# High resolution multi-marker DNA metabarcoding reveals sexual dietary differentiation in a bird with minor dimorphism

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## Abstract

Although sexual dietary differentiation is well known in birds, it is usually linked with significant morphological dimorphism between males and females, with lower differentiation reported in sexually monomorphic or only slightly dimorphic species. However, this may be an artefact of poor taxonomic resolution achieved in most conventional dietary studies, which may be unable to detect subtle intraspecific differentiation in prey consumption. Here we show the power of multi-marker metabarcoding to address these issues, focusing on a slightly dimorphic generalist passerine, the black wheatear Oenanthe leucura. Using markers from four genomic regions (18S, 16S, COI and trnL), we analysed faecal droppings collected from 93 adult black wheatears during the breeding season. We found that sexes were rather similar in bill and body features, though males had a slightly thicker bill and longer wings and tail than females. Diet was dominated in both sexes by a very wide range of arthropod species and a few fleshy fruits, but the overall diet diversity was higher for males than females, and there was a much higher frequency of occurrence of ants in female (58%) than male (29%) diets. We hypothesise that the observed sexual differentiation was likely related to females foraging closer to their offspring on abundant prey, while males consumed a wider variety of prey while foraging more widely. Overall, our results suggest that dietary sexual differentiation in birds may be more widespread than recognised at present, and that multi-marker DNA metabarcoding is a particularly powerful tool to unveiling such differences.

## Keywords

bird; diet; high-throughput sequencing; multi-marker; *Oenanthe leucura* ; resource differentiation.

## Introduction

Sexual partitioning of food resources is known to occur in many animal species, but the extent and ecological significance of this phenomenon are still poorly understood (Ruckstuhl & Neuhaus, 2006). In birds, differences in diet indicative of resource differentiation have mostly been studied in birds with considerable sexual dimorphism in body size (Bravo, Ponce, Bautista, & Alonso, 2016; Catry, Alves, Gill, Gunnarsson, & Granadeiro, 2012; Donals et al., 2007; Gonzalez-Solis, Croxall, & Wood, 2000; Thalinger, Oehm, Zeisler, Vorhauser, & Traugott, 2018) or in bill size or shape (Smith, 1990; Summers, Smith, Nicoll, & Atkinson, 1990; Temeles, Mazzotta, & Williamson, 2017; Temeles & Roberts, 1993). As a consequence, intraspecific dietary differentiation in birds has been largely attributed to morphological differences, with more sexually dimorphic species expected to show higher resource differentiation (Alarcón et al., 2017; Fonteneau, Paillisson, & Marion, 2009; Lewis et al., 2005; Phillips, McGill, Dawson, & Bearhop, 2011; Selander, 1966). However, it is possible that sexual food resource differentiation also occurs in monomorphic or only slightly dimorphic birds, but this idea remains little explored (but see Botha, Rishworth, Thiebault, Green, & Pistorius, 2017; Cleasby et al., 2015; Elliott, Gaston, & Crump, 2010; Hedd, Montevecchi, Phillips, & Fifield, 2014).

One of the obstacles to understand eventual sexual partitioning of food resources is related to limitations of widely used diet analysis methods, which often are unable to provide enough taxonomic resolution to detect subtle differences in prey consumption (e.g., Mata et al., 2016). This is the case, for instance, of methods widely used in avian ecology, including for instance the morphological identification of the remains of ingested food items (Bravo et al., 2016; Fonteneau et al., 2009; Hunter, 1983; Hunter & Brooke, 1992), direct observation (Catry et al., 2012), fatty acids and alcohols analysis (Owen et al., 2013), or stable isotope analysis (Blanco-Fontao, Sandercock, Obeso, McNew, & Quevedo, 2013; Cleasby et al., 2015; Elliott et al., 2010; Hsu, Shaner, Chang, Ke, & Kao, 2014; Ludynia et al., 2013; Paiva et al., 2018; Phillips et al., 2011). The advent of high-throughput DNA sequencing is making it possible to overcome the limitations of these methods, providing the ability to identify virtually all prey species consumed with unprecedent taxonomic resolution (Hope et al., 2014; Nielsen, Clare, Hayden, Brett, & Kratina, 2017; Razgour et al., 2011; Soininen et al., 2009). As a consequence, this approach has been increasingly used to describe the diets of a wide range of animals (Brown, Jarman, & Symondson, 2012; Kaunisto, Roslin, Sääksjärvi, & Vesterinen, 2017; Macías-Hernández et al., 2018; Mata et al., 2016; Soininen et al., 2009), including birds (Coghlan et al., 2013; Deagle, Chiaradia, McInnes, & Jarman, 2010; Jedlicka, Vo, & Almeida, 2017; Liu et al., 2018; Sullins et al., 2018; Trevelline et al., 2018). The high taxonomic resolution provided by high-throughput sequencing has already been used to describe sexual dietary differences that otherwise would be almost impossible to detect (Mata et al., 2016). However, previous studies have focused on specialists with a relatively narrow feeding niche, while this methodology remains underexplored in testing sexual dietary in more generalist species such as many omnivorous passerines. Dietary generalists are more challenging to study using metabarcoding because they require a combination of markers to fully encompass the full spectrum of food resources used (da Silva et al., 2019a).

Here we aim to show the power of multi-marker metabarcoding to investigate differences in diet between sexes, by focusing on a generalist passerine judged to have minimal sexual dimorphism, the black wheatear (*Oenanthe leucura* ). To address this general goal, the study first documents differences in morphology (bill and body features) between sexes, and then uses a previously developed approach for integrating metabarcoding dietary data across multiple markers (da Silva et al., 2019a) to describe the diets of both sexes. Using this data we then tested the hypothesis that diet varies between sexes in terms of (i) diet diversity and (ii) frequency of occurrence of the main food items, and that (iii) sexual dietary differentiation can only be detected at the high taxonomic resolution provided by metabarcoding. Results were used to discuss the potential of multi-marker metabarcoding to provide a detailed understanding of intraspecific variation in bird diets.

## Material and Methods

### Study area and species

The study was conducted in northeast Portugal, along the Douro river valley and surrounding areas, which corresponds to the last stronghold of the black wheatear in the country. This population occurs mainly in traditional vineyards and olive groves (terraces with stone walls) and is spatially isolated from the remaining Iberian population.

The black wheatear is a highly territorial passerine that occurs in arid and semiarid regions of the Iberian Peninsula and North Africa. Although the species is not globally threatened, European populations are declining, and the species is now considered regionally Vulnerable in Europe (BirdLife International, 2015) and Critically Endangered in Portugal (Cabral et al., 2005). Previous studies using conventional morphological approaches have shown that the species feeds on a wide range of animal and plant food items, no study has shown any sexual dietary differences.

### Field sampling

To document the morphology and diet of black wheatears we carried out captures throughout the study area, during the entire breeding season from April to August of 2014 to 2016, using spring traps baited with mealworms (*Tenebrio molitor* ). Birds were removed from the traps immediately after being captured,

placed in a cotton bag, and afterwards ringed and measured. Birds were retained for less than 15 minutes and all procedures were made with the required permits from national authorities. We made a total of 143 captures, but for this study we only considered the first capture of adult individuals, i.e. 2$^{nd}$ calendar year or more identified following Svensson (1992), totalling 110 adult black wheatears, 79 males and 31 females. For each individual, a number of morphometric measures were taken following Svensson (1992): maximum cord wing length; 3$^{rd}$ primary length; tail length; tarsus length; bill length, depth and width at the distal edge of the nostril; and body mass. Wing, 3$^{rd}$ primary and tail were measured using a ruler to the nearest 0.5 mm, tarsus and bill measurements were made with a calliper to the nearest 0.1 mm, and body mass with a digital balance to the nearest 0.1g. All measures were taken by LPS and when feathers were not fully developed (i.e. moulting birds) the measurements affected were not recorded (Table S1).

Droppings for molecular analysis were collected from bird handling bags, or directly from small rocks used to disguise the bottom of the spring traps (McInnes et al., 2017; Oehm, Juen, Nagiller, Neuhauser, & Traugott, 2011). Bags were soaked in 10% bleach for 1 hour and then washed between each use to minimize contamination. From the 93 droppings thus collected, 62 from males and 31 from females, three were obtained from birds that defecated inside the traps but were not captured. Droppings were stored in 2ml tubes with 98% ethanol at 4$^{o}$C until laboratory analysis (da Silva et al., 2019a).

**Diet analysis**

The samples used in this study were previously analysed by da Silva et al. (2019a) to describe the limitations of single markers in metabarcoding analysis of generalist birds, and to describe a novel method to integrate metabarcoding dietary data from multiple markers. Here we use a subset of that data corresponding to the first capture of 110 adult birds, thereby avoiding biases that might result from including data from a few birds captured more than once (pseudo-replication), as well as eventual confounding effects of including a small number of 1$^{st}$ calendar year birds. Laboratory analysis and bioinformatic processing followed the procedures described in da Silva et al. (2019a). Shortly, the DNA of the droppings was extracted in batches of 23 samples plus a negative control, using the Stool DNA Isolation Kit (Norgen Biotek Corporation) and following the manufacturer's protocol. DNA extracts were then subjected to four independent PCR reactions, each targeting a different gene region: 18S (Jarman et al., 2013), 16S (da Silva et al., 2019a), COI (Zeale, Butlin, Barker, Lees, & Jones, 2011) and *trn* L (Taberlet et al., 2007). PCR products were diluted 1:4 and amplified again to incorporate Illumina indexes. Resulting fragments were purified using AmPure Beads, quantified in Nanodrop, normalized and pooled per primer. Each library was further quantified using qPCR, normalized to 4nM and pooled. The final pooled library was sequenced in an Illumina MiSeq using a partial V2 2x250bp kit with an expected sequence coverage of 12,000 reads/primer/sample. Bioinformatic procedures were done using ObiTools and consisted in pairwise alignment of reads, removal of primer sequences, collapsing of reads into exact sequence variants (ESVs), and removal of non-target and potential spurious sequences using obigrep and obiclean (more detailed methods in da Silva et al., 2019a). Finally, reads were assigned to a prey item by blasting each ESV against BOLD and NCBI online databases and COI sequences from arthropods collected in Portugal (Ferreira et al., 2018). Each possible taxon was checked for its occurrence in the Iberian Peninsula and discarded if not known to occur in either Portugal or Spain. Species level identifications were usually made at identity levels above 98.5% with a single species, except for rare cases where no other species of the genus were known to exist. If the same ESV matched different species, genus, or families, identifications were made to the lowest taxonomic level possible that encompassed all the closest hits. Whenever different ESVs matched the same taxa they were joined into a single molecular unit.

For diet analysis, we only considered molecular operational taxonomic units (MOTU's) of prey identified to the order, family, genus or species levels. We excluded all items that were likely sampling contaminations (e.g. human, fungi and mealworm DNA), and other items not likely to be intentionally ingested by wheatears, as bird parasites and plants that do not have ripe fleshly fruits during the sampling period and are likely the detection of secondary consumption (da Silva et al., 2019a; Sheppard et al., 2005). We integrated all the dietary items obtained from the four molecular markers into a single dataset (Table S2) using the Python script provided by da Silva et al. (2019a).

## Data analysis

All statistical analysis were performed in R v3.5.2 (R Core Team, 2018) using packages car (Fox & Weisberg, 2011), iNEXT (Chao et al., 2014), MASS (Venables & Ripley, 2002) and mvabund (Wang, Naumann, Eddelbuettel, & Warton, 2018). A significance level of $\alpha = 0.05$ was considered. To test for sexual size dimorphism, we compared all the adult bird's measurements (wing, 3$^{rd}$ primary, tail, tarsus, weight, bill length, depth and width) using a MANOVA and subsequent univariate tests. Dietary analysis and comparisons were all done at 3 taxonomic levels: highest prey resolution (all prey items to the most resolved possible taxonomic levels, which varied across taxonomic groups), family and order. To compare the average number of prey taxa detected per dropping of males and females, we used a GLM with a Poisson error distribution. The overall richness of prey ingested by both sexes was estimated using Hill numbers with the double of the reference sample size to avoid extrapolation bias (Chao et al., 2014). We compared the estimated richness considering sample coverage and not sample size (Chao & Jost, 2012). Instead of comparing the 95% confidence interval, a very conservative approach, we considered that differences were significant if the 84% confidence interval (a proxy for $\alpha = 0.05$) of both estimates did not overlap (MacGregor-Fors & Payton, 2013). Finally, we also compared the diet composition between sexes using Generalized Linear Models for Multivariate Abundance Data with a binomial distribution (*manyglm* and *anova.manyglm*functions). We did not include in diet analysis possible confounding variables as sampling day or sample collection localization, because they do not differ between sexes (sampling day (1$^{st}$April = day 1), GLM with negative binomial distribution: LR Chisq = 1.066, df = 1, p = 0.302; latitude, GLM with Poisson distribution: LR Chisq = 2.149, df = 1, p = 0.143; longitude, GLM with negative binomial distribution: LR Chisq = 2.056, df = 1, p = 0.152).

## Results

### Morphology

Black wheatears showed significant sexual dimorphism in the studied measurements (MANOVA: Pillai's trace = 0.502, $F_{1,91} = 10.594$, $p < 0.001$). The univariate tests showed that females had on average a shorter wing (4%), 3$^{rd}$ primary (5%) and tail (2%), as well as a thinner bill (2%), while the other measurements (tarsus, body mass, bill length and width) were similar between sexes (Table 1).

### Diet

The diet of black wheatears was very diverse, with 337 prey items of 96 families and 29 orders (Table S3). Arthropods were detected in all samples and belonged to 5 classes and 22 orders, of which 17 orders were Insecta. The main prey belonged to the order Hymenoptera (Frequency of Occurrence: 83%), mainly ants (family Formicidae; 75%). Frequent animal orders that were detected in more than half of the samples included Lepidoptera (67%), mainly belonging to families Noctuidae (30%), Pterophoridae (25%) and Geometridae (15%); Coleoptera (62%), mainly Tenebrionidae (28%) and Carabidae (13%); Orthoptera (54%), mainly Acrididae (42%); and Diptera (51%), with 10 families identified but none detected in more than 10% of droppings. There were also other important arthropods as Hemiptera (40%), mainly from the family Pentatomidae (16%); and Araneae (34%), mainly Salticidae (11%). The only vertebrates found were lizards (Squamata) detected in two droppings. The vegetal component of the diet was less diverse, but also very common (60% of the droppings), with *Solanum nigrum* (order Solanales, family Solanaceae) being the most frequently detected (35%) (Figure 1; Table S3).

We found no differences between sexes in the average number of prey items detected per sample, irrespective of taxonomic resolution: highest prey item resolution (? = 8.344; LR Chisq = 0.232, df = 1, p = 0.630), families (? = 5.739; LR Chisq = 0.130, df = 1, p = 0.718) or orders (? = 5.226; LR Chisq = 0.083, df = 1, p = 0.773). However, the overall prey richness was higher for males than females for the analysis carried out at the highest prey item resolution (even if a 95% confidence interval was considered), while no significant differences between sexes were detected for analysis based on identifications at the family or order levels (Figure 2).

Regarding diet composition, we found a significant difference between sexes at the highest prey item resolution

(Res. Df = 91, Deviance = 427, p = 0.006), family level (Res. Df = 90, Deviance = 139.9, p = 0.021), but not at the order level (Res. Df = 91, Deviance = 44.52, p = 0.054). The univariate tests showed that the differences found were due to 11 prey items and 6 families (Table S3). The prey item most important for compositional differences was one unidentified Myrmicinae species, that was also the prey most often detected in black wheatear droppings. This ant species was detected in 58% of females' droppings, while in males it was only detected in 29% of droppings (Table S3). At the highest resolution level, all other prey had differences in frequency of occurrence between sexes smaller than 10% (Table S3). At the family level, the differences were mainly due to the families Pentatomidae, Formicidae, Tettigoniidae, that were preyed 24%, 21% and 11%, respectively, more often by females, while males preyed 23% more often on Tenebrionidae (Figure 1; Table S3). There were also 2 orders that differed between sexes (Hymenoptera and Santales), despite the overall effect of sex being non-significant when analysing prey composition at the order level (Table S3).

**Discussion**

Our results confirmed all our hypothesis and showed that although black wheatears exhibit only minor sexual size dimorphism there was dietary differentiation between both sexes, by (i) males having an overall higher diet diversity and (ii) females preying more often on some ant species than males. This is the first time such intra-specific differences are either studied or found in birds using metabarcoding techniques. As expected, the differences found in the diet composition and estimated richness were smaller or not significant using higher taxonomic ranks, suggesting that if lower taxonomic resolution methodologies had been used, these differences would not have been detected. This methodology could be particularly relevant for birds as passerines and near passerines, that feed on hyper diverse taxonomic groups that are often difficult to identify, as insects and other arthropods, and in which diets are often evaluated to the order or family level through conventional techniques (Araújo, Lopes, da Silva, & Ramos, 2016; Catry et al., 2019; Hodar, 1995).

The morphometric differences between sexes observed in our study were related to the thicker bill and longer wings and tail of males. In previous studies conducted in Alicante (Pérez-Granados & Seoane, 2018) and Hoya de Guadix (Møller, Lindén, Soler, Soler, & Moreno, 1995), Spain, males were described not only as having longer wings (wing length and 3$^{rd}$ primary) and tail, but also as being heavier and with a longer tarsus than females. This indicates that sexual size dimorphism on this species may differ across its distribution. The fact that our males showed longer wings and tail, but similar body mass and tarsus, a proxy for body size (Freeman & Jackson, 1990; Pérez-Granados & Seoane, 2018; Rising & Somers, 1989), suggests a higher flight capability of males compared to females. It has been suggested that the larger wings and tail of male black wheatear's could be related to their stone-carrying behaviour (Pérez-Granados & Seoane, 2018; Soler, Soler, Møller, Moreno, & Lindén, 1996) that is mainly done by males (Aznar & Ibáñez-Agulleiro, 2016; Moreno, Soler, Møller, & Linden, 1994). Males also move more often in their territories than females, especially for territory defence, not only against conspecifics, but also against other birds of different sizes (Møller, 1992; Prodon, 1985). Regarding the thicker bill of males, it could also be an adaptation to the stone-carrying behaviour and higher aggressivity.

The dietary composition of black wheatear observed in our study was largely similar to that documented elsewhere. In particular, the large dietary spectrum of arthropod groups and the ability to hunt relatively large prey such as reptiles was already reported from natural habitats of Spain, where the most frequent prey were also ants (Hodar, 1995; Richardson, 1965). The highest difference found between previous dietary studies of this species and our work, is the high frequency of berries detected in our study. To some extent this could be due to the different methods used for the identification of the droppings remains (da Silva et al., 2019a). However, it is more likely related to differences in habitat, since the Portuguese population occurs mainly in traditional agricultural habitats (vineyards and olive groves) where *Solanum nigrum* is a very widespread and abundant herb, providing a high number of ripe fruits, while the studied Spanish populations were located in shrub steppe areas, presumably with a lower availability of berry-bearing plant species during the wheatear's breeding season (Hodar, 1995).

The differences in diet composition observed in our study are likely more related to sexual behavioural

differences during the breeding season than to the morphometric differences observed. Although males have a more robust bill than females, its length and width is similar, which in principle allows both sexes to capture and swallow similar prey items. In some birds it has been reported that females tend to forage closer to their offspring than males (Sunde, Bølstad, & Møller, 2003). This behaviour could lead females to prey more often on abundant and predictable prey like ants, even if these are smaller and less nutritious (Dean & Milton, 2018). On the other hand, the higher mobility of males within territories could explain the lower frequency of some less nutritious prey (e.g., ants), and the wider range of other prey, likely less predictable and abundant.

As far as we could find, this is the first example of a monomorphic (or minor dimorphic) passerine species exhibiting dietary differences between sexes, during the breeding season.

Usually, the more sexually dimorphic a bird species is a higher resource differentiation is expected (Fonteneau et al., 2009; Lewis et al., 2005; Phillips et al., 2011; Selander, 1966). Nevertheless, on some monomorphic seabirds species, different foraging areas have been described between sexes, especially in the beginning of the breeding period (Cleasby et al., 2015; Hedd et al., 2014; Pinet, Jaquemet, Phillips, & Le Corre, 2012). On two New Guinean whistlers, passerine species with little sexual dimorphism, vertical segregation was also found between sexes and attributed to male territory defence and intersexual food resource differentiation (Freeman, 2014). Nonetheless, it is not clear how spatial segregation translates into dietary segregation, and there seems to be little evidence of dietary segregation in monomorphic species (Catry et al., 2019; Phillips et al., 2011), despite some exceptions (Cleasby et al., 2015). Regardless of the main cause for the dietary differentiation found in our study, it shows a sexual dietary differentiation during the breeding period, which may help lowering intraspecific competition, which can be especially important in the (semi-)arid landscapes where black wheatears occur.

Overall, our study shows how even minor dimorphic bird species can have subtle differences in diet during their breeding season. The differences found were most likely related to sexual differences in behaviour rather than morphology, which means that this pattern might be far more common than what is currently recognized in birds. Moreover, this pattern was only possible to detect thanks to the high taxonomic resolution offered by metabarcoding, as analyses at higher taxonomic ranks were not able to identify such differences. At a time when metabarcoding is starting to be used to re-visit and assess the diet of many species, as well as to study other species interactions like pollination, it becomes increasingly important to understand the impact of taxonomic resolution in ecological studies (Renaud, Baudry, & Bessa-Gomes, 2020). Finally, this study is an example of how the development of new techniques, such as metabarcoding, can help ecological studies go a bit further and gain better insights into fine ecological patterns that could otherwise go unnoticed.

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## Data Accessibility

Morphometric and dietary matrices used for analysis are available in supporting information (Table S1 and Table S2). Raw sequencing data is available at Dryad: 10.5061/dryad.26vr077 (da Silva et al., 2019b).

## Author Contributions

LPS designed the study and led the writing of the manuscript with contributions from all authors. LPS, VAM, PBL and RJL collected the data. LPS and VAM led the data analysis with contributions from the other authors.

## Supporting information

Table S1, Table S2 and Table S3

## Tables

Table 1 – Biometric differences between adult black wheatear sexes. All measures are in mm except body mass that is in grams. Average +- 95% confidence interval and MANOVA univariate tests (F and $p$ value). Significant values are in bold.

| Measurement | Female | Male | Univariate test |
|---|---|---|---|
| Wing | 95.318 ± 0.976 | 99.648 ± 0.479 | **F = 73.756, p < 0.001** |
| 3$^{rd}$ primary | 70.955 ± 0.783 | 74.514 ± 0.463 | **F = 58.078, p < 0.001** |
| Tail | 68.595 ± 0.917 | 69.824 ± 0.489 | **F = 9.182, p = 0.003** |

| Measurement | Female | Male | Univariate test |
|---|---|---|---|
| Tarsus | 27.159 ± 0.387 | 27.215 ± 0.216 | F = 0.065, p = 0.799 |
| Body mass | 34.875 ± 1.274 | 35.531 ± 0.502 | F = 1.344, p = 0.249 |
| Bill length | 12.659 ± 0.330 | 12.928 ± 0.159 | F = 2.554, p = 0.114 |
| Bill width | 4.232 ± 0.086 | 4.285 ± 0.061 | F = 0.783, p = 0.379 |
| Bill depth | 4.409 ± 0.092 | 4.517 ± 0.048 | **F = 4.653, p = 0.034** |

**Figures**

Figure 1 – Frequency of occurrence network showing the families ingested by black wheatear males and females. On the bottom, animal orders are in grey and plant orders in green. Red interactions indicate orders consumed in significantly different proportions by both sexes, as revealed by univariate tests. Only the names of the most frequent families (more than 10% frequency) are shown.

Figure 2 – Rarefaction curves showing the observed (full line) and estimated (dashed line) richness, until double the reference sample size, and respective 84% confidence interval (a proxy for $\alpha = 0.05$) by sample coverage.