Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Biologia da Conservação

Dissertação

Diet study of the domestic cat (Felis catus) in the ecological Park of Funchal, Madeira Island

Alexandra Sofia Vieira Galão

Orientador(es) | Catarina Castela dos Santos Rato

Nuno M. Pedroso

Évora 2023

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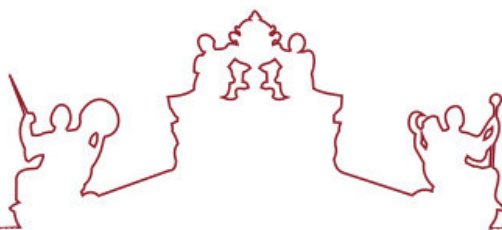
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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente | Paulo Sá-Sousa (Universidade de Évora)

Vogais | Catarina Castela dos Santos Rato () (Orientador)
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Estudo da dieta do gato-doméstico (*Felis catus*) no Parque Ecológico do Funchal, Ilha da Madeira

Resumo

Os ecossistemas insulares são particularmente vulneráveis ao impacto dos predadores introduzidos, como os gatos (*Felis catus*), que representam uma ameaça à fauna insular. Neste estudo, investigou-se a dieta dos gatos ferais e com acesso ao exterior no Parque Ecológico do Funchal, Ilha da Madeira, utilizando *DNA metabarcoding* e análises morfológicas. Nesta pesquisa identificou-se 19 taxa de presas, estando incluídos alimentos de origem antropogénica, taxa nativos e não nativos. A análise molecular obteve uma maior resolução taxonómica, identificando uma maior variedade de presas, e revelando que os gatos não dependem exclusivamente de recursos fornecidos pelo Homem. Também, revelam variações sazonais na dieta destes predadores, com répteis e aves a serem mais predados no verão, enquanto no outono há uma maior dependência de roedores introduzidos. Este trabalho realça a importância do uso de métodos complementares para o estudo da dieta de carnívoros invasores em ecossistemas insulares, como os gatos. Adicionalmente, este trabalho enfatiza a necessidade de estratégias de conservação para proteger as espécies nativas do impacto destes predadores.

Palavras-chave: *Felis catus*, espécies invasoras, análise de dieta, sequenciamento de nova geração, Ilha da Madeira.

Diet study of the domestic cat (*Felis catus*) in the Ecological Park of Funchal, Madeira Island

Abstract

Insular ecosystems are particularly vulnerable to the impact of introduced predators, such as free-ranging cats (*Felis catus*). Here, we investigated the diet of free-ranging cats in the Ecological Park of Funchal, Madeira Island, using both DNA metabarcoding and morphological analyses. Our research identified 19 distinct prey taxa, including anthropogenic food, native species, and non-native taxa. Molecular analysis enhanced taxonomic resolution, unveiling specific prey species and indicating that cats don't heavily rely on human food resources. Additionally, we identified seasonal variations in cat diet, with lizards and birds being more prevalent in summer, while cats were particularly reliant on non-native rodents in autumn. Our findings underscore the importance of employing complementary methods for investigating carnivorous mammals' diets, particularly invasive species in insular ecosystems, such as free-ranging cats. Furthermore, its emphasis the necessity for evidence-based conservation strategies to protect vulnerable native taxa from the impact of these predators.

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ESVs: Exact sequence variants

FO: Frequency of occurrence

MOTUs: Molecular Operational Taxonomic Units

NC: Negative control

OTUs: Operational taxonomic units

Introduction

Insular ecosystems tend to have fewer species than their mainland counterparts. However, they are important for their high number of endemic taxa, a result of their evolutionary isolation (Cronk 1997; Whittaker & Fernández-Palacios 2007; Conenna *et al.* 2017). Due to this isolation, island species tend to evolve somewhat sheltered from of many of the main ecological stressors affecting mainland species, such as high levels of interspecific competition, parasitism, and predation/herbivory (Nogales & Marrero 2006). Therefore, insular ecosystems are particularly sensitive to the pervasive effects of the introduction of exotic species, especially mammals introduced by humans (Doherty *et al.* 2016; Russell *et al.* 2017; Russell & Kueffer 2019).

For millennia, cats (*Felis catus*) have been important companions, pest controllers, and part of our cultural heritage, currently being found in most continental and island ecosystems inhabited by Humans (Crowley *et al.* 2020; Magle & Crowther 2023). However, due to competition with native species for food and resources, transmission of diseases, hybridation and predation, they constitute an important threat to native biodiversity (Herrera *et al.* 2022; Loss *et al.* 2022; Fardell *et al.* 2023; Nussberger *et al.* 2023; *Szentivanyi et al.* 2023). Their impact as predators of native species is particularly problematic (Trouwborst *et al.* 2020), as cats are opportunistic and generalist predators, which prey on a wide variety of species, including mammals, reptiles, birds, and insects (Nogales & Medina 2009; Bonnaud *et al.* 2011; Medina *et al.* 2011; Mori *et al.* 2019). They are also known to consume wild prey even when they are regularly fed by humans (Hernandez *et al.* 2018; Cecchetti *et al.* 2021; Plimpton *et al.* 2021), which puts increased pressure on wildlife communities. In isolated and already vulnerable populations, like those often found on islands, even relatively low predation pressure by cats can have profoundly negative consequences, leading to significant declines in abundance and, in some cases, even resulting in local or global extinctions (Bonnaud *et al.* 2011; Moseby *et al.* 2015; Alho *et al.* 2022). Indeed, it is estimated that cats have contributed to the extinction of approximately 14% of the world's mammals, birds and reptiles listed on the IUCN Red List (Courchamp *et al.* 2003; Medina *et al.* 2011).

On islands, unowned and owned free-ranging cats are a key threat to island vertebrates (Zino *et al.* 2001; Bonnaud *et al.* 2011; Medina *et al.* 2011; Russell *et al.* 2018;

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Alves *et al. in press*). This is largely due to the fact that insular native species have evolved in the absence of mammalian terrestrial predators, and thus have not developed natural defences to avoid them (Baeckens & Van Damme, 2020; Choeur *et al.* 2022). Therefore, studies of the diet of free-ranging cats are important to understand the impact they have on island fauna. These studies can provide data on the prey that cats consume, which can help to identify which native species are most at risk (Bonnaud *et al.* 2011; Carrión & Valle 2018).

In the Macaronesian islands, including the archipelagos of Azores, Madeira, the Canary Islands and Cabo Verde, free-ranging and feral cats are widely distributed across all inhabited areas (Silva *et al.* 2008; Masseti 2010). Although multiple studies have studied cat trophic interactions in the Canary Islands (Nogales 2006; Nogales & Medina 2009; Ravelo & Reyes 2021), research on cat diet in the other archipelagos remains somewhat limited. In the Canary Islands, cats mainly prey on introduced mammals such as rabbits, rats, and mice, but also consume native birds and reptile species (Nogales & Medina 2009; Medina *et al.* 2010; Ravelo & Reyes 2021). In Cabo Verde, while still consuming introduced mammals, cats' prey predominantly on reptiles (skinks and geckos), with birds being the least targeted group (Donald *et al.* 2005; Medina *et al.* 2010, 2012, 2021). In the Azores, cats primarily target introduced mammals like mice and rats; however, documented cases of predation on native species, including seabirds such as Cory's shearwater (*Calonectris borealis*), common and roseate terns (*Sterna hirundo* and *S. dougallii*, respectively), and the Azores Woodpigeon (*Columba palumbus azorica*), have been recorded (Hervías *et al.* 2013, 2014; Lamelas-López *et al.* 2020, 2021). Research on cat diet in the Madeira archipelago is limited (but see Cook & Yalden 1980; Medina *et al.* 2010); however, Medina *et al.* (2010) showed that cats in Madeira prey mainly in introduced mammals, passeriformes and reptiles, but instances of predation on other endemic and endangered taxa, seabirds, and bats, have also been documented (Zino *et al.* 2001; Rocha 2015).

Studies on cat diet typically analyse fecal samples, or focus on stomach or intestinal contents (e.g., Medina *et al.* 2008, 2011, 2021; Hervías *et al.* 2014). The analysis of diet from fecal samples – the most common methodology used to date to investigate cat diet - has traditionally relied on the morphological identification of undigested remains of prey (Bonnaud *et al.* 2011; Carrión & Valle 2018). Although useful, morphological studies generally have a limited resolution in terms of taxa identification. This limitation result from several

factors, such as the inability to detect rare prey, the challenge of correctly classifying prey from skeletons and reference collections, and the possibility of missing soft-bodied organisms (Shehzad *et al.* 2012). Additionally, morphological studies are also limited when there are no digested remains or when these remains are rare (Massey *et al.* 2021). This limitation hinders the identification of food items that do not leave behind hard remains, such as pet food or leftovers composed only of soft tissues (Forin-Wiart *et al.* 2018). Moreover, the results of prey identification also rely on the experience of the person conducting the analysis, and it is a time-consuming process (Alemany *et al.* 2023). These limitations often lead to the underestimation of predation rates (Shehzad *et al.* 2012). Previous studies have shown that the use of morphological techniques alone considerably underestimates the rate of predation and consumption of certain taxa, such as birds (Zarzoso-Lacoste *et al.* 2016; Oja *et al.* 2017; Massey *et al.* 2021).

Next-generation sequencing (NGS) techniques have been used to study the diet of multiple carnivorous mammals (e.g., Massey *et al.* 2021; Shi *et al.* 2021; McLennan *et al.* 2022; Woo *et al.* 2022, 2023; Groen *et al.* 2023; Lino *et al.* 2023). However, to date, only Plimpton *et al.* (2021) has used molecular methods to study the diet of free-ranging cats in mainland USA, and no studies have used this technique in island ecosystems. Through the analysis of 134 samples from cats living in five Trap-Neuter-Return colonies situated in urban parks on Staten Island, New York, USA, Plimpton *et al.* (2021) identified a total of 16 different vertebrate prey items in their diet.

DNA metabarcoding is a non-invasive method that can be used to identify the taxa consumed by a given predator through sequencing of standardized DNA fragments (Pompanon *et al.* 2012; Martins *et al.* 2022). This technique offers several advantages over morphological analyses when studying trophic interactions, allowing for the collection of extensive datasets without the time or effort required by traditional methods (Gil *et al.* 2020). Additionally, DNA metabarcoding is more likely to identify a higher proportion of prey species consumed and is less prone to misclassifying species (Massey *et al.* 2021). Also, the level of prey identification does not depend on the analyst's experience (Yoccoz 2012; Massey *et al.* 2021). Although DNA isolated from highly degraded samples may be of lower quality, making it impossible to accurately identify some taxa, molecular methods can still be as, or even more effective than morphological analyses (Massey *et al.* 2021). Nonetheless, molecular methods

can overlook species due to the lack of adequate primer-binding sites due to mutations in the primer-binding regions and due to incomplete molecular databases (Schenk *et al.* 2020). Consequently, species with multiple reference sequences are more likely to be identified accurately compared to those without (Schenk *et al.* 2020). Therefore, due to the limitations presented by both methods, some studies recommend employing both methodologies as complementary approaches to describing a species' diet (Gil *et al.* 2020; Alemany *et al.* 2023).

Here, we use both DNA metabarcoding and morphological analyses, to investigate the trophic ecology of free-ranging cats in the peri-urban Ecological Park of Funchal, in the sub-tropical oceanic island of Madeira, Portugal. Specifically, we address the following questions:

- Which taxa are consumed by free-ranging cats? Based on previous studies both in Madeira (Medina *et al.* 2010), and other oceanic islands (e.g., Carrión & Valle 2018; Medina *et al.* 2010, 2021), we anticipate that the diet of free-ranging cats will consist of both wild preys, including both native and introduced vertebrates, as well as anthropogenic food sources.

- Does prey species composition and prey species richness differ between the summer and autumn seasons? We hypothesize that during the summer, cats will exhibit a higher propensity to prey upon birds and the Madeira wall lizard, due to the greater availability of more naive, immature birds and the higher activity of ectothermic species. In contrast, during the autumn, we predict that there will be a reliance on introduced mammals.

- How different are the dietary patterns obtained using DNA metabarcoding and morphological analysis? Based on previous studies contrasting both methodologies (Gil *et al.* 2020; Massey *et al.* 2021; Alemany *et al.* 2023) and considering the relatively good representation of vertebrates from Madeira in molecular databases, we anticipate that DNA metabarcoding will provide a greater number of prey species consumed and a higher resolution identification compared to morphological analyses.

Methodology

1. Study Area

Fieldwork was conducted in the Ecological Park of Funchal, located on the subtropical Island of Madeira, Portugal (Fig. 1). Madeira is an oceanic island in the eastern Atlantic Ocean, situated about 900 km from mainland Portugal and 600 km from Morocco. It is the largest island of the Madeira Archipelago, with an area of 737 km² and a population of over 250,000 people (2021). The capital, Funchal, is home to about 55% of the island's population (DREM 2021). The Ecological Park of Funchal is a 7.5 km² protected area located at the north of the municipality of Funchal and half of the area is included in the Natura 2000 Network (3.6 km²). The park ranges from 470 m to 1818 m above sea level (Fagundes *et al.* 2008) and is traversed by two main water courses: Ribeira de Santa Luzia to the west and Ribeira das Cales on the center (Fig. 1).

The Ecological Park of Funchal is home to several species of vertebrates, with birds being the most representative group. There are 26 confirmed breeding species of birds in the park, of which three are endemic to Madeira (*Regulus madeirensis*, *Fringilla maderensis*, and *Columba trocaz*) (Nunes *et al.* 2021; Recuerda *et al.* 2021). Furthermore, the Ribeira de Santa Luzia valley hosts the only known breeding colony of the Max shearwater (*Puffinus puffinus*) in Madeira Island, a regionally threatened seabird, whose population size and ecology in Macaronesia is poorly known (Nunes *et al.* 2010; Rodríguez *et al.* 2020). Other than birds, the Ecological Park of Funchal harbors other vertebrate species such as the endemic Madeira wall lizard (*Teira dugesii*), and three species of bats (*Pipistrellus maderensis*, *Nyctalus leisleri verrucosus*, and *Plecotus austriacus*), one of which is endemic to Macaronesia (*P. maderensis*; Gonçalves *et al. in press*). Several species of introduced and invasive mammals are also present, in addition to the domestic cat, such as the Black and Norway rats (*Rattus rattus* and *R. norvegicus*, respectively), the domestic mouse (*Mus musculus*), the European rabbit (*Oryctolagus cuniculus*) and ferrets (*Mustela furo*) (Soto *et al. in press*).

2. Sample collection

Between July and November 2021, we randomly collected a total of 58 fresh cat scats throughout the Funchal Ecological Park (Fig. 1). Twenty five were collected during the summer (13 July and 22 September), while 33 were collected during the autumn months (23 September and 20 November). Only fresh scats (deposited < 1 day) were considered as we aimed to obtain relatively good quality DNA for the intended molecular analyses. We stored the samples in plastic bags or individual vials with silica gel, and labelled them with an ID, date, and respective coordinates. They were then refrigerated at -20 ºC until DNA extraction was performed.
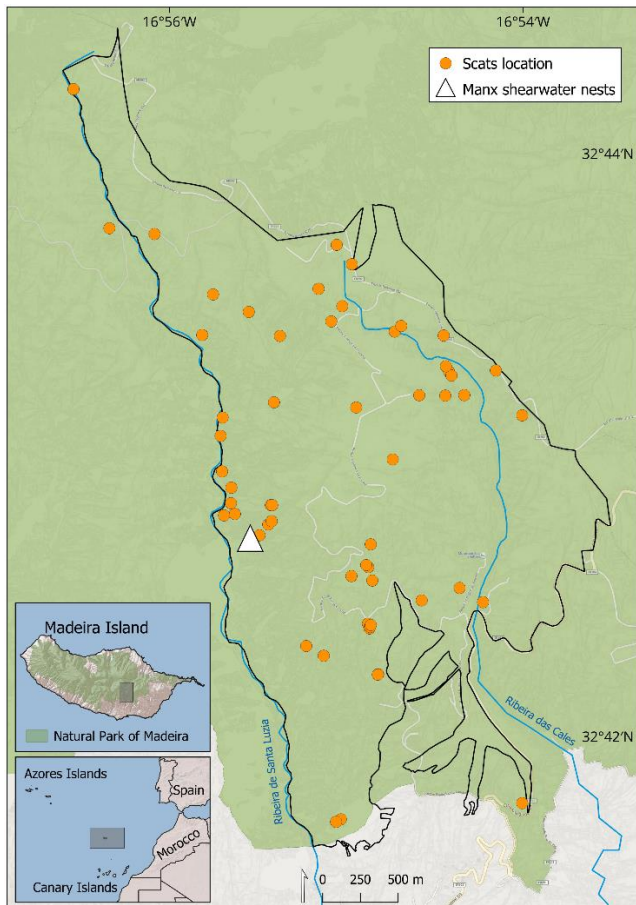
Figure 1: Location of the Ecological Park of Funchal in Madeira Island, Portugal. The distribution of cat scats collected is shown by orange dots. The white triangle indicates the location of the only known nesting colony of Manx shearwaters in the Madeira Archipelago.

3. <u>Molecular Study of the Diet</u>

3.1. DNA Extraction and Library Preparation

For the extraction of DNA, we used the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada). First, a subsample of 2.5 to 3 cm was extracted, taking care not to sample the external surface, as this will have a high concentration of predator epithelial cells (Waits & Paetkau 2005). Then, the sample was homogenized in 4 to 7 ml of PBS Solution, vortexed and centrifuged at room temperature for 30 s to 1 min. The remaining steps followed the kit recommendations, except that the DNA was eluted twice. This resulted in two 50 μL elutions per sample and three extraction control samples, which were stored in plates at -20°C until amplification.

To analyse the vertebrate component of the cat diet, the V5 variable region of the mitochondrial 12S gene (73–110 bp) was amplified using the 12SV5F/12VS5R primers and an adapted PCR from Plimpton *et al.* (2021). This marker has been shown to have great resolution power for genus and species identification across numerous vertebrate taxa (Riaz *et al.* 2011). Both primers were modified to include Illumina adaptors, and a 0 – 5 bp shift made of Ns was added between the adaptor and the primer to increase sequencing diversity and quality. The different primer variations were then combined before the PCR reactions, resulting in mixed forward and reverse primer single solutions.

PCR reaction was comprised by 6.25 μL of QUIAGEN Multiplex PCR Master Mix (Quiagen, Crawley, UK), 0.625 μL mix of 10 mM 12SV5 F primers, 0.625 μL mix of 10 mM 12SV5 R primers, 2.5 μL of a 20 nM blocking primer (Plimpton *et al.* 2021) to reduce the prevalence of cat DNA, 0.5 μL of ultra-pure water and 2 μL of DNA extract. Cycling conditions consisted in an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 98°C denaturing for 30 s, annealing at 58°C for 30 s, extension at 72°C for 10 s, and a final extension at 72°C for 1 min. A PCR negative control (NC) was included in the amplification to check for potential contaminations. Amplification success and quality was checked by running the PCR products in 2% agarose gels stained with GelRed.

The library preparation began by conducting an initial PCR clean-up, utilizing Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), to eliminating primer dimer. Subsequently, an indexing PCR was performed to accurately label each amplified product. For

the indexing PCR, 2.8 μL of ultra-pure water, 7 μL of 2 x Kapa HiFi, and 2.8 μL of the cleaned PCR product were employed. The cycling conditions involved an initial denaturation at 95°C for 3 m, followed by 10 cycles of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. To eliminate any remaining primer dimer, nucleotides, and enzymes that could interfere with the sequencing reaction, a second bead clean-up of the PCR products was carried out. Following these procedures, all purified PCR products were quantified using Epoch, normalized to 20 nM, and pool sampling. The purified and normalized PCR products were pooled, and the library was quantified using qPCR (KAPA Library Quant Kit qPCR Mix; Bio-Rad iCycler) and then diluted to 4 nM. Ultimately, the library was equimolarly pooled and sequenced using a MiSeq Reagent Micro Kit v2 (300 cycles) to achieve an expected average of 25,000 paired-end reads per sample-marker combination.

3.2. Analyses of DNA Sequence Data

Initially, the paired-end reads underwent alignment using PEAR (Zhang *et al.* 2014), where base-pairs with q-scores below 26 were discarded. Subsequently, reads were assigned to respective samples, and primer sequences were eliminated utilizing the *ngsfilter* command from OBITools (Boyer *et al.* 2016), allowing a maximum of four mismatches to the expected primer sequence. Following this step, reads were de-replicated into unique sequences or exact sequence variants (ESVs), and singletons were excluded using the *obiuniq* command. Fragments shorter than 73 bp (as in Plimpton *et al.* 2021) and those occurring ≤10 times were removed using the *obigrep* command. The *obiclean* command was then employed to enhance data accuracy by eliminating potentially spurious sequences with an 'r' level of one. This means that any 'A' ESV differing one base-pair from a 'B' ESV, with an absolute read count lower than 'B', and that was not found without the presence of 'B' in any PCR product, was removed as it was most likely a PCR or sequencing error. An Operational Taxonomic Unit (OTU) table was produced using the *obiannotate*

Ultimately, the *--usearch_global* command from VSEARCH (Rognes *et al.* 2016) was employed to create a matchlist containing all internal matches of OTUs. The obtained OTU table and sequences were further cleaned using the R package LULU (Frøslev *et al.* 2017) to remove potential mtDNA nuclear copies and persisting PCR and sequencing errors. ESVs with a read count < 1% of the total reads in each PCR product were removed (Mata *et al.* 2016),

aiming to eliminate remaining PCR and sequencing errors that had passed the *obiclean* denoising step. Subsequently, reads identified in the extraction and PCR controls were subtracted from the respective sample batch (Evans *et al.* 2021).

The taxonomic classification of OTUs was accomplished using the BLAST function within the NCBI database. Sequences displaying less than 90% similarity were categorized only at the taxonomic class level, while those falling within the 90% to 95% similarity range were placed in the corresponding taxonomic family level. Sequences exhibiting more than 95% similarity were precisely identified at the species or genus level. In situations where there were multiple potential matches at the genus or species level, OTU assignments were determined based on species records from Madeira (Borges *et al.* 2008; Fagundes *et al.* 2008). For ESVs that could not be determined to the species level, a neighbor-joining tree was constructed using Geneious Prime software (Drummond *et al.* 2010). Subsequently, a visual inspection of the alignment was conducted, looking for co-occurrence patterns among similar ESVs to cluster them (~98%) into distinct taxa, such as *Rattus* sp.1, *Rattus* sp.2, and so on, also known as Molecular Operational Taxonomic Units (MOTUs). After this process, taxa that did not belong to the animal kingdom, including bacteria (Proteobacteria), as well as mammals (such as cats, dogs, and humans), were excluded.

4. <u>Morphological Study of the Diet</u>

Following Medina *et al.* 2010, after the scats were saturated in water, they were broken and inspected under the stereo microscope. All biological material that could be prey items, such as bones, skin, hair, feathers, scales, and exoskeletons, were meticulously extracted using tweezers. These remains were stored in petri dishes and cross-referenced with available reference collections and literature sources (Brom 1986; Teerink 1991; Valente *et al.* 2015) and identified at the species level whenever possible (Medina *et al.*, 2010; Medina *et al.*, 2021). Additionally, the taxa identification was complemented by specialist consultation whenever necessary and possible. For this approach, the number of occurrences of each specific prey category was recorded, considering the minimum number of individuals for each taxon, given the highly fragmented nature of the prey remains.

5. <u>Statistical analyses</u>

Dietary analyses were performed at three taxonomic levels: order, family and OTU, this final taxonomic level refers to all identified taxonomic units resolved to the highest possible level, even if the classification was limited to family or order level. We used OTUs as the most resolved taxonomy instead of species because many taxa could not be identified to that level due to gaps in reference databases. Additionally, we categorized the OTUs into two primary groups: wild food and anthropogenic food, the latter encompassing pet food or food remnants. The relationship between the amount of ingested wild and anthropogenic food was assessed through a linear regression.

To evaluate the effects of different variables on the average number of prey taxa detected per fecal sample (i.e. richness), we conducted a General Linear Model (GLM), using the seasons and diet type (wild or anthropogenic food) as explanatory variables. When using seasons as the predictor, we excluded the anthropogenic food items because our objective was to determine if the richness of wild prey changes between seasons. To perform this analysis, we employed the *glm* function with a Poisson distribution, as the data did not exhibit a normal distribution (OTU: Shapiro-Wilk normality test: $W = 0.839$, $p < 0.001$; Family: Shapiro-Wilk normality test: $W = 0.787$, $p < 0.001$; Order: Shapiro-Wilk normality test: $W = 0.820$, $p < 0.001$). Subsequently, to assess the significance of the model, we employed the *anova* function from the car package (Fox *et al.* 2019). Graphical representation of the relationships depicted from the results of the GLMs, we used the *effect* function from the effects R package (Fox 2003), where richness means and standard errors were derived from the model's parameters estimates. For morphological analyses, we only used season as an explanatory variable since, in this method, it was often challenging to identify prey taxa originating from anthropogenic food sources.

To calculate dietary niche-width across different seasons and methods, we built prey rarefaction and extrapolation curves using the R package iNEXT version 2.0.20 (Hsieh *et al.* 2016). These analyses were conducted with incidence frequencies of prey taxa. We compared the estimated richness considering completeness (i.e., sample coverage) instead of sample size (i.e., number of samples), to avoid biases of communities with different levels of richness requiring different sampling efforts to be sufficiently characterized (Chao & Jost 2012). We

considered differences were significant if the 84% confidence interval (a proxy α = 0.05) of both estimates did not overlap, as the use of a 95% confidence interval is a very conservative approach (MacGregor-Fors & Payton 2013).

Permutational multivariate analysis of variance (PERMANOVA) was used to compare the diet composition between seasons, in both methods, and the diet composition between methods with the function *adonis* from the vegan package (Oksanen *et al.* 2013). First, presence or absence of each prey item in each sample was used to build a Jaccard dissimilarity matrix using the *vegdist* function from the vegan package. A homogeneity of dispersion test (function *betadisper*) was carried out to assess if the observed differences in PERMANOVA could be due to unequally dispersed values across the different groups (Anderson 2006). Finally, the command *simper* was used to identify the prey items that contributed the most to dietary composition disparities across seasons and methods. The comparison of diet composition between the two methods was conducted exclusively at the order taxonomic level. This limitation arose because with the morphological analysis, many of the prey remains could not be classified beyond the order level.

The frequency of occurrence (FO) of each diet item for molecular analyses was calculated as the number of occurrences of the diet item (limited to a maximum of 1 occurrence per sample) divided by the total number of sequenced cat scats for molecular analyses (Plimptom *et al.* 2021). For morphological analysis, the division was performed using the total number of scats with preys remains. However, it is important to note that a sample can have more than one prey from a certain taxonomic group, so the sums of FO for species will never equal the value of FO for the order. The frequencies of occurrence of these identified prey items were then represented in a stacked histogram. The FO for diet type (wild or anthropogenic food) was calculated based on the results of the molecular analyses. And the FO for prey type (native or non-native/introduced) was determined for each approach.

In this study, the FO for each prey item was calculated using Excel, while the remaining statistical analyses were performed using R version 4.1.2 (R Core Team 2022).

Results

Overall diet description

A total of 19 prey taxa were identified using both methods, from five classes (Mammalia, Aves, Reptilia, Actinopteri, and Insecta) and ten orders (Rodentia, Lagomorpha, Artiodactyla, Passeriformes, Galliformes, Anseriformes, Squamata, Testudines, Scombriformes and Orthoptera) (Table 1). However, all prey items from the orders Artiodactyla, Anseriformes, and Scombriformes were likely derived from anthropogenic resources (such as pet food or food remnants), and the chicken (*Gallus gallus*) was the only prey item from the Galliformes order considered anthropogenic food. Conversely, all prey from the orders Rodentia, Lagomorpha, and Testudines correspond to non-native species, while Squamata included one introduced specie (*Tarentola mauritanica*) and one native species (*Teira dugesii*). Finally, all prey items from the Passeriformes and Orthoptera orders were considered native species.

Diet characterization using DNA metabarcoding

We were able to amplify and sequence DNA material from all the 58 fecal samples collected. The libraries generated *ca.* 8 million raw sequence reads, which were reduced to 2,038.749 reads during the bioinformatic processing and to 109 OTUs. Non-target amplification from diverse sources was observed in samples, extractions, and PCR negative controls representing 50.35% of the total reads. Bacteria represented most of the non-target OTU diversity (44%). An expected amount of cat reads was also obtained, corresponding to a frequency of occurrence of 26.67%, and 9.87% belonged to human. After negative controls, singletons, replicates, and taxa filtering the cats' final diet consisted in 910517 reads and 18 OTUs.

The 18 vertebrate prey OTUs identified belong to four classes, nine orders, 12 families, 14 genera, and 13 species (Fig. 2; Supplementary Data SD1). Mammalia was the predominant class (98.28%), followed by Aves (56.90%), Reptilia (34.48%), and Actinopteri (8.62%). Among the orders detected, Rodentia (94.83%) was the most prevalent, followed by Passeriformes (37.93%), Galliformes (32.76%), Lagomorpha (25.86%), Artiodactyla (24.14%), Testudines (18.97%), Squamata (17.24%), Scombriformes (8.62%), and Anseriformes (3.45%). However,

it is important to note that several of the detected prey items were derived from anthropogenic sources. Consequently, when excluding the taxa associated with them, a total of just 13 wild preys taxa were detected, resulting in a total of 179 occurrences of wild preys (Table 1). Anthropogenic food was present in 37.93% of the scat samples, while wild prey items were present in 98.28% of the scats, just only one sample did not contain wild prey items. Native prey species were present in 43.86% of the samples, whereas non-native species were identified in all the samples. Not surprisingly, no invertebrates were detected in this analysis since the 12SV5 primers are specific for vertebrate DNA.

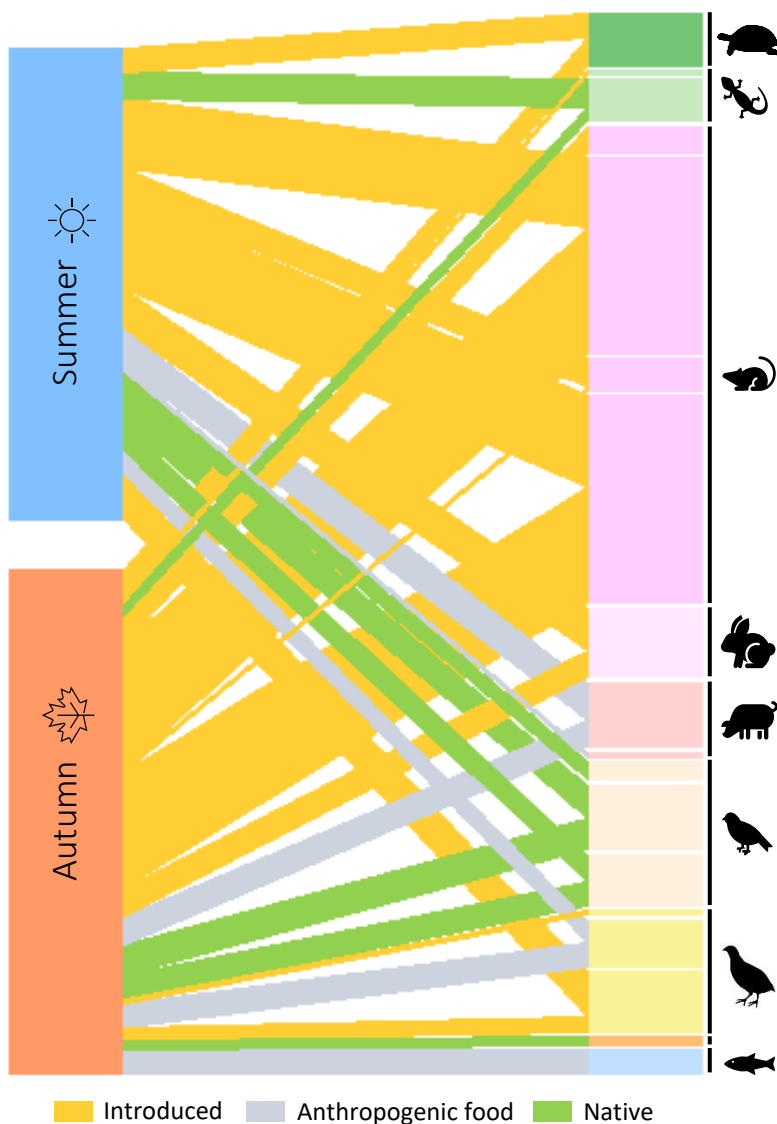**Figure 2:** Foodweb displaying the Operational Taxonomic Units (OTUs) consumed by *Felis catus* in the Ecological Park of Funchal on Madeira Island, Portugal. Link width between seasons (summer, above; autumn, below) and their prey detection is proportional to its frequency of occurrence in scats samples. White lines separate the different OTUs and various colors indicate different orders.

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When comparing the average prey diversity per sample between diet types, it was higher for wild prey than for anthropogenic food (see Supplementary Data SD2), and these differences were statistically significant for all taxonomic levels analysed (order: $p < 0.05$; family: $p < 0.05$; OTU: $p < 0.01$). Results from the linear regression show no relationship between the amount of consumed wild and anthropogenic food items ($R^2 = 0.05$, $p = 0.08$).

Diet characterization using morphological analyses

Through the morphological analyses, a total of 58 fecal samples were examined and remains were detected in 57 of them. We identified 8 prey taxa (Table 1) from four classes, six orders, four families, five genera and four species. The class Mammalia was the most prevalent with a FO of the 85.96%, followed by Reptilia (45.61%), Insecta (36.84%) and Aves (33.33%). Among the orders, Rodentia was the most frequently detected (80.70%), followed by Squamata (45.61%), Orthoptera (36.84%), Passeriformes (31.58%), Lagomorpha (10.53%) and non-Passeriformes (3.51%). In our analysis, we employed the term "non-Passeriformes" to collectively represent all avian remains that did not fall within the passerine category, as our methodology allowed only these two distinct classifications. Additionally, we considered that identified non-Passeriformes prey related to introduced species, as non-native partridges are the most abundant and, due to its behaviour, more likely to be preyed non-passerine in our study area. From the taxonomic family level and below, prey information is more limited (Supplementary Data SD3), due to the impossibility of reaching a lower taxonomic level based on the remains of the preys. However, at the family level taxonomy, Muridae was the most frequently consumed family, followed by Lacertidae, Leporidae, and Muscicapidae, the latter being detected only once (Table 1). At the genus level, we could discern only five taxa, with *Mus* sp. emerging as the most predominant, succeeded by *Rattus* sp., *Teira* sp., *Oryctolagus* sp. and *Erithacus* sp., the latter also having only one detection. In this approach, native prey species were identified in 77.19% of the samples, whereas non-native species were present in 85.96% of the scats. However, there were samples containing two or more non-native or native taxa, leading to 71 occurrences of non-native prey and 65 occurrences of native prey.

Table 1: Results of the diet of free-ranging cats (*Felis catus*) on Ecological Park of Funchal through molecular(n=57) and morphological analyses (n=57). N – Number of occurrences of a particular prey category; FO – frequency of occurrence in total (%); S - frequency of occurrence in summer (%); A – frequency of occurrence in autumn (%)

	Molecular Analyses (n=57)				Morphological Analyses (n=57)			
	N	FO	S	A	N	FO	S	A
MAMMALIA	115	96.49	42.11	54.39	69	85.96	33.33	52.63
Rodentia	100	96.49	42.11	54.39	63	80.70	29.82	50.88
Rattus sp.	-	-	-	-	30	52.63	14.04	38.60
Rattus sp.2	6	10.53	0	10.53	-	-	-	-
Rattus rattus	42	73.68	26.32	47.37	-	-	-	-
Rattus norvegicus	7	12.28	10.53	1.75	-	-	-	-
Mus musculus	45	78.95	35.09	43.86	33	57.89	24.56	33.33
Lagomorpha	15	26.32	15.79	10.53	6	10.53	3.51	7.02
Oryctolagus cuniculus	15	26.32	15.79	10.53	6	10.53	3.51	7.02
AVES	43	52.63	28.07	24.56	21	33.33	17.54	15.79
Passeriformes	29	38.60	19.30	19.30	18	31.58	15.79	15.79
Sylvia sp.1	4	7.02	7.02	0	-	-	-	-
Sylvia atricapilla	14	24.56	12.28	12.28	-	-	-	-
Erithacus rubecula	11	19.30	10.53	8.77	1	1.75	0	1.75
Non Passeriformes	-	-	-	-	2	3.51	3.51	0
Galliformes	14	24.56	17.54	7.02	-	-	-	-
Phasianidae 1	1	1.75	0	1.75	-	-	-	-
Alectoris rufa	13	22.81	17.54	5.26	-	-	-	-
REPTLIA	21	35.09	19.30	15.79	26	45.61	28.07	17.54
Squamata	10	17.54	10.53	7.02	26	45.61	28.07	17.54
Teira dugesii	9	15.79	10.53	5.26	26	45.61	28.07	17.54
Tarentola mauritanica	1	1.75	0	1.75	-	-	-	-
Testudines	11	19.30	8.77	10.53	-	-	-	-
Pelusios castaneus	11	19.30	8.77	10.53	-	-	-	-
INSECTA	-	-	-	-	21	36.84	12.28	15.79
Orthoptera	-	-	-	-	21	36.84	12.28	15.79
TOTAL	179				137			

Seasonal variation in the richness and composition of cat diet

The average prey diversity per sample, in the molecular analyses, was slightly higher for summer than for autumn (see Supplementary Data SD4a). However, these differences were not statistically significant for any of the taxonomic levels analysed (order: p = 0.098; family: p = 0.053; OTU: p = 0.154). The rarefaction curve suggests overlapping niches breadths in cat diet between the two seasons (Fig. 3). Furthermore, this analysis shows high sample coverage for both seasons (<95%), meaning that we have identified a large proportion of the available prey present in both seasons.

Figure 3: Rarefactions curves for seasons at different taxonomic levels in molecular analyses. Showing the observed (full line) and estimated (dashed line) richness, and respective 84% confidence interval by sample coverage.

Likewise, in the morphological analyses, the average prey diversity per sample was also slightly higher for summer than for autumn (see Supplementary Data SD4b), but differences were non-significant at any of the taxonomic levels analysed (order: p = 0.592; family: p = 0.829; OTU: p = 0.898). Regardless of the analysed taxonomic level, we detected substantial dietary niche overlap between both seasons (Fig. 4). Sample coverage for these analyses was also high (<95%).
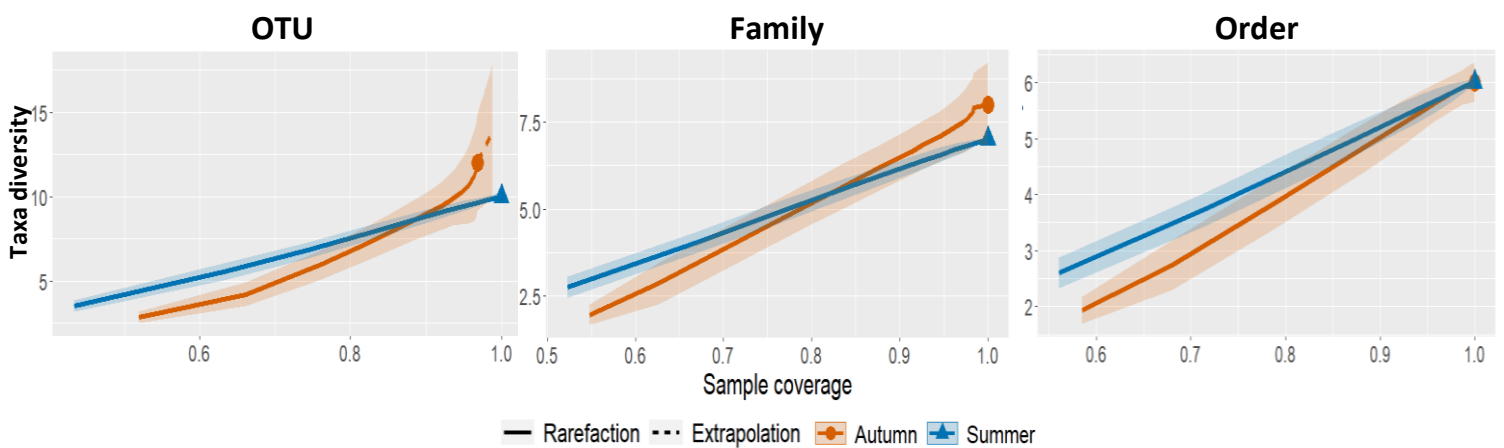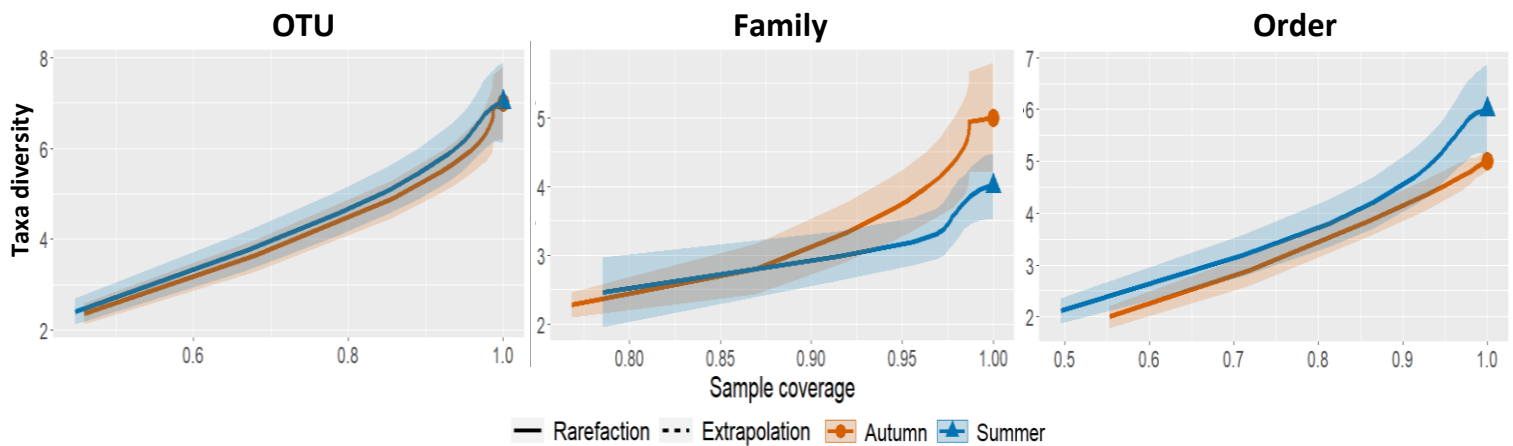
Figure 4: Rarefactions curves for seasons at different taxonomic levels in morphological analyses. Showing the observed (full line) and estimated (dashed line) richness, and respective 84% confidence interval by sample coverage.

Regarding diet composition, the molecular analyses suggest the existence of significant seasonal effects only at the OTU level (OTU: $p < 0.05$; family: $p = 0.109$; order: $p = 0.14$). However, the homogeneity of multivariate dispersion test suggested that this was due to a lack of homogeneity in group variances ($p < 0.05$). Simper analysis at the order level revealed a higher consumption of Galliformes during summer compared to autumn ($p < 0.01$; Fig. 5, Table 1). Moreover, Phasianidae detections were more frequent in sampled collected over the summer at the family level ($p < 0.05$). At the OTU level, differences between seasons were attributed to four OTUs. *Rattus rattus* ($p < 0.05$) was detected more frequently in autumn than in summer. In contrast, *Alectoris rufa* ($p < 0.01$) and *Rattus norvegicus* ($p < 0.05$) were detected more in summer than in autumn and *Sylvia* sp.1 was detected only in summer ($p < 0.05$).

As for the morphological analyses, significant seasonal effects on diet compositions were observed across the OTU, family, and order levels, as indicated by the PERMANOVA results (OTU: $p < 0.05$; family: $p < 0.01$; order: $p < 0.05$). Simper analysis at the order level revealed a higher consumption of Squamata during summer compared to autumn ($p < 0.01$; Fig. 5; Table 1). Additionally, Lacertidae were more frequent in summer samples at the family level ($p < 0.01$). Conversely, Muridae prevalence increased in autumn samples ($p < 0.01$). At the OTU level, *Teira dugesii* was consumed more in summer than in autumn ($p < 0.01$). Meanwhile, *Rattus* sp. was detected more frequently in the autumn than in summer ($p < 0.05$).

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Method-associated differences in the richness and composition of cat diet

More OTUs and families were detected using DNA metabarcoding than the morphological method. However, the two approaches have similar niche breadths at the order level (Fig. 5).
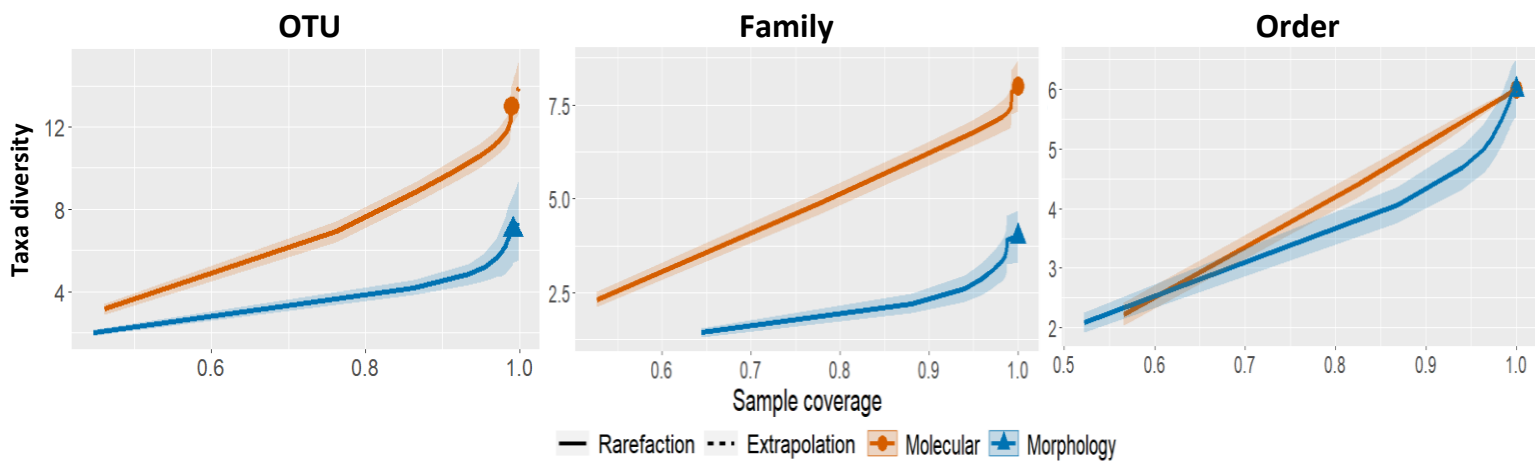
Figure 5: Rarefactions curves for the two methods at different taxonomic levels. Showing the observed (full line) and estimated (dashed line) richness, and respective 84% confidence interval by sample coverage.

Moreover, the type of prey detection method has a significant effect on diet composition at the order level ($p < 0.01$). Simper analysis at the order level revealed that composition differences between methods were due to five orders (Fig. 6; Table 1). Squamata was detected more frequently in morphological analyses than in molecular analyses ($p < 0.05$; 45.61% vs 17.54%, respectively), while the Rodentia was detected more frequently in molecular analyses than in morphological analyses ($p < 0.05$; 96.49% vs 80.70%, respectively). Testudines ($p < 0.01$) and Galliformes ($p < 0.001$) were detected in molecular analyses but were absent in morphological analyses, while Orthoptera was detected only in morphological analyses ($p < 0.001$).
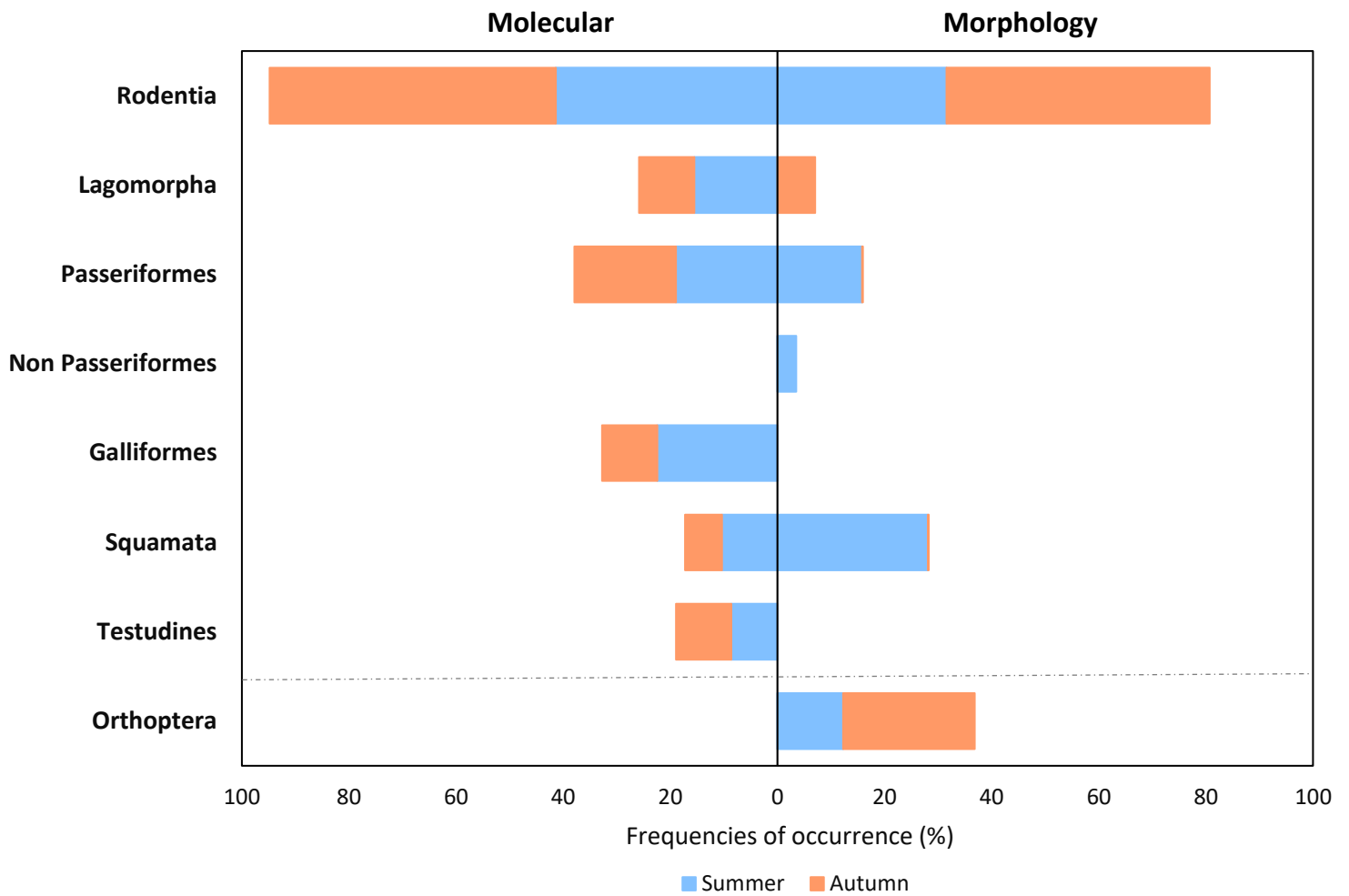
Figure 6: Orders identified using molecular (left) and morphological methods (right). Results from summer are represented in blue, and autumn results are in orange. Orthoptera is separated by the dashed line because it could not be detected using the molecular approach. For further details, see Table 1.

Discussion

This is the second study to use DNA metabarcoding to investigate the diet of free-ranging cats and the first to do so in insular ecosystems. Free-ranging cats exert significant adverse effects on insular wildlife, making it crucial to understand their diet for conservation efforts (Bonnaud *et al.* 2011). Despite this, studies investigating cat diet on island ecosystems are scarce, and the Madeira archipelago is no exception (but see Cook & Yalden 1980; Medina *et al.* 2010). Notably, existing studies for the Madeira archipelago are over 10 years old, and, while one encompassed two distinct locations on Madeira Island (Pico do Areeiro and Paul da Serra; Medina *et al.* 2010), the second one was carried out in the remote Desertas Islands (Cook & Yalden 1980). Here, we investigated the diet of free-ranging cats in the Ecological Park of Funchal, a peri-urban protected area with a high density of adult free-ranging cats (estimated 36 adult individuals, in an area of *ca*. 7.5 km²; Soto *et al.* 2023). We identified 19 distinct prey taxa, among which, five were linked to anthropogenic food sources, five were native species, and the remainder correspond to non-native taxa.

Previous studies have highlighted the precision of metabarcoding in prey identification, indicating its potential to increase the number of identifiable prey taxa (Zarzoso-Lacoste *et al.* 2016; Dawson *et al.* 2020; Gil *et al.* 2020; Massey *et al.* 2021). This was demonstrated in our research, where molecular analysis yielded a higher number of taxa at both the species and genus levels, compared to morphological analysis (Table 1). This enabled the identification of consumed taxa not identified in the previous cat diet study in Madeira (Medina *et al.* 2010), nor in our morphological analysis (e.g., taxa associated with anthropogenic food sources). This is due to the inability of morphological analysis to identify food items that do not leave durable remnants, such as pet food or leftovers composed solely of soft tissues (Forin-Wiart *et al.* 2018). These findings suggest that certain free-ranging cats in this area have access to pet food or food leftovers of anthropogenic origin. Moreover, even when excluding taxa related to anthropogenic food, our results demonstrated significantly higher diversity of consumed taxa determined through molecular techniques (at the OTU and family levels, although not at the order level), compared to results based solely on morphological analyses. This disparity is mostly due to fewer taxa being identified at the species and genus levels through the morphological method, which only allowed for the identification of most prey at the order level. This was especially true for certain groups such

as Passeriformes, non-Passeriformes, and Orthoptera. It is noteworthy that the identification of species or genera using the morphological approach was mainly possible due to the limited diversity of vertebrate species within specific orders on Madeira Island (Borges *et al.* 2008). This is exemplified by species such as *Mus musculus*, *Oryctolagus cuniculus*, or *Teira dugesii*, which represent the sole representatives of their respective genera (Borges *et al.* 2008).

As previously mentioned, our observations indicated that cats in our study area primarily feed on wild prey. Despite occasional consumption of anthropogenic food, our results did not reveal any significant correlation between the consumption of anthropogenic food and wild prey, suggesting that the intake of pet food or leftovers does not influence the consumption of wild prey. This aligns with similar patterns documented in several studies (Hernandez *et al.* 2018; Cecchetti *et al.* 2021; Plimpton *et al.* 2021). Furthermore, Soto *et al.* (2023) suggested that free-ranging cats in our study area do not heavily rely on anthropogenic food resources and might avoid human interaction. This avoidance behaviour is supported by the observation of higher activity levels among cats in areas of the park away from human resource subsidies (Soto *et al.* 2023).

Also, we did not observe differences in prey diversity per sample across the analysed taxonomic levels between seasons. There were overlapping niche breadths in the cat diet during two seasons, likely due to the predation of most taxa during both seasons (Table 1). Upon analysing compositions, no significant differences were found. However, minor variations in the consumption of specific taxa were noted between summer and autumn. *Alectoris rufa*, *Teira dugesii* and *Rattus norvegicus* were more prevalent in the summer, and the OTU Sylvia sp.1 was exclusively detected during this season, while *Rattus rattus* and *Mus musculus* were more frequently consumed in autumn. This dietary pattern can be attributed to the opportunistic and generalist nature of cats, allowing them to consume a diverse range of prey based on availability and abundance in their environment, a phenomenon well supported by previous studies (Fitzgerald & Turner 2000; Nogales & Medina 2009; Hervías *et al.* 2014; Széles *et al.* 2018; Plimptom *et al.* 2021; Ravelo & Reyes 2021). During periods of reduced small mammal populations or bird nesting seasons, cats might alter their prey preference from small mammals to birds or lizards (Fitzgerald 1988; Peck *et al.* 2008; Széles *et al.* 2018). This phenomenon can explain the prevalence of *Teira dugesii* and *Alectoris rufa* in the summer samples, or the slight increase in the frequency of occurrence of *Erithaculus rubecula.*

Furthermore, this aligns with previous studies, suggesting that the presence of introduced species adapted to cat predation (with high population growth and effective anti-predator strategies) can promote an increase in cat numbers and exacerbate their impact on native prey species through hyperpredation (Fig. 7; Courchamp *et al.* 1999; Ringler *et al.* 2015).

Figure 7: Cats photographed at the Ecological Park of Funchal. Photo (a) shows cats carrying, respectively, an endemic Madeira Wall lizard (*Teira dugesii*) and a rodent (*Rattus* sp.). Photos by Elena J. Soto.

Comparing diet compositions between methods, we found significant differences. These differences were primarily attributed to five orders: Rodentia, Squamata, Testudines, Galliformes, and Orthoptera (Table 1; Fig. 6). In the case of Rodentia, there were slight differences, but this order had the highest frequency of occurrence in both methods. In molecular analyses, this value was higher. However, this approach was limited to establishing the presence or absence of vertebrate prey in each fecal sample. Relying solely on presence or absence data can underestimate the importance of given prey by assuming that each occurrence represents a single predation event. Conversely, it can also overestimate its relevance if DNA from the same predation event is detected in multiple fecal samples (Plimptom *et al.* 2021). Conversely, Squamata was more frequently detected in morphological analyses compared to molecular analyses. Despite these differences in frequency of occurrence between methods, molecular analysis alone underestimated the predation of *Teira dugesii,* the only species of this order identified in the morphological approach. Yet, although molecular analyses seem to have underestimated the predation on this species, it was the only method to detect the consumption of another Squamata, the introduced *Tarentola mauritanica*, whose population in Madeira has been expanding since the mid-1980s (Silva-Rocha *et al.* 2022) and of the order Galliformes. This, alongside the limitations of

molecular analyses in the quantifications of the number of individual preys, highlight the benefits associated with using both methods (Gil *et al.* 2020; Plimptom *et al.* 2021). On the point of prey quantification, during sample collection, a cat vomit containing at least five adult Madeira wall lizards and a domestic mouse was observed, highlighting that free-ranging cats can consume multiple vertebrates, in a single meal (Fig. 8). Furthermore, several jawbones of Madeira wall lizards were found in the same scat, emphasizing the importance of complementing both methodologies to effectively analyse the impact of cats on native populations.

Figure 8: Cat vomit containing at least five adult Madeira Wall lizards (*Teira dugesii*), photographed in August 2021 in the Ecological Park of Funchal, Madeira Island. Photo by Elena J. Soto.

Employing morphological methods, we were limited to two distinct classifications of birds: Passeriformes and non-Passeriformes. This limitation arose due to the impossibility of reaching a lower taxonomic level based on the remains of the prey and the of distinctive identifying features. This restricted accuracy in identification, mainly limited to order level, was mostly due to the challenge of discerning unique diagnostic traits among a multitude of species that exhibit morphological similarities (Zarzoso-Lacoste *et al.* 2016). In contrast, the order Orthoptera was only detected in morphological analysis. This result was anticipated because the 12SV5 primers used in molecular analysis are specific for vertebrates (Riaz *et al.* 2011; Plimptom *et al.* 2021; McLennan *et al.* 2022).

An intriguing finding from our molecular analysis was the detection of *Pelusios castaneus* in multiple samples (Table 1). This terrapin is not known to have any established

populations in Madeira (Borges *et al.* 2008). However, the species has multiple non-native populations in oceanic islands (Guedes *et al.* 2023) and it is kept as a pet in Madeira, with at least one individual already been found in the wild (*pers. comm*). The samples containing *P. castaneus* were found near water bodies; however, this may not be a significant factor since cats can easily cover distances exceeding 1 km (Turner & Bateson 2000). A more plausible hypothesis, which we consider to be the most valid, is that some cats might have access to water used by pet *P. castaneus*, as at least one house surrounding our study area contains a pound with several terrapins.

Notwithstanding some key differences, our findings generally align with the previous study analysing the diet of free-ranging cats in Madeira (Medina *et al.* 2010). The most frequently detected group in our samples was non-native mammals, with an occurrence frequency of 98%, followed by birds and reptiles, consistent with the patterns observed by Medina *et al.* (2010) and in studies conducted in the Canary Islands and Azores (Nogales & Medina, 2009; Hervías *et al.* 2014; Ravelo & Reyes 2021). Notably, our invertebrate data only yielded the order Orthoptera (Table 1), contrary to our expectations of detecting more invertebrate orders such as Coleoptera (Medina *et al.* 2007), due to the fact that morphological analyses tend to more easily detect hard-bodied groups like Coleoptera (Gil *et al.* 2020). However, our data aligns with Medina *et al.* 2010, where most invertebrate items were Orthoptera (108 prey items), and only six Coleoptera and one Hymenoptera were detected. Nevertheless, due to the absence of primers capable of detecting invertebrates in our work, we cannot compare the diversity and frequency of occurrence of this group between the two methodologies. Previous studies have shown that morphological methods tend to underestimate the frequency of occurrence of prey with fully digested parts, such as some soft-bodied arthropods like Zygentoma and Lepidoptera, which were only detected using DNA-based methods (Brown *et al.* 2012; Gil *et al.* 2020).

Consistent with the findings of Medina *et al.* (2010), we did not find any evidence of seabird predation. However, several studies have documented cat predation on seabirds in Madeira and other oceanic islands (including in Macaronesia), highlighting the significant negative impact of free-ranging cats on seabird populations (Cook & Yalden 1980; Zino *et al.* 2001; Medina *et al.* 2011; Hervía *et al.* 2013; Dias *et al.* 2019; Lamelas-López *et al.* 2021). In our case, the absence of seabirds as prey, specifically the Manx shearwater (*Puffinus puffinus*),

can be justified by the fact that sample collection did not occur during the nesting season or the time of fledging of this species (Nunes *et al.* 2010). This, combined with the reduced estimated populations size of this species in Madeira (Rodríguez *et al.* 2020), might explain their absence in our samples. According to Bonnaud *et al.* (2011), while breeding, seabirds can become the preferred prey item of insular cats. Indeed, the current low population of *P. puffinus* in Madeira might reflect the impacts of non-native mammals, including cats, which are likely to have been associated with island-level extirpation of *Puffinus* species (e.g., Boyd's shearwater *Puffinus lherminieri boydi* from Santa Luzia, Cabo Verde; Alho *et al.* 2022).

Conclusion

By employing both DNA metabarcoding and morphological analyses, this study advances the understanding of the free-ranging cat diet in oceanic islands. Our findings confirm that free-ranging cats consume a wide variety of prey, encompassing both native and non-native vertebrates, as well as anthropogenic food sources, validating our initial hypotheses. Interestingly, occasional consumption of anthropogenic food does not seem to significantly influence their predation on wild prey, suggesting that free-ranging cats in our study area do not heavily rely on human-provided food resources. Moreover, our hypothesis predicting potential differences in diet richness or composition between seasons was somewhat supported, as our study revealed nuanced seasonal variations in cat diet, with the Madeiran wall lizard and red-legged partridge being more prevalent in samples during the summer, as anticipated. During autumn, cats exhibited a greater reliance on non-native rodents such as domestic mice and black rats, emphasizing their opportunistic behaviour. This suggests that cats likely adjust their prey preferences based on the seasonal availability of different prey species.

Additionally, our research highlighted significant differences in the taxa detected between methods, as anticipated. Metabarcoding proved to be a method with superior taxonomic identification resolution, surpassing morphological techniques in identifying specific prey species and genera. This higher resolution enabled the detection of taxa associated with anthropogenic food, emphasizing the use of both human- and non-human-associated food sources by free-ranging cats. However, it was noted that at least for one prey species (the Madeira wall lizard), metabarcoding potentially underestimated cats' predation, highlighting the benefits of employing both molecular and morphological methods. Yet is it worth noting that the morphological method also underestimated predation of several taxa.

We recommend that future research exploring the diet of free-ranging cats uses primers for both vertebrates and invertebrates. This approach would certainly yield the identification of more prey species, especially considering the endemic-rich invertebrate fauna of Madeira (Borges *et al.* 2008). Furthermore, conducting a year-round study on the diet of this predator is crucial, aiming to obtain data for all four seasons and assess potential variations in both diversity and composition. Another pivotal aspect justifying the study

throughout the four seasons is the opportunity to investigate if Manx shearwaters and other seabirds are preyed upon by free-ranging cats during their nesting or fledgling periods.

Altogether, this study emphasis the benefits of adopting both molecular and morphological methods in diet studies, thus providing a more comprehensive understanding of trophic food webs. Furthermore, our study highlights that, while primarily preying on introduced rodents, free-ranging cats exert significant pressure on native species, including the endemic Madeira wall lizard. This stresses the importance of evaluating conservation impacts of free-ranging cats on endangered and geographically restricted species, especially considering the potential implications of hyperpredation processes associated with non-native prey.

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Supplementary material

Supplementary Data SD 1: List of 18 OTUs identified through molecular analyses of cat diet. The list includes details about their previous knowledge about their presence in Madeira and their colonization status on the island, according to Borges *et al.* (2008). Additionally, the percentage of scat samples in which each prey item was detected is provided. The abbreviation 'MAC' signifies Macaronesian endemic species/subspecies.

Class	Order	Family	OTU	Status	Frequencies of occurrence
Mammalia	Rodentia	Muridae	*Mus musculus*	Introduced	77,59%
			Rattus norvegicus	Introduced	12,07%
			Rattus Rattus	Introduced	72,41%
			Rattus sp.2	Introduced	10,34%
	Lagomorpha	Leporidae	*Oryctolagus cuniculus*	Introduced	25,86%
	Artiodactyla	Bovidae	*Bos* sp.1	Anthropogenic food	1,72%
		Suidae	*Sus scrofa*	Anthropogenic food	24,14%
Aves	Passeriformes	Muscicapidae	*Erithacus rubecula*	Native	18,97%
		Sylviidae	*Sylvia atricapilla heineken*	MAC	24,14%
			Sylvia sp.1	Native	6,90%
	Galliformes	Phasianidae	Phasianidae 1	Introduced	1,72%
			Alectoris rufa	Introduced	22,41%
			Gallus gallus	Anthropogenic food	17,24%
	Anseriformes	Anatidae	*Cairina moschata*	Anthropogenic food	3,45%
Reptilia	Squamata	Lacertidae	*Teira dugesii*	Endemic	15,52%
		Phyllodactylidae	*Tarentola mauritanica*	Introduced	1,72%
	Testudines	Pelomedusidae	*Pelusios castaneus*	Introduced	18,97%
Actinopteri	Scombriformes	Scombridae	*Thunnus* sp.1	Anthropogenic food	8,62%
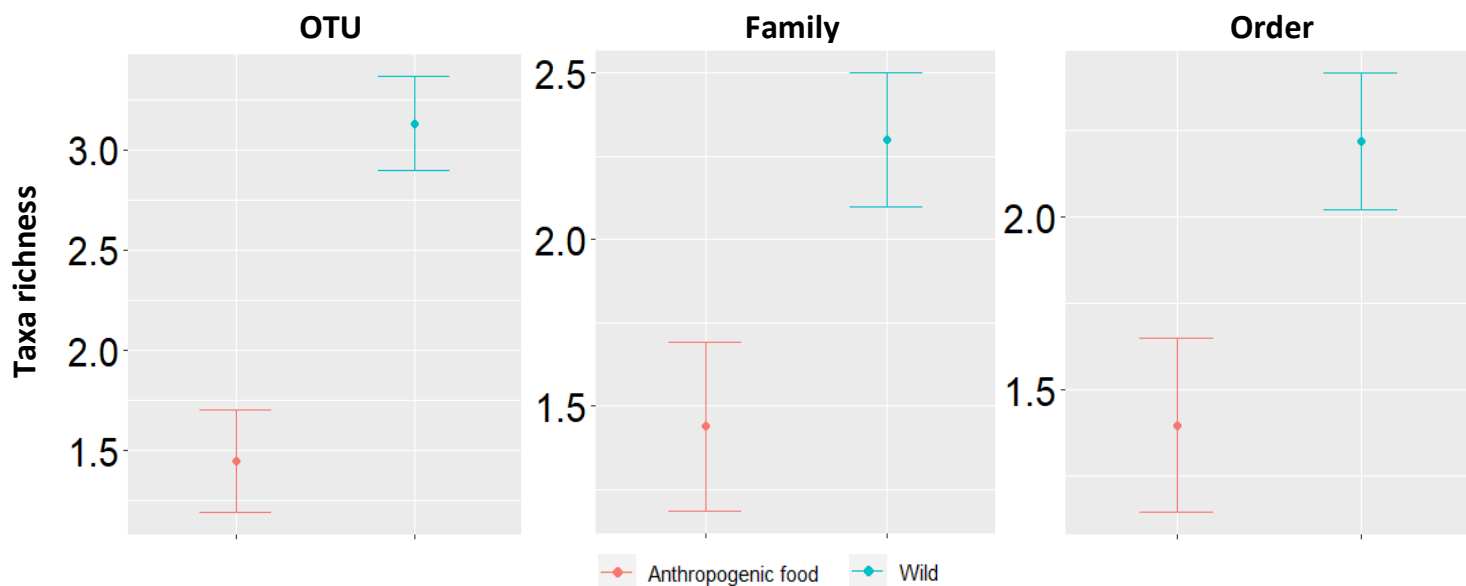
Supplementary Data SD 2: Modelled effect of diet type on the average prey richness per sample across various taxonomic levels.

Supplementary Data SD 3: List of 8 OTUs identified through morphological analyses of cat diet. The list includes details about their previous knowledge about their presence in Madeira and their colonization status on the island, according to Borges *et al.* (2008). Additionally, the percentage of scat samples in which each prey item was detected is provided.

Class	Order	Family	OTU	Status	Frequencies of occurrence
Mammalia	Rodentia	Muridae	*Mus musculus*	Introduced	57,89%
			Rattus sp.	Introduced	52,63%
	Lagomorpha	Leporidae	*Oryctolagus cuniculus*	Introduced	10,53%
Aves	Passeriformes	-	*Passeriformes*	Native	29,82%
		Muscicapidae	*Erithacus rubecula*	Native	1,75%
	non - Passeriformes	-	*Non - Passeriformes*	Introduced	3,51%
	Squamata	Lacertidae	*Teira dugesii*	Endemic	45,61%
Insecta	Orthoptera	-	Orthoptera	Native	36,84%
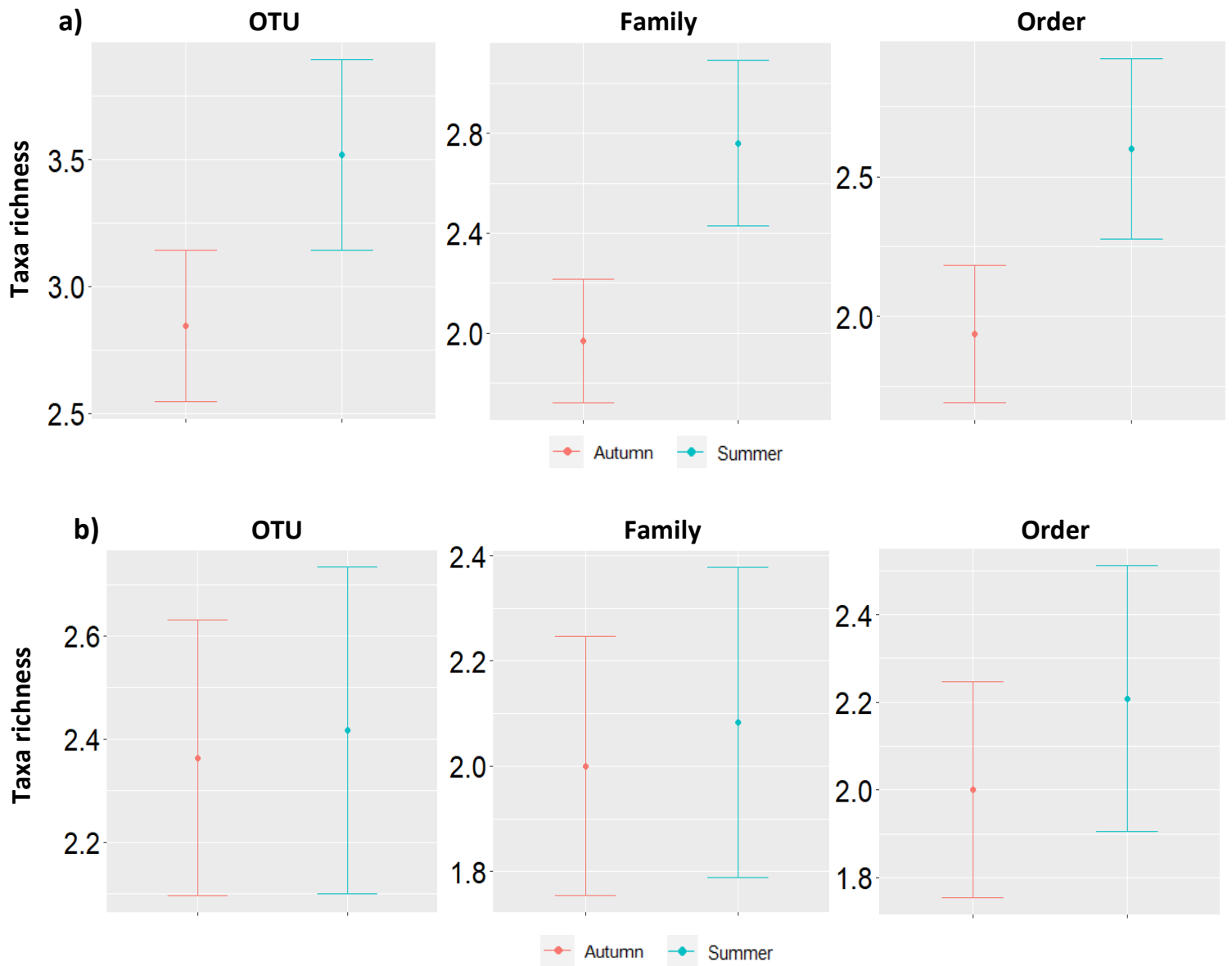
Supplementary Data SD 4: Modelled effect of seasons on the average prey richness per sample at different taxonomic levels in a) molecular analyses b) morphological analyses