simmons, 2004; Guallar et al., 2010).

The diet of the Sardinian Warbler is known to consist predominantly of insects, with an increase of fruit consumption during autumn and winter (Tejero et al., 1983; Herrera, 1984). They mostly forage on low shrubs, but also on the canopy and ground. Several studies suggest Hymenoptera, Hemiptera, Coleoptera and Stylommatophora as common orders on the diet of the Sardinian Warbler (Debussche & Isenmann, 1983; Tejero et al., 1983). An observational study of their feeding behavior in southern Spain during winter and spring indicated that frugivory was uncommon, corresponding roughly to 8% of the feeding events (Cuadrado, 1988). In contrast, Herrera (1984) found fruits on 80% of the droppings of Sardinian warblers in central and southern Spain, which was on lower frequency and with less diversity than larger sylviid warblers, like the Blackcap (*Sylvia atricapila*) and the Garden Warbler (*Sylvia borin*).

On the south of the breeding range, where fleshy fruits are available the whole year, Sardinian Warblers seem to constantly feed on them (Herrera, 1984).

In the past few years, high-throughput sequencing (HTS) and metabarcoding techniques have allowed the identification of ingested items with a high taxonomic resolution, leading to a finer scale discrimination among species' diets (Soininen et al., 2014). Even though the diet of the Sardinian Warbler has already been somewhat studied using traditional methods, feeding habits have been drawn mostly from studies of frugivory comparing several bird species (Jordano, 1982; Debussche & Isenmann, 1983; Herrera, 1984; Izhaki & Safriel, 1985; Izhaki et al., 1991; Rey, 1995; Rey, 2011) and field observations of foraging behavior (Jordano, 1982; Izhaki et al., 1991; Bensusan et al., 2011), rather than a complete examination of the ingested plants and arthropods. Furthermore, the few studies exhaustively describing its diet through stomach content analysis have only analyzed autumn and winter individuals, thus failing to describe any seasonal variation (Tejero et al., 1983). This way, we aim to produce a detailed description of the annual diet of the Sardinian Warbler and to test for differences in diet richness and composition between sexes and age categories. A higher overall diet richness is expected for males, especially adults, since their more bold and active behavior could express in a wider range of potential prey (Breitwisch & Hudak, 1989; Møller, 1992), while young birds have less foraging experience (Rutz et al., 2006) and tend to feed on more predictable food resources like fruits (Stevens, 1985). We also predict differences in diet richness and composition along the year as a response to fluctuations in food availability, with individuals showing a higher diet overlap in autumn and winter months, when plant species have more fleshy fruits available (e.g., Herrera, 1984) and arthropod prey taxa diversity and availability is lower (da Silva et al., 2019).

Methods

Field sampling

The study was conducted near the Tua River mouth, on the Douro Valley, Northeastern Portugal. The region has a typical Mediterranean climate and the landscape is dominated by a matrix of vineyards, olive groves, Mediterranean maquis (with high plant diversity dominated by *Quercus* spp., *Arbutus unedo*, *Pistacia terebinthus, Phillyrea* spp, *Erica* spp., etc.), with some pine trees (*Pinus pinaster*) and small villages and farms. Birds were mist netted every month between April 2018 and March 2019, on four sampling sites within a 1,500 m radius (41.2153°, -7.4152°). Mist nests were operated for 5 hours in the morning after sunrise and for 4 hours in the afternoon before dusk in all months except in April 2018, when they were operated only in the afternoon, due to weather constraints. From May to September the morning captures were done the day after the afternoon captures, but between October and March they were done on the same day. Mist nests were checked every 30 min and birds held in cotton bags for 15 to 30 minutes. All birds were ringed and we recorded the ring number, sex (when possible, because young birds cannot be sexed based on coloration before their first molt) and age (1y: first year birds; or 2y+: second year or older birds) following Svensson (1992). Droppings were collected from handling bags, which were sterilized with 10% bleach for 1 hour and washed after every use, and stored in 2 ml tubes with 98% ethanol (da Silva et al., 2019).

Laboratory procedures

DNA was extracted from bird droppings using 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; Maudet et al., 2002), followed by inhibitor removal using Inhibitex tablets (QIAGEN), DNA precipitation and washing using E.Z.N.A. Tissue Kits (Omega). The extraction protocol started by adding 800uL of lysis buffer to the dropping. Samples were homogenized with a spatula, vortexed, and left in a dry bath at 70ºC for 30 min. Afterwards, samples were short-spined and up to 700uL 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 500uL of supernatant was transferred to a new tube and 25uL of OB Protease was added. The remaining steps followed the kit recommendations, except that DNA was eluted two times in 50uL into different extracts. DNA was extracted in batches of 23 samples plus one negative control in which no dropping was added. Extracted DNA was distributed in 96-well plates where the last well was left empty for PCR negative control. DNA extracts were further purified using Agencourt AMPure XP beads (Beckman Coulter), before PCR amplification. Invertebrate prey and consumed plants were independently amplified using the FwhF2-R2n (Vamos et al., 2017) and UniPlantF-R (Moorhouse-Gann et al., 2018) primer-sets modified with Illumina adaptors. These primers target the COI and ITS2 region, respectively, and have been shown to amplify a wide diversity of invertebrates and plants, while providing a high taxonomic resolution of the amplified taxa (Moorhouse-Gann et al., 2018; Elbrecht et al., 2019). PCR reactions consisted in 5uL of Qiagen Multiplex Master Mix, 0.3uL of each 10nM primer, 3.4uL of water, and 1uL of DNA extract. Cycling conditions consisted in a 15 min period at 95ºC, 35 cycles of 30 sec denaturation at 95ºC, 30 sec annealing at 50ºC, and 30 sec extension at 60ºC, and a final extension period of 10 min at 72ºC. PCR products were diluted 1:4 and went through a second PCR reaction to incorporate 7bp long indexes and P5+P7 Illumina 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 55ºC. 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 two libraries were then individually quantified using qPCR (KAPA Library Quant Kit qPCR Mix, Rocher) and diluted to 4 nM. Finally, libraries were pooled equimolarly and sequenced in a HiSeq Rapid SBS Kit v2 (500 cycles) along with samples from other bird species.

Bioinformatic analysis

Bioinformatic processing of generated Illumina reads was done using standard bioinformatic packages for metabarcoding data. First, paired reads were merged with Flash (Magoč & Salzberg, 2011), followed by primer removal and sample tagging of reads with the command ‘ngsfilter’ of Obitools (Boyer et al., 2016). Reads were then dereplicated per sample using ‘obiuniq’, and singletons of each sample, as well as amplicons without the expected length (202-208bp for fwh2; 187-387bp for uniplant) were also removed with the command ‘obigrep’. Afterwards, samples were merged and the reads denoised using the command ‘--cluster_unoise’ of VSEARCH (Rognes et al., 2016). Resulting ZOTUs were further inspected for chimeras using the command ‘--uchime3_denovo’ and then clustered at 99% similarity using ‘--cluster_size’. Reads were then mapped back to the retained OTUs using the command ‘--usearch_global’ with an identity level of 99%. Finally, LULU (Frøslev et al., 2017) was used to merge similar OTUs (identity >84%) with high co-occurrence levels (>95% of samples), this way greatly reducing the number of retained PCR artifacts, sequencing errors, as well as nuclear copies of the mitochondria, that tend to artificially inflate the number of OTUs present in each sample. The number of reads observed per OTU present in extraction and PCR blanks was further subtracted to the respective samples associated with each extraction batch and PCR plate, in order to remove potential lab contaminations.

OTUs were then compared to online databases (BOLD and NCBI) and identified to the lowest taxonomic rank possible, taking in account the likelihood of occurrence in Portugal and Iberia in the case of arthropods, and in the study area in the case of plants. Whenever an OTU matched several species, genus, or families at similar identity levels, we selected the most inclusive taxonomic rank. OTUs assigned only to a genus, family, order or class, that matched the same group of sequences, and usually diverged less than 2%, were further clustered with the help of a neighbour-joining tree (Mata et al., 2018) into distinct OTUs (e.g., Carabidae 1, Carabidae 2, etc). Each OTU was also categorized as either being “diet” (i.e., most arthropods and plants with fleshy fruits) or “not diet” (e.g., birds, fungi, human, internal parasites). For plant taxa, because there have been intensive flora surveys in the region, we discarded all plant taxa not recorded in those surveys or during fieldwork. Furthermore, for each plant taxon, we evaluated whether it produced fleshy fruits and in which months the fruits were available (González-Varo et al., 2021a), and categorized each bird-plant interaction per month as “likely” (i.e., plant taxon produces fleshy fruits and interaction was observed in fruiting season) or “unlikely” (i.e., plant taxon does not produce fleshy fruits or the interaction was observed outside the fruiting season). This was done in order to avoid incorporating secondary detections in ecological analysis, a problem known to occur frequently in omnivorous bird species (da Silva et al., 2019; Tercel et al., 2021). Also, interactions potentially arising from flower-visitation behavior were not considered, as no evidence of pollen or nectar was found on the sampled birds (da Silva et al., 2014; 2017). Finally, samples that did not have at least 100 reads belonging to dietary items were considered to have failed and were discarded. From each sample we further removed all taxa representing less than 1% of the total number of dietary reads of that sample (Mata et al., 2018).

Data analysis

All statistical analyses were performed on R v4.0.3 (R core Team 2020). Dietary analyses were based on presence/absence of prey items on each sample at the OTU and family level. We used OTUs as the most resolved taxonomic level instead of species, since many taxa were identified up to family or order and then clustered into groups. Family identification level was used because families are often considered surrogates of functional and monophyletic groups (Lovell et al., 2007; Hawkins et al., 2012; Laiolo et al., 2020). Months were grouped in pairs for analyses to guarantee an adequate sample size per level of factor.

The richness of ingested prey items was estimated using rarefaction curves based on Hill numbers with the function ‘*iNEXT*’ of the package ‘*iNEXT’* (Hsieh et al., 2016) that were extrapolated for two times the observed sample size. Prey richness was estimated for the entire population using the asymptotic diversity estimate, while for month, sex and age categories, prey richness was estimated across equal levels of sampling coverage by selecting the lowest value observed in each variable (Chao & Jost, 2012). For comparing the richness of ingested prey items between variable categories, we considered that they differed significantly if the 95% confidence intervals did not overlap.

Differences in dietary composition between sexes, ages and months were tested using a permutational multivariate analysis of variance (PERMANOVA) with the function ‘*adonis*’ of the package ‘*vegan’* (Dixon, 2003) based on a ‘Jaccard’ dissimilarity matrix calculated with the function ‘*vegdist’* of the same package. Identity of the bird (ring number) was included in the model to control for repeated measurements. To identify the prey items that mostly contributed to differences in dietary composition, the function ‘*simper’* of the package ‘*vegan’* was used. Statistical significance was considered at an alpha value of 0.05.

To test for differences in diet overlap across months, we compared the mean dissimilarity values in prey composition of warbler individuals to that of simulated dissimilarity values of 1,000 null models. Z-scores were calculated by subtracting the mean dissimilarity values of the null models to the observed dissimilarity values and then dividing it by the standard deviation of the null models (Z-score = (Observed dissimilarity – Average-nullmodels)/ SD-nullmodels). Niche overlap among individuals was calculated using only prey families, because the OTU sampling coverage per pair of months was low. Only one sample per individual for each pair of months was included in this analysis in order to avoid pseudo-replication. Null models were simulated using the functions ‘*nullmodel’* of the package ‘*vegan’* (Dixon 2003) and *‘simulate’* of the package ‘*stats’* (R core Team, 2020). We used methods ‘r1’ (RANDOM1: Patterson & Atmar, 1986) and ‘c0’ (RANDNEST: Jonsson, 2001) to simulate the null models. Both methods use non-sequential algorithms, but r1 maintains sample richness and selects prey items using marginal column sums as probabilities, while c0 only maintains prey item frequency (Dixon, 2003). The former should thus help identify months in which niche overlap among individuals was different from expected given the observed prey diversity, possibly indicating months of similar prey selection, with specialized individuals preying on a subset of what generalists consumed. Contrarily, c0 allows for prey richness to vary among samples (assuming a normal distribution centred on the observed average), and is thus able to detect differences in niche overlap caused by differences in prey richness among individuals. This should help detect months in which niche overlap was higher/lower among individuals than what would be expected if they were all feeding on a similar number of prey, indicating months in which individuals showed distinct patterns of niche width. Significance was assessed using the function *oecosimu* of the package *vegan.*

Results

For the dietary analysis we used a total of 234 samples belonging to 146 Sardinian Warbler individuals (Table 1), resulting from 292 captures of 165 individuals and 273 collected droppings. An average of 39 samples ± 6.81 (Standard Deviation) were used for each pair of months, corresponding to 20.67 ± 2.58 males and 12.5 ± 4.68 females, and 25.5 ± 8.59 first year birds (1y) and 13.5 ± 8.12 second year birds or older (2y+). After all filtering steps, samples showed an average of 13,026 reads ± 22,966 for the arthropod and 9,515 ± 17,416 for the plant component of the diet.

Table 1 – Number of samples with successful amplification and sequencing results per sex and age class. In brackets is the number of corresponding individuals of Sardinian Warblers.

Age	Males	Females	Unknown sex	Total
1y	84 (49)	34 (22)	35 (35)	154 (105)
2y+	41 (26)	40 (26)	0 (0)	81 (52)
Total	125 (68)	74 (43)	35 (35)	234 (146)

Overall diet description

We identified 617 OTUs in the diet of the Sardinian Warbler, belonging to 179 different families and 38 orders. Rarefaction curves indicated an estimated richness for the entire population of 1,327 OTUs (95% CI: 1,146-1,569), 258 families (95%CI: 220-331), and 42 orders (95%CI: 39-64). The most commonly observed OTU was *Rubus ulmifolius* (23% of the samples), followed by *Camponotus cruentatus* (22%) and *Nysius graminicola* (17%; Figure 1A). Regarding families, Formicidae was the most common (35%), followed by Noctuidae (31%), Chironomidae (26%), Cicadellidae (26%) and Lygaeidae (25%; Figure 1B). Finally, the most common orders were Hemiptera (78%), Lepidoptera (52%), Diptera (52%), Hymenoptera (47%) and Coleoptera (30%; Figure 1C). Plant occurrence in samples was the highest during August and September, and happened for every pair of months, except for April and May (Figure 2).

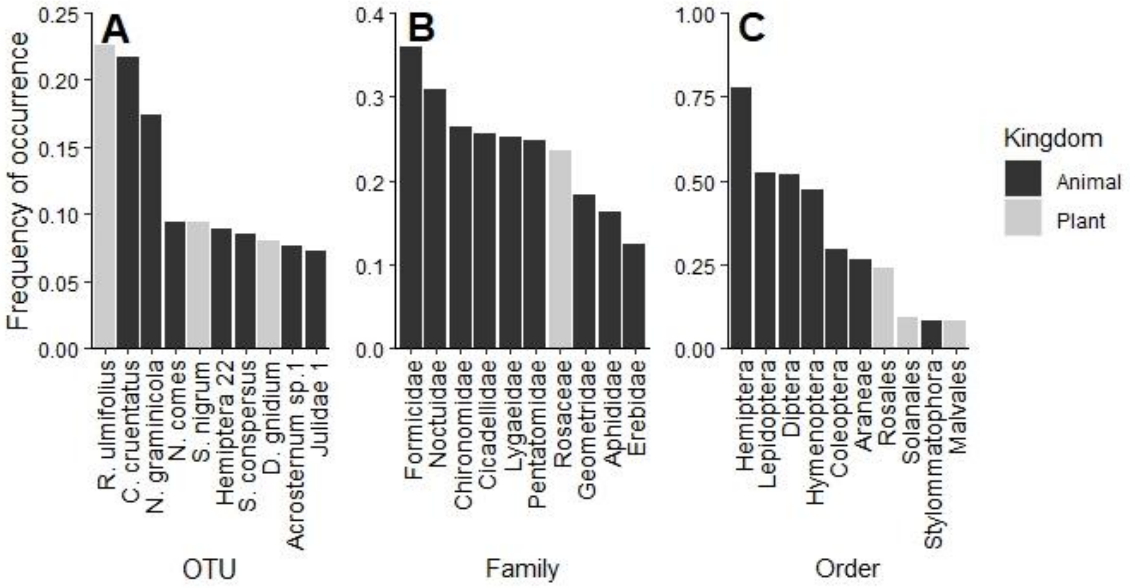

Figure 1. Frequency of occurrence of mostly consumed OTUs (A), families (B), and orders (C) by warblers throughout the study period.

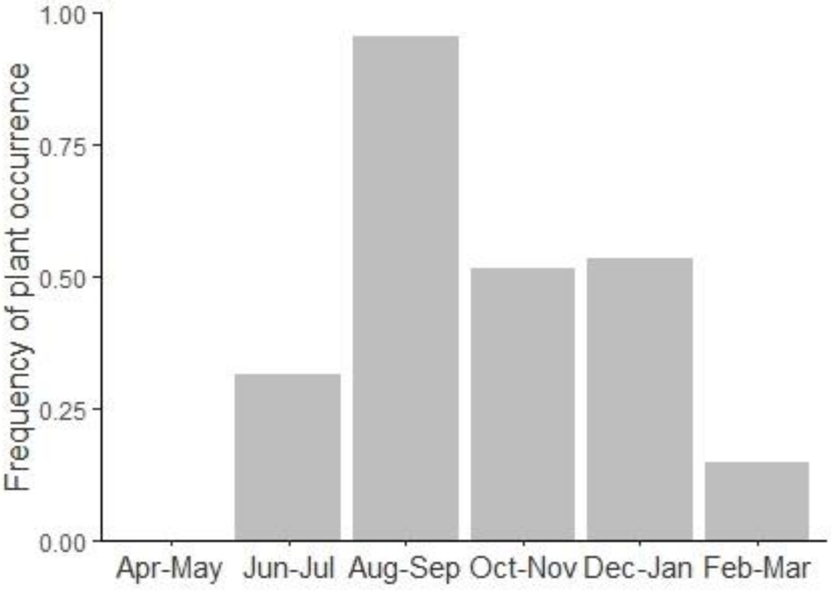

Figure 2. Frequency of occurrence of plants in the diet of the Sardinian Warbler throughout the year.

Variation in dietary richness

Overall diet richness was significantly different between sexes, age categories and pairs of months (Figure 3). For OTUs, from October to March (95% CI: 202-270) individuals presented a richer diet than in April-May (141-194) and August-September (89-110). Furthermore, August-September presented a significantly lower dietary richness than all the other pairs of months. Finally, males ingested a significantly higher number of prey taxa (522-594) than females (417-500), while first calendar year birds did not show a significant difference from second year birds or older (1y: 437-495; 2y+: 465-556). Regarding the number of different prey families consumed, in winter months, from December to March, individuals showed a higher dietary richness (90-124) than in spring and summer (April to September: 52-83). In contrast to the number of OTUs, different sexes consumed a similar number of prey families (males: 142-153; females 145-162), whereas 1y birds ingested a higher number of prey families (173-211) than 2y+ (130-170).

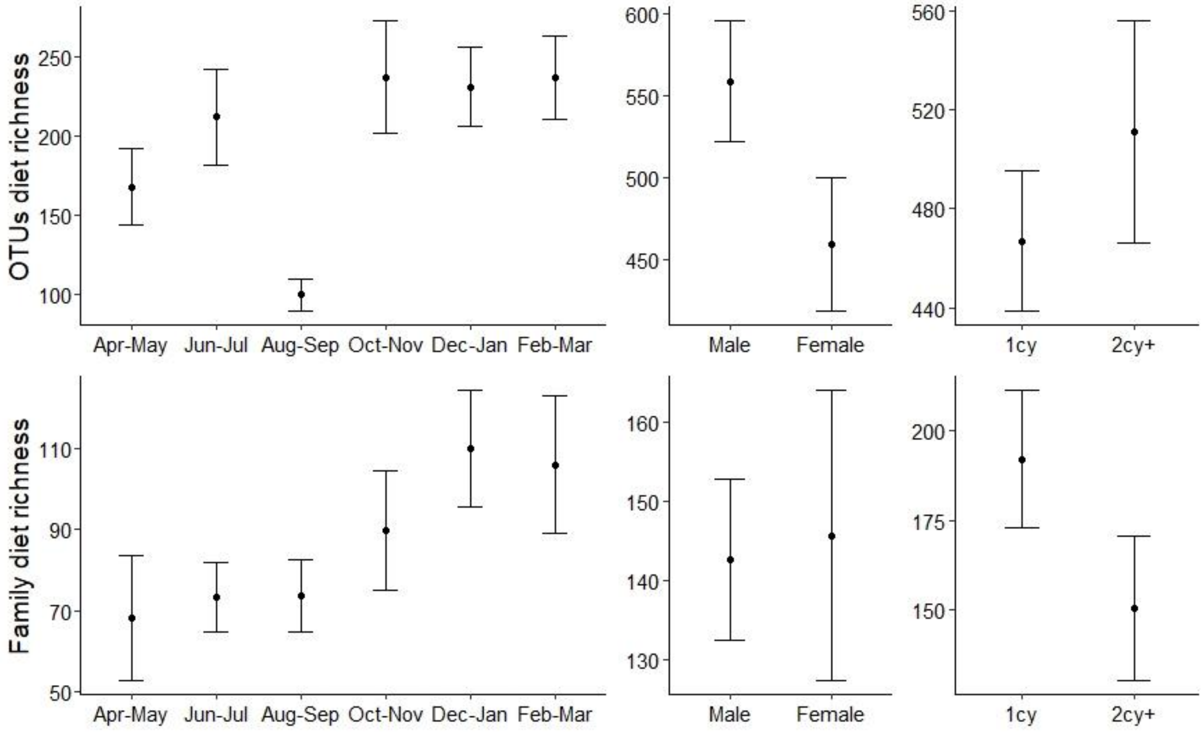

Figure 3. Estimated dietary richness of OTUs and families for different months, sexes and age classes. OTU richness was estimated with a sample coverage of 65% for months, 75% for sexes, and 74% for age classes. Family richness was estimated with a sample coverage of 90% for months, 92% for sexes, and 95% for age classes. Whiskers represent 95% confidence intervals based on 1,000 bootstraps.

Variation in dietary composition

The PERMANOVA at OTU level revealed differences in dietary composition between age categories (df = 1, 0.005, p-value = 0.011), sexes (df = 1, 0.006, p-value = 0.001) and months (df = 5, 0.092, p-value = 0.001), while for family level there were differences only among months (df = 5, 0.11, p-value = 0.001). Simper analysis evidenced that the differences at OTU level for age and sex were mostly related to taxa occurring at very low frequency (<5%) in one group and not occurring at all in the other. On the other hand, all of the ten most common OTUs and families partially explained the variance in dietary composition across months. The families Formicidae, Pentatomidae, Lygaeidae, Aphididae and Rosaceae were most common from June to November, while Cicadellidae, Chironomidae and some moths, like Noctuidae and Geometridae, were ingested the most in winter months (Figure 4**)**.

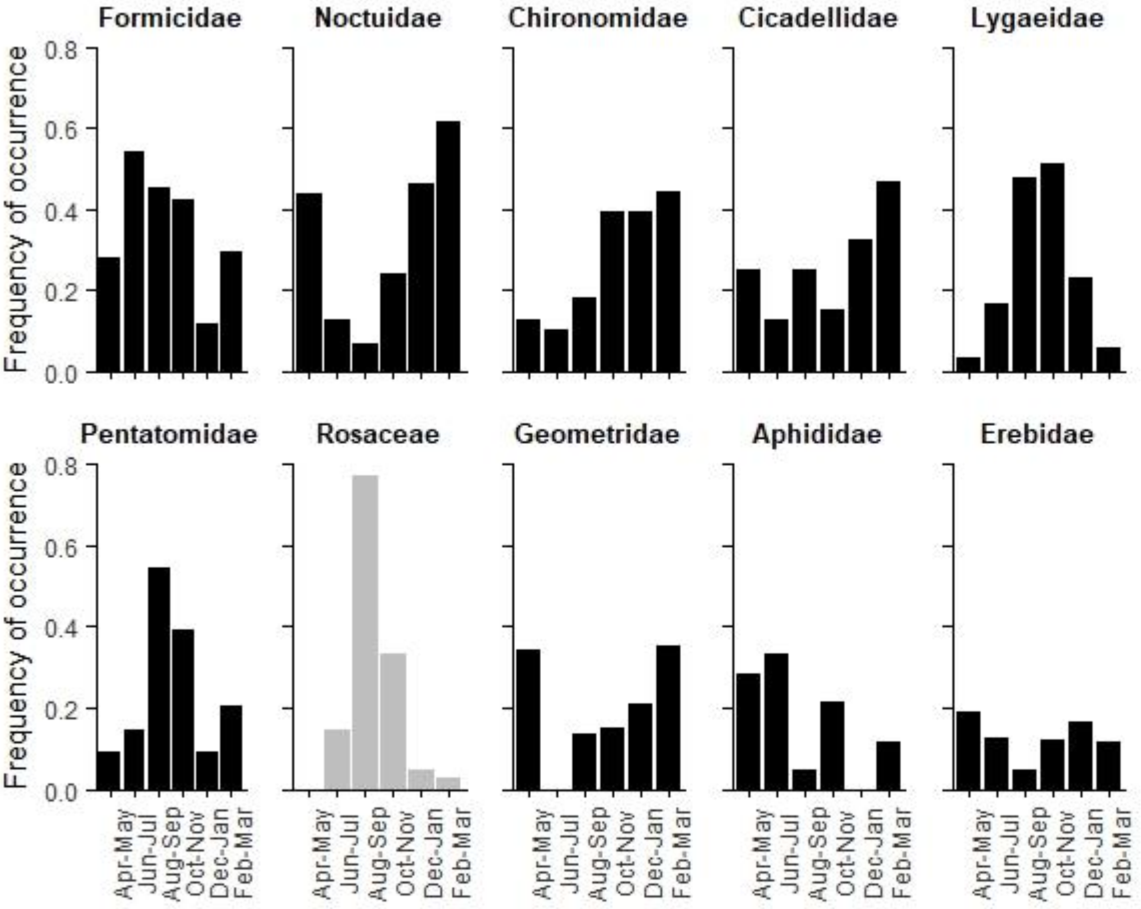

Figure 4. Annual fluctuation in the frequency of occurrence of the ten most common prey families consumed by Sardinian Warblers. Black plots represent arthropod families, and grey plots plant families.

Seasonal variation in intraspecific niche-overlap

Mean diet dissimilarity varied across the year according to both methods used for simulating null models, indicating that niche-overlap was not constant throughout the year. For method r1, August-September showed a significantly lower average dissimilarity between pairs of samples than the null models (0.83; *p-value* = 0.015), suggesting that diet composition between individuals was more similar than expected given the observed prey richness. When using the method c0, December-January (0.93; *p-value* = 0.019) and February-March (0.90; *p-value* = 0.001) had a higher dissimilarity than the simulated null models, indicating that on average individuals shared less prey taxa than what would be expected if they were all feeding on a similar number of prey (Figure 5).

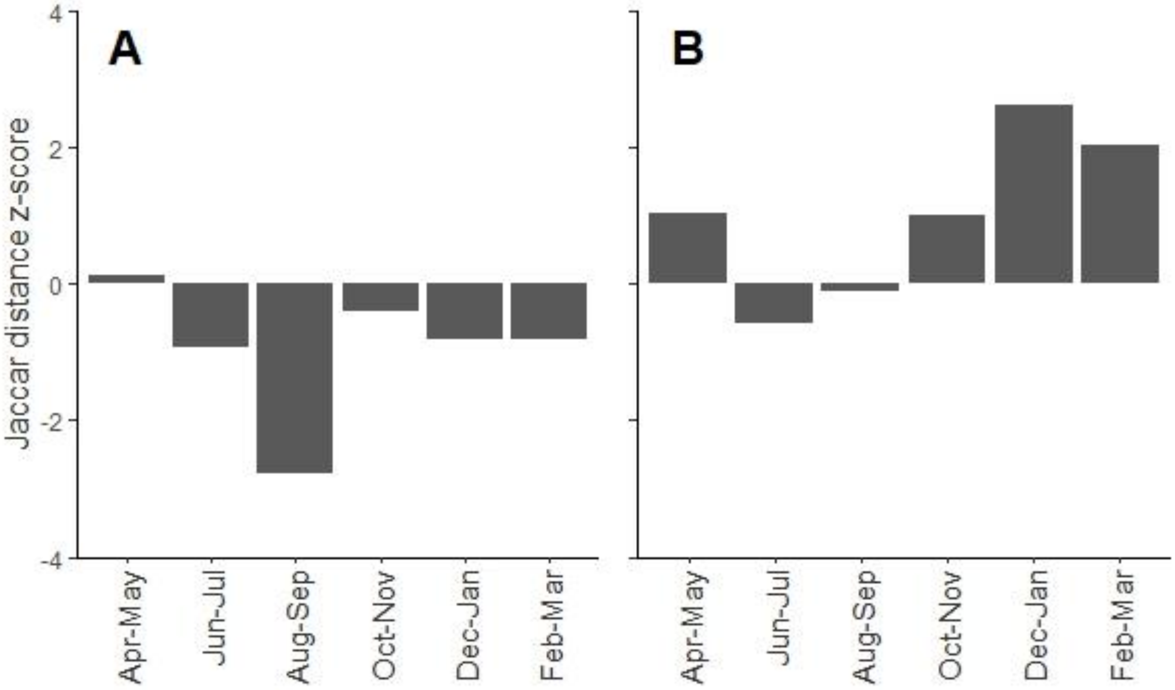

Figure 5. Dissimilarity z-scores comparing the observed diet dissimilarity per pair of months with the simulated diet dissimilarity of 1,000 null models with methods r1 (A) and c0 (B).

Discussion

This study provides a highly resolved taxonomic identification of the diet of the Sardinian Warbler in northeast Portugal, along with its seasonal variation during an entire year. Previously, trophic ecology has been seldom approached with a monthly recording of the diet, but rather with a static description or a comparison of spaced periods of time. Our results indicate that during winter months, when arthropods become scarcer (da Silva et al., 2019), Sardinian Warblers widen their niche and show lower levels of niche overlap. Additionally, in congruence with our initial hypothesis and with past findings (da Silva et al., 2020), we detected a higher richness of ingested prey items for males than for females, but not prey families, which suggests a broader dietary niche as a result of the differences in behavior, but not of functional diversity. On the other hand, contrary to what we expected, younger individuals exhibited a higher richness of prey families in their diet than older birds. Besides detailing the natural history of the Sardinian Warbler, we found evidence supporting the optimal foraging theory and contribute to the knowledge on intraspecific variance in trophic ecology.

The overall dietary composition of the Sardinian Warbler obtained in this study is similar to that reported in previous studies based on both foraging behavior (Debussche & Isenmann,

1983) and stomach content (Tejero et al., 1983), with Hymenoptera, Hemiptera, Coleoptera, Lepidoptera, Diptera and Stylommatophora dominating the diet. We found evidence of fruit consumption throughout the entire year except in April and May, when there are no ripe fruits in the study area, with a frequency peak in August and September (95% of fruit prevalence) followed by a steady decrease until February and March (15%). However, we found a different proportion of fruit consumption than that recorded in previous studies. Jordano (1982) reported the presence of *Rubus* on 25% of the samples of Sardinian Warbler in Southern Spain between August and November, while during the same period of time we registered the presence of *R. ulmifolius* on 58% of the samples. Contrarily, we report a lower prevalence of plants on droppings during the whole fruiting season (June-March) than what has been described in Southern Spain. Herrera (1984) found evidence of frugivory on 79-82% of the samples, while we found it on 50% of the samples. These differences in fruit consumption are likely related to the availability of adequate size fruits, which are known to vary greatly among years and sites (e.g., González-Varo et al., 2021b; Molina et al., 2011).

Sex specific differences in dietary niche width have been reported previously for several taxa, like other birds (Phillips et al., 2011; da Silva et al., 2020), mammals (Kernaléguen et al., 2015; Voigt et al., 2018) and reptiles (Houston & Shine, 1993). However, size dimorphism has been a prevalent explanation for sex differences in diet, with commonly contrasting diets in highly dimorphic species (e.g., Houston & Shine, 1993; Phillips et al., 2011; Kernaléguen et al., 2015). Differently, body measurements of the Sardinian Warbler are similar between sexes, with males only having slightly longer wings (Guallar et al., 2010). We suggest that the sex specific niche width is mostly explained by differences in behavior, rather than dimorphism. For other passerines, it has been reported that males are more risk-takers (Breitwisch & Hudak, 1989), they habituate faster (Ensminger & Westneat, 2012), and move more often inside their territory (Møller, 1992), which could be translated into more opportunities for encountering different prey taxa. The fact that this difference was not observed for prey families and that prey family composition did not differ between sexes, further supports that males and females are foraging on similar types of prey.

We registered higher dietary richness at family level for younger birds, despite the pattern not being sustained at the OTU level. Although it has been thought that birds suffer a selection for yearlings to develop as fast as possible (Cheng & Martin, 2012), individuals of some species can present an ontogenetic foraging behavior associated with learning (Rutz et al., 2006; Riotte-Lambert & Weimerskirch, 2013). If juveniles spend more time handling and searching for prey due to their inexperience than adults (Wheelwright & Templeton, 2003), it is expected that they compensate with longer foraging periods and a more opportunistic diet (Wunderle, 1991). Further, it has been noted that restricted foraging behavior is less common for juveniles since the knowledge of specific favorable zones comes with experience (Riotte-Lambert & Weimerskirch, 2013). If first year Sardinian Warblers have a less efficient foraging behavior, it is possible that they spend more time foraging and on a wider range of (micro-)habitats, thus feeding on suboptimal prey items, and therefore expanding their dietary niche. On the other hand, the differences detected in prey OTU composition between sexes and age classes, but not families, were most likely the result of a statistical artifact and seem to have no ecological relevance. Our results, however, revealed a strong annual fluctuation in the consumption of dietary resources, with month showing a significant effect on diet richness and composition. We registered an increase in the overall richness of the diet along the year, reaching a peak in February and March. This change in diet richness was accompanied by differences in diet composition both at the OTU and family level. The pattern of ingestion was distinct between families, with some families being mostly consumed between June and September and others December to March (Figure 4).

Mean dissimilarity between pairs of samples revealed that the degree of niche overlap fluctuates along the year. In contrast to what we predicted, we found a lower niche overlap among individuals from December to March. This was observed for the c0 model, but not the r1, and thus likely reflects differences in niche width among individuals. In fact, a disproportionately high number of individuals showed extremely high values of prey families (>10) during winter in comparison to the rest of the year ($x^2 = 18.809$, $p < 0.001$), while lower values were equally observed. This pattern does not seem to have been driven by juvenile individuals, as the proportion of juveniles and adults with extreme values of prey family richness in winter did not differ ($x^2 = 0.730$, $p = 0.393$). We hypothesize that individuals are eating more distinct prey items during winter than in other seasons probably because since the abundance of arthropods is lower (da Silva et al., 2019), a higher proportion of individuals need to widen their niche width to secure the same energetic intake (Sargeant, 2007), thus supporting the optimal foraging theory (Pyke, 1984). Another possible explanation is that the dietary niche expansion is a response to reduce competition with other ecologically similar species that arrive during winter, like the Blackcap (*Sylvia atricapilla*; Bolnick et al., 2010). On the other hand, from August to September niche overlap was higher than expected, given the observed niche width of individuals. During this period the family Rosacea was present on 77% of the samples, mainly due to the high occurrence of the very common and abundant *R. ulmifolius*. This was the highest frequency value recorded for any family during any pair of months, thus likely increasing the average degree of niche overlap between samples. Probably, the high availability and consumption of *R. ulmifolius* during this time is responsible for the overall low prey richness observed in August-September (Figure 2), since Sardinian Warblers concentrate on this predictable, easy to catch, sugar rich, and abundant dietary item.

In addition to being an abundant and widespread bird, we identified several important pest arthropods in the diet of the Sardinian Warbler. Among the common agricultural and forestry pests that were detected in the diet of the Sardinian Warbler are the moths *Lymantria dispar* and *Catocala nymphagoga*, both pests of *Quercus* species (Branco et al., 2014); *Empoasca vitis,* a leafhopper pest of the common grape vine (Böll & Herrmann, 2004); *Bactrocera oleae,* a phytophagus fly that preys on the fruit of the olive tree (Malheiro et al., 2015); *Cydia pomonella* an important *Citrus* pest (Pajač et al., 2011); *Aphis spiraecola* a common pest of apple trees (Pfeiffer et al., 1989); and *Myzus persicae*, a pest of peach trees and many other agricultural species (Blackman & Eastop, 2000). Taking into consideration that the study area is composed of a matrix of maquis, vineyards, olive plantations, and other forms of agriculture, conservation strategies for insectivorous birds, like the Sardinian Warbler, may be critical for a future understanding between environmental protection and economic development (Wenny et al., 2011).

It is noteworthy that distinct results are reached when analyzing the diet at different taxonomic levels. First, sample coverage was higher for families than OTUs, which means that more robust results were obtained for the former (Chao & Joost, 2012). Also, the information given by the ingestion patterns of different taxonomic levels is distinct. It has been noted that species-level taxonomic resolution can distract from the emerging ecological patterns (Hector et al., 2001), and in that case, using higher taxa with a reliable phylogenetic relationship could help refocus the analysis (Reiss et al., 2009). It is possible that in our study for example, the significant difference in richness of prey families but not OTUs between age classes is related to older birds consuming more functionally redundant species that form a more optimal diet (Timms et al., 2013).

Our results provide support for differences in diet between sexes and age categories, and detail an important annual fluctuation in the diet of the Sardinian warbler, its overall richness and composition, as well as intra-specific niche overlap. This study also contributes to the natural history knowledge of the species and the region, with evidence supporting the optimal foraging theory and suggesting that Sardinian Warblers can deliver important pest reduction services in complex landscapes. Further, the observed seasonality in prey consumption indicates that the ecosystem services provided by insectivorous birds also shift throughout the year, like has been reported for other taxonomic groups (e.g., Koch et al., 2009; Vasconcellos et al., 2010). Next-generation sequencing proved to be a useful tool to produce solid and cost-effective ecological information, but we encourage future studies to measure the annual changes in resource availability for a deeper understanding of diet fluctuations.

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Manuscript II

Intra-individual temporal consistency and sex-specific specialization in the diet of a bird revealed by DNA metabarcoding

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Abstract

Individual specialization describes for some generalist species that within the total population's niche width, individuals use restricted subsets of resources with low overlap between them. To evaluate the influence of individual traits we use as a model species the Eurasian Blackcap, a common generalist passerine, during autumn and winter in northern Portugal. We use primers targeting the COI and ITS2 regions to identify arthropods and plants on 880 blackcap droppings. We assessed if dietary variance within individuals was significantly lower than between individuals, and if diet specialization was explained by phenotypic traits, as sex, age, or body size and condition. Our results reveal that individuals are consistently selecting over time a subset of the generalist diet of the population, supporting the individual specialization theory. We found no relationship between size nor age and dietary specialization, but males presented a more generalist diet than females. Our findings reflect the usefulness of DNA metabarcoding for obtaining multiple detailed dietary profiles during the lifetime of an individual and revealing temporal dietary patterns both within and between individuals.

Keywords

Individual based network; trophic ecology; resource partitioning; insectivorous; dietary consistency; high-throughput sequencing.

Introduction

Species partitioning resources has been extensively observed in population and community ecology studies, most often occurring through the differential use of a heterogeneous resource, like foraging in different microhabitats (Pulliam, 1985; Symes et al., 2013), feeding on different prey items (Frere et al., 2008; Knickle & Rose, 2014), or avoiding temporal overlap (Hirai & Matsui, 2002; Newell et al., 2014). Traditionally, it served to explain the sympatric distribution of ecologically similar species, but further research revealed that individuals within a population are not ecologically equivalent and can present high variance in their pattern of resource use (Violle et al., 2012). For some trophic generalist species, within the same population, individuals only consume a small subset of the spectrum of resources (Rutz et al., 2006; Meik et al., 2012).

Bolnick et al. (2003) proposed that niche width is a property of the individual phenotype, and therefore should be defined at the individual level. Further, the total niche width of a population could be partitioned in the variance of the diet within individuals and between individuals. Therefore, the population's niche is the combined interactions of the individuals with heterogeneous phenotype, and it is predicted that this variability will be expressed in a differential use of the available resources. For some species, sex and age classes present morphological and behavioral differences, which could be translated on different potential prey items (Rutz et al., 2006; da Silva et al., 2020). However, variance in diet not only occurs across discrete intraspecific morphs, but also when phenotypic variation is continuous, like in some morphological traits (Jung, 1992; Simpfendorfer et al., 2001), behavioral measurements (Griffen et al., 2012), or when the between individual variance in diet is not linked to a trait but it is consistent through different contexts (Araújo & Gonzaga, 2007; Toscano & Griffen, 2014).

Although in order to maximize fitness, individuals will show flexibility across ecological conditions (Ménard et al., 2014; Stofberg et al., 2019), research suggests that behavioral traits and diet present low variation within individuals, both across ecological context and over time during the life of the organism (Estes et al., 2003; Sih et al., 2004; Toscano et al., 2016). However, measuring temporal or ecological consistency requires a longitudinal following of the individuals with several dietary observations, which can be difficult for elusive species (Newmaster et al., 2013; Tournayre et al., 2021). Stable isotopes have been used to measure long-time foraging trends and to assess temporal consistency (Mizukami et al., 2005; Marcoux et al., 2012), however, isotopic analysis have serious limitations on the quantity of prey items that can be individually identified and to distinguish similar isotopic profiles (Layman et al., 2012). Otherwise, DNA metabarcoding allows a finer scale discrimination of the resources that compose a diet, with high taxonomic resolution and in a cost-effective manner (Soininen et al., 2014; Elbrecht et al., 2019). This technique facilitates the production of large data sets of detailed dietary profiles, that when including repetitive measurements of the same individual could help to understand the variation within and between individuals of the same population (Nichols et al., 2016; Thuo et al., 2019).

The Eurasian Blackcap (*Sylvia atricapilla*) is a very common and widespread generalist warbler that inhabits deciduous forests and mixed woodlands, as well as gardens and plantations, of most of Western Palearctic. Populations range from long-distance migrators in northern Europe to sedentary on Mediterranean and Atlantic islands, with many partially migratory populations on the Mediterranean basin (Cramp & Simmons, 2004; Hera et al., 2012). Individuals from northern and migratory populations are generally bigger than their Southern and resident counterparts (Lo Valvo et al., 1988; Ożarowska et al., 2021) and both co-occur during the wintering period in the Mediterranean basin (Cramp & Simmons, 2004; Hera et al., 2012). The Blackcap is a well-known species and is particularly important for plant dispersion (González-Varo et al., 2021). Previous studies show a strong seasonality on the diet, with an arthropod-based diet during the breeding season and shifting to mainly plant material for the rest of the year (Rey & Valera, 1999; Schaefer & Schmidtt, 2002). This study aims to detail the diet of the Blackcap during the wintering period (October-March), when resident and wintering populations are sympatric. In particular, we aim to understand if bird's individual traits (size, condition, sex, and age) influence diet specialization, as well as if diet variation within individuals is lower than between individuals. We hypothesize intrapopulation differences in specialization according to sex and age, with males and adults presenting richer diets due to behavioral differences and ontogeny-related proficiency of foraging techniques (Møller, 1992; Rutz et al., 2006; Ensminger & Westneat, 2012). Further, we expect birds with a smaller body size to show a less generalist diet, due to restrictions in resource use caused by body size limitations (Pineda-Munoz et al., 2016) and that birds with a better body condition can more efficiently select and eat more profitable resources. We expect evidence of individual specialization through lower variance in diet within individuals than between individuals (Bolnick et al., 2003).

Methods

Sample collection

The sampling of Blackcap was conducted in the Douro Valley, near the Tua River mouth, in Northeastern Portugal. The landscape is covered by olive groves, vineyards, Mediterranean maquis, pine trees and small farms and villages. Birds were captured with mist nets at four close locations (called A, B, C and D), all within a 1,5 km radius, every month between October 2018 and March 2019. This sampling period was chosen because during this period there is a mix of resident and wintering migrant populations from across Europe (Cramp & Simmons, 2004; Hera et al., 2012), that usually have distinct body sizes (Lo Valvo et al., 1988; Ożarowska et al., 2021). Mist nets were operated in the morning and afternoon of the same day for each site, for the first five hours with sunlight, and for four hours before dusk. Mist nets were examined every 30 min, and captured birds held for a maximum of 30 min in clean bird handling bags. All captured birds were ringed and we recorded sex and age following Svensson (1992), wing length (maximum wing chord, 0.5mm precision) and weight (0.1g precision). Droppings were collected from the handling bags and stored in 2ml tubes filled with ethanol. After every capture, handling bags were sterilized with 10% bleach for 1 hour and washed (da Silva et al., 2020).

Laboratory procedures

DNA extraction from bird droppings was performed using a custom protocol with an initial incubation period consisting of a lysis buffer (0.1 mEDTA, 0.01 mNaCl, 0.1 mTris–HCl, 1% N-lauroylsarcosine, pH 7.5-8; Maudet et al., 2002), followed by inhibitor removal using Inhibitex tablets (QIAGEN), and DNA washing and precipitation with E.Z.N.A. Tissue Kits (Omega). The extraction protocol begun by incorporating 800uL of lysis buffer to the dropping. A spatula was used to homogenize the samples. Then the tubes were vortexed, and put in a dry bath at 70ºC for 30 min. Subsequently, samples were short-vortexed. 700uL of supernatant was relocated to a new tube that contained one quarter of an inhibitex tablet. Afterwards, samples were vortexed for 1 minute and then centrifuged at 8,000 rpm for 30 sec. 500uL of supernatant was relocated to a tube with 25uL of OB Protease. For the rest of the extraction the kit recommendations were followed, with the exception that DNA was diluted twice in 50uL. The extraction was performed in groups of 23 samples, with an additional negative control without a dropping. DNA extracts were allocated into 96-well plates with the last well empty to be used as PCR negative control. Before PCR amplification, extracted DNA was purified utilizing Agencourt AMPure XP beads (Beckman Coulter). Consumed plants and invertebrate prey were amplified independently by applying the UniPlantF-R (Moorhouse-Gann et al., 2018) and FwhF2-R2n (Vamos et al., 2017) primer-sets altered with Illumina adaptors. The primers target the ITS2 and COI region, and have been shown to amplify an extensive diversity of plants and invertebrates, while still providing high taxonomic resolution (Moorhouse-Gann et al., 2018; Elbrecht et al., 2019). The PCR mixture contained 1uL of cleaned DNA extract, 5uL of Qiagen Multiplex Master Mix, 0.3uL of each 10nM primer, and 3.4uL of water. Cycling conditions consisted of an initial period of 15 min at 95ºC, followed by 35 cycles of 30 sec at 95ºC for denaturation, 30 sec at 50ºC for annealing, and 30 sec at 60ºC for extension; and a period of 10 min at 72ºC for a final extension. PCR products were diluted at 1:4 and underwent a second PCR to integrate P5+P7 Illumina adaptors containing 7bp long indexes. Conditions for the second PCR were similar to the first reaction, except that KAPA HiFi HotStart ReadyMix (Rocher) was used, and cycling included only 8 cycles with annealing at 55ºC. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using Nanodrop. Finally, PCR products were diluted to 15nM and pooled per marker. The obtained libraries were individually quantified with qPCR (KAPA Library Quant Kit qPCR Mix, Rocher), followed by a dilution to 4 nM. Lastly, the two libraries were grouped equimolarly and sequenced using a HiSeq Rapid SBS Kit v2 (500 cycles).

Bioinformatic analysis

Flash (Magoč & Salzberg, 2011) was used for merging the paired reads. Afterwards, primers were removed and reads were tagged with ‘ngsfilter’ of Obitools (Boyer et al., 2016). For the dereplication of reads it was used the command ‘obiuniq’, and then singletons and amplicons shorter than expected (202-208bp for fwh2; 187-387bp for uniplant) were removed with ‘obigrep’. Denoising of reads was performed with ‘--cluster_unoise’ of VSEARCH (Rognes et al., 2016). The obtained ZOTUs were examined for chimeras with ‘--uchime3_denovo’, and clustered with ‘--cluster_size’ at 99% similarity. Reads were mapped to the OTUs using ‘--usearch_global’ with 99% of identity level. Finally, to merge OTUs with identity higher than 84% and co-occurrence levels higher than 95%, LULU was used (Frøslev et al., 2017), thus decreasing the number of sequencing errors, PCR artifacts and nuclear copies of the mitochondria. To exclude possible sources of laboratory contamination, the number of observed reads per OTU in the extraction and PCR blanks was subtracted to the respective samples that belonged to each extraction batch and PCR plate.

The obtained OTUs were compared to BOLD and NCBI online databases and identified to the lowest possible taxonomic rank. For arthropods, the taxonomic identification was constrained to taxa occurring in Iberia, and for plants to taxa in the study area. If an OTU matched several species, genus, or families at similar identity levels, it was assigned to the most inclusive taxonomic rank. OTUs assigned to a higher taxonomic level than species and with less than 2% of divergence, were clustered with a neighbor-joining tree (Mata et al., 2018) into distinct OTUs (e.g., Carabidae 1, Carabidae 2, etc). OTUs were further categorized as “diet” (i.e., plants with fleshy fruits) or “not diet” (e.g., fungi, birds, human, internal parasites) depending on whether they were likely to be taxa actively foraged by birds. Plant taxa that were not registered in plant surveys of the region or during fieldwork were discarded. Further, for each registered plant taxon we assessed if it produced fleshy fruits and during which months, and then categorized the interactions per month as “likely” (plant taxon produced fleshy fruits during the month of the interaction) or “unlikely” (plant taxon does not produce fleshy fruits, or it does not produce fleshy fruits during the month of the interaction). This was performed to avoid including secondary detections in the ecological analysis, a frequent obstacle in omnivorous species (da Silva et al., 2019, Tercel, 2021). As there was no evidence of nectar or pollen on the sampled birds, potential interactions from flower-visitation behavior were excluded (da Silva et al., 2014; 2017). Samples with less than 100 reads of dietary items were discarded. Finally, for each sample, taxa with less than 1% of the total number of dietary reads of that sample were also discarded (Mata et al., 2016).

Data analysis

Data analysis was conducted using R v4.0.3 (R core Team, 2020). We used for all dietary analysis the presence/absence of prey items on samples at both OTU and family level. We used OTUs instead of species as the most resolved taxonomic level, since some taxa were identified only to genus or family and later clustered into discrete OTUs. Family level was included in the analysis as a proxy of functional groups (Lovell et al., 2007; Hawkins et al., 2012; Laiolo et al., 2020).

Differences in diet specialization between individuals were tested using two indices of specialization, d’ (Blüthgen et al., 2006) and nestedrank (Alarcon et al., 2008), and two of centrality, closeness and betweenness (Blüthgen, 2010). Indices were calculated with the function “species level” of the package “bipartite” (Dormann et al., 2021). Generalized Linear Mixed Models (GLMMs) were then used to assess which individual traits help explaining specialization levels. GLMMs were performed with the function “glmmTMB” of the package “glmmTMB” (Magnusson et al., 2021), using a beta distribution and month as a random variable. This was done to control for temporal differences in diet due to fluctuations in resource availability. Identity of the bird (ring number) was not included in the model, since the low variance compromised the results of the models. The residuals of an ordinary least squares regression between Body Mass (g) and wing length (mm; Labocha & Hayes, 2012) was used as a surrogate of body condition. The following factors were included as explanatory variables in the GLMMs: sex, age, body condition and wing length. Statistical significance was considered at an alpha value of 0.05. For all indices we used the function “model.sel” of the package “MuMIn” to select the best fitted model according to the Akaike weights.

To assess if the variance in dietary composition within-individuals was significantly lower than between-individuals, the Jaccard distance was calculated between all pairs of samples using the function “vegdist” of the package “vegan” (Oksanen et al., 2020). Pair-wise comparison values were then categorized as “within individuals” if resulting from samples belonging to the same individual or “between individuals” if resulting from samples belonging to different individuals. The independence between the two sets of values was then assessed using the function "independence_test" of the package "coin" (Hothorn et al., 2021). Statistical significance was assessed using Monte Carlo resampling. This analysis was performed for the entire dataset, but also for each of the four sampling sites to assess if the result was due to samples from the same locality being more similar.

Results

For the dietary analysis, after all the filtering process, we used data from a total of 807 samples belonging to 640 Eurasian Blackcap individuals (Table 1), from 938 captures of 724 individuals and 880 collected droppings. After all filtering steps, samples showed an average of 12,133.89 reads ± 21,124.91 for the arthropod and 7,384.807 ± 10,030.17 for the plant component of the diet.

Table 1. Number of samples with successful amplification and sequencing results per sex and age class. In brackets is the number of corresponding individuals of Eurasian Blackcap.

Age	Males	Females	Total
1y	367 (305)	238 (178)	605 (483)
2y+	100 (83)	102 (76)	202 (159)
Total	467 (387)	340 (253)	807(640)

Overall diet description

We identified 1,180 OTUs in the diet of the Eurasian Blackcap, belonging to 237 different families and 46 orders. Rarefaction curves indicated an estimated asymptotic richness of 2,240 OTUs (95% CI: 2,030 – 2,502) and 236 families (95% CI: 273- 367). Sample completeness for OTU was 0.94 (0.933 - 0.948) and for family 0.994 (0.991 - 0.997). Average OTU and Family richness per sample was 7.22 and 5.41, respectively. The most commonly observed OTU was *Smittia sp.1* (19% of the samples), followed by *Arbutus unedo* (17%) and *Gymnometriocnemus brumalis* (17%; Figure 1A). Regarding families, Chironomidae was the most common (68%), followed by Cicadellidae (27%), Mycetophilidae (25%), Sciaridae (25%) and Ericaceae (17%; Figure 1B). Finally, the most common orders were Diptera (89%), Hemiptera (47%), Lepidoptera (27%), Hymenoptera (26%) and Coleoptera (22%; Figure 1C).

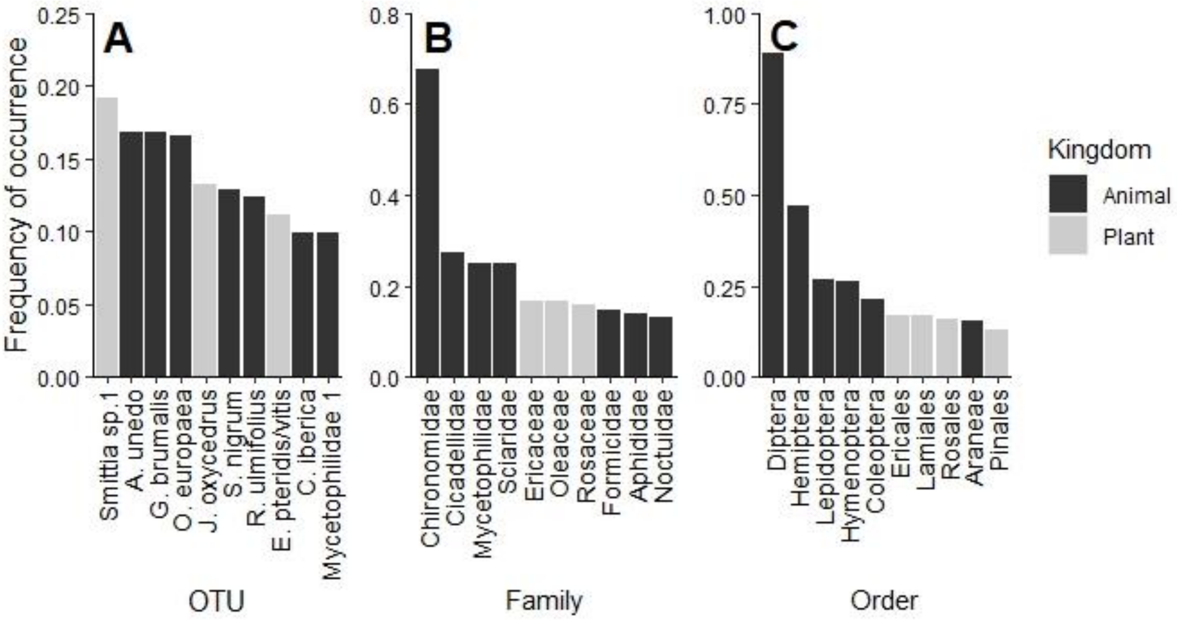

Figure 1. Frequency of occurrence of mostly consumed OTUs (A), families (B), and orders (C) by blackcaps throughout the study period.

Individual specialization and centrality

GLMMs supported the factor sex as the only individual trait that explained the variance in specialization or centrality (Table S1 and Table S2). For OTU level, the specialization index d’ (df=1; p-value=0.017) and the centrality index closeness (df=1; p-value=0.007) showed a significant difference between sex categories (Figure 2, Table S3), with females showing higher values of specialization and lower values of centrality. For family level, no individual trait influenced the variance in diet specialization or centrality significantly (Table S4), with closeness showing an alpha value close to significant for the trait sex (df=1; p-value=0.072).

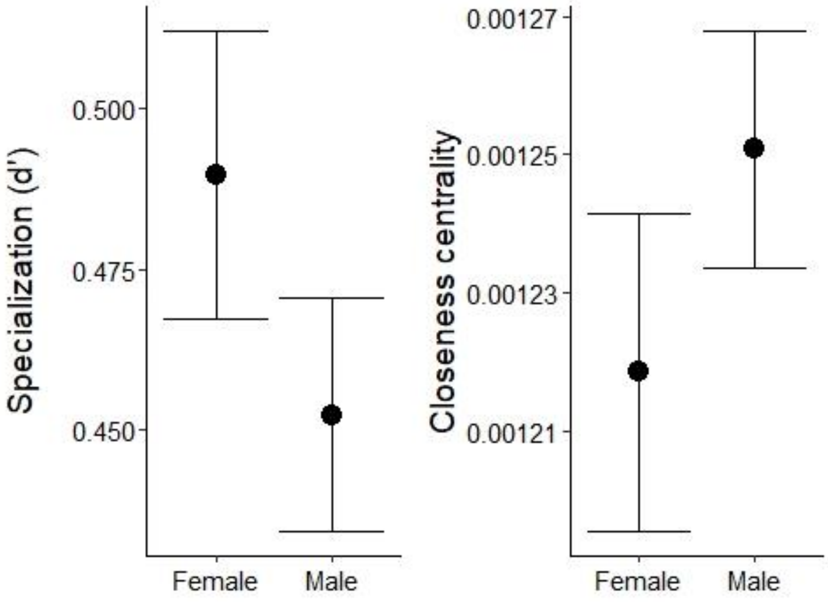

Figure 2. Specialization (d') and closeness centrality for female and male Eurasian Blackcap individuals. Points show means and whiskers 95% confidence intervals.

Within- and between-individual variation

Both, for OTU and Family level analyses, within-individuals' dissimilarity values were lower than between-individuals' dissimilarity values. Mean dissimilarity for within-individuals at OTU level was 0.958, in contrast with 0.977 for between-individuals (Z=5.298; p-value<0.001). At family level, mean dissimilarity for within-individuals was 0.885 and between-individuals 0.903 (Z=2.476; p-value=0.007). When analyzing for each sampling site, only sites with a high number of captures and recaptures reported differences between both groups. In the case of OTU level, site C and D showed significant differences, with 138 and 85 samples from recaptures, respectively. For family level only C showed differences between within-individual and between-individual dissimilarity values. Sites A and B had 32 and 42 samples from recaptures respectively. Overall, 75% of the comparisons between individuals and 65% of comparisons within individuals resulted in no common prey items (Jaccard dissimilarity of 1).

Discussion

This study presents a detailed taxonomic dietary profile of the Blackcap, during the wintering period in a Mediterranean region. It also reports a lower intra-individual variance in diet than between individuals, suggesting some degree of temporal consistency within individuals, and sex-specific differences in dietary specialization. To our knowledge only one study has compared intra-individual and inter-individual variance using several samples per individual and with DNA-based identification of prey items (Fedriani & Kohn, 2001). However, this is the first study that implements it in an omnivorous diet, with one marker targeting arthropods and other plants, or in a bird species. Previous research has attempted to measure intra-individual variance in diet through time, mostly using stable isotopes (Matich et al., 2011; Cryan et al., 2012; Vander Zanden et al., 2013) and behavioral observations (Woo et al., 2008; Balme et al., 2020), and predominantly for carnivorous predators (Newsome et al., 2009; Matich et al., 2011; Vander Zanden et al., 2013; Yurkowski et al., 2016; Voigt et al., 2018; Balme et al., 2020). Stable isotopes have proven to be successful for longitudinal analysis of diet, since different tissues provide distinct time scales (e.g., Bearhop et al., 2006; Yurkowski et al., 2016), yet, the resolution obtained does not allow to discriminate against several prey items (Layman et. al, 2012), hampering the study of species with highly diverse diets. This study shows the potential of DNA metabarcoding in providing large and detailed dietary data for exploring patterns of intra-individual variance along with inter-individual differences in diet of generalist species.

The diet of Blackcap during autumn and winter is strongly dominated by Diptera, which is present in 89% of the samples, a percentage considerably higher than the second most frequent order, Hemiptera, present in 47% of the samples. Plant taxa was constantly present in the diet of the individuals during the sampling period. Within the 10 most common prey items in the diet of the Blackcap half are plant taxa (Figure 1). The overall dietary composition of the Blackcap obtained in this study is similar to that reported in previous studies based on both foraging behavior (Garcia, 1981; Herrera, 1984) and stomach content analysis (Jordano & Herrera, 1981). We observed a generalist diet with 1,180 OTUs ranging over 46 orders, however, the lower intra-individual variance and the low richness of prey items in each sample suggest that the diet of each individual encompasses only a small subset of the population’s diet with low overlap between individuals.

Sex specific differences in diet have been previously reported for many taxa, including other birds (da Silva et al., 2020), reptiles (Houston & Shine, 1993) and mammals (Voigt et al., 2018). Size dimorphism has been used as the main explanation for differences in diet between sexes, usually with comparisons in highly dimorphic species. Otherwise, the Blackcap shows virtually no sexual size dimorphism (Cramp & Simmons, 2004). We propose that rather than dimorphism, sex-specific specialization is due to differences in behavior. Previous research reports that males habituate faster (Ensminger & Westneat, 2012), take more risks (Breitwisch & Hudak, 1989) and move more inside of their territory (Møller, 1992), which could result in a broader range of prey items. This pattern does not seem to hold when analyzing the diet at the family level, which could indicate that individual males cover a larger subset of the diet of the population, however increasing the richness through ecologically redundant species (Timms et al., 2013).

Contrary to what we expected, we did not find evidence of specialization in response to body measurements, such as body condition or size, nor signs of age-specific specialization. Previous studies on granivorous birds have found a relationship between the range of preferred seed sizes and bill size, but not with body mass (Díaz, 1994). King Cormorant showed no differences in foraging behavior according to body mass (Kato et al., 2000). Additionally, in some species it has been reported a negative relationship between body size and range of resources used, as bigger individuals dismiss smaller prey items (Costa et al., 2008), and similarly, in *Periparus ater* smaller individuals can exploit more patches due to their more frequent use of hanging postures (Barbosa et al., 2000). Previous evidence does not support a consistent pattern between resource used and body size, but instead population specific inter-individual differences.

Despite the increasing evidence for foraging individuality, and its importance for understanding ecological dynamics, intra-individual variation in diet is still poorly understood due to the difficulties of longitudinal recording (Clutton-Brock & Sheldon, 2010). Using DNA metabarcoding we obtained the dietary profile of 136 birds with at least one recapture, and compared the variance within individuals and between individuals. Results show some degree of temporal consistency in the diet within individuals. It seems that individuals of Blackcap are consistently selecting over time a subset of the generalist diet of the whole population, which suggests individual specialization. Bolnick et al., (2003) defined individual specialization as the resource use variance within the residuals error of a model explained by sex, age or other morphotype; or when variance within individuals covers only a small fraction of the total variance of the population. Studies on individual specialization should specify consistency on individual specialization, whether across time (e.g., Newsome et al., 2009; Vander Zanden et al., 2013) or a continuous phenotypic trait (e.g., Magalhães de Oliveira et al., 2020). With lack of evidence of consistency, it becomes troublesome to account for random foraging or differences in availability of resources, and it could be reflecting the specialization in individual foraging events rather than in individual diet, overestimating the degree of variance between-individuals (Bolnick et al., 2002). Although we evidenced an individual consistency in the use of a subset of prey items, we did not find a correlation with a phenotypic trait. Future studies on Blackcap should consider additional individual traits, including behavioral syndromes, that have been recently proposed as an additional cause of individual specialization characterized by temporal consistency in the behavior (Toscano et al., 2016), finer morphological measurements, or physiological estimators.

Our results provide evidence of sex-specific specialization and temporal consistency within individuals in the diet. Understanding dietary patterns has proven insightful for both, the development of ecological and evolutionary theory, as well as the natural history of species which helps to enlighten about ecosystem functions. Specially, generalist species can act as community regulators through the interaction with numerous species (Palacio et al., 2016; Bastazini et al., 2019). Further, the elusive behavior of many generalist species, the wide range of foraging techniques used, and the high richness of their diet complicate producing detailed dietary descriptions (Piñol et al., 2014; Quéméré et al., 2021). Evidence of individual specialization within a generalist population informs about the importance to recognize intraspecific variance in conservation strategies and resource management, rather than using mean values of the population for decision making (Newton et al., 1999; Des Roches et al., 2021). This study evidences the usefulness of DNA metabarcoding to produce large databases of generalist species, and the strength to target temporal patterns, at a cost and time-efficient manner. We encourage future studies to include surveys of resource availability to add another essential dimension to the model that could give a clearer picture of the intraspecific dietary patterns.

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Supplementary materials

Table S1. Summary of model selection for the effects of the explanatory variables (sex, age, wing length and body condition) in specialization and centrality with OTU dietary composition of Eurasian Blackcap (Sylvia atricapilla) in northern Portugal. All models included “month” as random factor. We show the three best-ranked models, and for each one we provide: k, the number of variables in the model; logLik, maximized log-likelihood values; delta Akaike information criteria; weight, relative Akaike weights.

Model/Predictor	K	logLik	AICc	delta	weight
specialization d'					
Sex	4	97.2	-186.36	0	0.572
Sex+Age	5	97.25	-184.43	1.929	0.218
Sex+Wing	5	97.22	-184.36	1.999	0.21
nestedrank					

Age	4	18.41	-28.77	0	0.374
Sex+Age	5	19.3	-28.53	0.231	0.334
Sex	4	18.16	-28.27	0.497	0.292
betweenness					
Sex	4	4411.48	-8814.9	0	0.525
Sex+Age	5	4411.7	-8813.33	1.576	0.239
Body condition+Sex	5	4411.69	-8813.31	1.598	0.236
closeness					
Sex+Age	5	5682.88	-11355.68	0	0.393
Sex	4	5681.69	-11355.33	0.357	0.329
Body condition +Sex+ Age	6	5683.55	-11354.99	0.689	0.278

Table S2. Summary of model selection for the effects of the explanatory variables (sex, age, body size and body condition) in specialization and centraliy with Family dietary composition of Eurasian Blackcap (Sylvia atricapilla) in northern Portugal. All models included “month” as random factor. We show the three best-ranked models, and for each one we provide: k, the number of variables in the model; logLik, maximized log-likelihood vaues; delta Akaike information criteria; weight, relative Akaike weights.

Model/Predictor	**K**	**logLik**	**AICc**	**delta**	**weight**
specialization d'					
Sex	4	506.24	-1004.43	0	0.43
Sex*Age	6	508.07	-1004.03	0.4	0.352
Sex+Wing	5	506.57	-1003.06	1.369	0.217
nestedrank					
Sex	4	20.52	-32.99	0	0.353
Age	4	20.46	-32.87	0.12	0.332
Wing	4	20.41	-32.77	0.22	0.316
betweenness					
Sex	4	4483.77	-8959.5	0	0.469
Sex+Age	5	4483.86	-8957.65	1.854	0.186
Body condition +Sex	5	4483.8	-8957.51	1.985	0.174
closeness					
Sex	4	5671.7	-11335.35	0	0.473
Body condition +Sex	5	5672.37	-11334.66	0.69	0.335
Sex+Wing	5	5671.81	-11333.54	1.806	0.192

Table S3. Generalized linear mixed model results of specialization indices with OTU in Eurasian Blackcap (Sylvia atricapilla) of northern Portugal. Asterisks indicate significant values ($p < 0.05$).

Model/Predictor	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
specialization d'				
Intercept	0.121	0.147	0.821	0.412
SexM	-0.155	0.065	-2.382	0.017 *
nestedrank				
Intercept	0.076	0.134	0.562	0.574

Age.cat2y+	-0.122	0.089	-1.380	0.167
betweenness				
Intercept	-6.370	0.071	-89.870	0.000 *
SexM	0.063	0.041	1.517	0.129
closeness				
Intercept	-6.316	0.020	-322.611	0.000 *
SexM	0.022	0.008	2.682	0.007 *
Age.cat2y+	0.014	0.009	1.549	0.122

Table S4. Generalized linear mixed model results of specialization indices with Family in Eurasian Blackcap (Sylvia atricapilla) of northern Portugal. Asterisks indicate significant values ($p < 0.05$).

Model/Predictor	Estimate	Std. Error	z value	Pr(>|z|)
specialization d'				
Intercept	-1.144	0.086	-13.252	0.000 *
SexM	-0.092	0.055	-1.665	0.096
nestedrank				
Intercept	0.067	0.148	0.453	0.651
SexM	-0.040	0.078	-0.516	0.606
betweenness				
Intercept	-6.338	0.047	-136.080	0.000 *
SexM	0.054	0.036	1.491	0.136
closeness				
Intercept	-6.300	0.012	-531.986	0.000 *
SexM	0.014	0.008	1.795	0.073

General Conclusions

This thesis provides a detailed taxonomic identification of the diet of the Sardinian Warbler and the Eurasian Blackcap, assessing temporal patterns in the diet and testing for intraspecific differences across sexes, ages and body size. Results indicate a marked shift in the diet of Sardinian Warblers, at frequency of consumption of families, dietary niche width of the population and degree of intraspecific overlap in diet. Specifically, during summer months, the population's niche width is at its lowest, with high overlap between individuals due to a focus on consumption of berries. In winter, when arthropods become infrequent, dietary niche width increases while intraspecific overlap decreases. These results support the Optimal Foraging Theory (OFT), which predicts that when resources are limited the diet will incorporate more suboptimal prey items and with lower degree of competition between individuals (Pyke 1984). On the other hand, the Blackcap shows evidence of temporal consistency in the diet within individuals, supporting the individual specialization theory (Bolnick et al., 2003). Individuals of Blackcap consistently select a narrow subset of the population's niche width. Previous studies perceive individual specialization theory as a departure from models of dietary optimization which predicts that individuals will actively modify their behavior to maximize fitness (Toscano et al., 2016). Although evidence for each theory comes from a different sylviid warbler species, it is possible both phenomena occur simultaneously within a population, with OFT driving a flexibility in diet to maximize energetic intake, while individual specialization constrains the flexibility at the individual level. Different species were used for each analysis because Blackcaps were captured in sufficient numbers for robust statistical analysis only during six months, precluding seasonality analysis, while less Sardinian Warblers droppings were collected, decreasing the power of a potential analysis of variation within individuals, but were collected more evenly throughout the year.

The use of COI and ITS2 allowed us to retrieve complementary information about the ingested prey items to reconstruct an omnivorous dietary composition. Few studies have performed molecular detections on omnivorous animals (but see De Barba et al., 2014; Robeson et al., 2018; da Silva et al., 2020; Bonin et al., 2020). The implementation of several markers and the potential biases of secondary detection make the identification of an omnivorous diet a challenging, costly and time-consuming process (Tercel et al., 2021). This study was benefited by the extensive floral surveys of the region that were used to assess geographical and temporal congruence in detection, and the several dietary reports of warblers to determine the likelihood of consumption (e.g., Jordano & Herrera 1981; Tejero et al.,1983; Herrera, 1984). Although the efficiency of the primer FwhF2-R2n has been tested in terrestrial invertebrate communities with a recovery of 95% of the community (Elbrecht et al., 2019), biological and technical biases lead to potential differential marker recovery rates so it is more cautious to use only presence/absence of prey items, rather than relative abundance of reads (Deagle et al., 2009; Pompanon et al., 2012). Therefore, all ecological network analyses do not contemplate the weight of interactions, resulting in no observable differences between a highly consumed item and a rare foraging event in the same sample. It is expected that different results arise when including an indicator of the degree in which a prey item was consumed by an individual. For example, it has been reported for studies tracking pollen consumption using the ITS2 region that for qualitative analysis the molecular method is superior, presenting higher sensibility and resolution, however, for quantitative analysis it is essential to include visual identification (Richardson et al., 2015).

This study presents the first extensive molecular identification of dietary items for two Sylviid warblers. Additionally, it evidences the potential of next-generation sequencing as a useful tool to produce large and cost-effective databases of ecological information with repeated samples per individual to assess intraspecific dietary patterns in a temporal perspective. In Chapter 1 it is presented the detailed annual fluctuation of the Sardinian Warbler, and it is used to test for temporal changes in total niche width and inter-individual overlap. Chapter 2 contributes to individual specialization theory using repeated samples of DNA-based identification, an approach seldomly used, which is essential to compartmentalize intraspecific difference in variance between individuals and variance between individuals. This is especially important for generalist species because of the difficulties of reporting dietary description due to the wide range of resources used and their elusive behavior, and their importance regulating community dynamics due to the centrality they present in the trophic webs. The information obtained reveals about the natural history of two generalist birds, the ecosystem functions provided, such as pest control services, and the annual fluctuation in its ecology. Further, it presents substantial support for two common theories of niche ecology, optimal foraging theory and individual specialization.

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