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Assessing mesocarnivores trophic niche using genetic identification of faecal samples

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Resumo

A competição entre predadores é um dos principais fatores estruturantes das comunidades e desempenha um papel fundamental no funcionamento e equilíbrio dos ecossistemas. O Princípio da Exclusão Competitiva pressupõe que espécies com nichos ecológicos idênticos não podem coexistir. No entanto, em sistemas de montado, é possível observar simpatria entre várias espécies de mesocarnívoros indicando uma coexistência estável, que possivelmente resulta da diferenciação entre as mesmas a nível espacial, temporal e/ou trófico. De modo a conseguir garantir a continuidade deste equilíbrio é importante entender estes mecanismos. Para mais, mamíferos carnívoros têm um papel fundamental no controlo das comunidades em que se inserem devido ao seu papel como predadores de topo. A sua conservação é de extrema importância, uma vez que tem uma influência direta e indireta em todo o ecossistema.

A recolha de dejetos é um método amplamente utilizado no estudo de mesocarnívoros. Este método permite obter uma grande diversidade de dados, é não-invasivo e apresenta custos baixos. No entanto, uma das maiores dificuldades na aplicabilidade deste método reside na identificação dos dejetos. A identificação das amostras é comumente feita utilizando características morfológicas das mesmas. No caso de as amostras fecais não serem atribuídas corretamente à espécie que as produziu, podem direcionar o investigador a conclusões erróneas.

Este estudo tem como principal objetivo avaliar o nicho trófico entre espécies de mesocarnívoros, em duas áreas de montado em Portugal (Companhia das Lezírias e Serra de Grândola), e qual a importância do recurso a métodos genéticos de identificação de dejetos. Desta forma, foi também possível avaliar o efeito da sazonalidade e do local na dieta destas espécies e possíveis fatores que podem levar à incorreta identificação de amostras fecais.

No total foram recolhidas 442 amostras inicialmente identificadas, recorrendo a métricas morfológicas, como pertencendo a *Vulpes vulpes*, *Mustela nivalis*, *Martes foina*, *Meles meles*, *Genetta genetta* e *Herpestes ichneumon*. De forma a confirmar a identidade das amostras, no laboratório foi realizada uma extração de ADN e o recurso a técnicas de PCR e a *primers* específicos para cada espécie. Posteriormente para cada amostra foi realizada a separação e identificação dos recursos consumidos. De forma a avaliar a dieta de cada espécie, apenas foram utilizados os dados provenientes das amostras identificadas geneticamente, tendo sido calculada a frequência de ocorrência de cada categoria de alimento. De modo a identificar a possível influência do conteúdo dos dejetos na sua correta identificação morfológica, foram utilizados dados relativamente à proporção da composição total do dejeto de cada categoria alimentar. Para avaliar a amplitude do nicho e a diversidade da dieta, foram calculados os índices de Levins e de Shannon-Wiener, e ainda o índice de Pianka de forma a avaliar a sobreposição de nichos.

Apenas cerca de 75% das amostras foram passíveis de ser identificadas geneticamente, registando-se uma clara distinção no sucesso da identificação genética entre locais e estações do ano. A dificuldade da identificação destas amostras residiu em particular nas amostras recolhidas na Serra de Grândola e durante a estação seca, possivelmente devido a condições meteorológicas adversas que podem ter levado à redução da quantidade e integridade do ADN presente nos dejetos.

Aproximadamente 65% das amostras foram corretamente identificadas morfologicamente, o que vai de acordo ao sucesso obtido em outros estudos. Os maiores erros na identificação dos dejetos ocorreram com dejetos de *M. foina,* em que cerca de 20% das amostras foram erroneamente identificados como pertencendo a *M. nivalis*. Dejetos produzidos por indivíduos juvenis de *M. foina* podem ter sido facilmente confundidos como pertencendo a *M. nivalis*, causando possivelmente este erro elevado. Efetivamente, verificou-se que os maiores erros de identificação foram entre espécies mais próximas filogeneticamente, que produzem dejetos morfologicamente semelhante. De modo a avaliar se o conteúdo dos dejetos poderia influenciar a identificação morfológica dos mesmos, tentou-se comparar se dejetos que apresentassem um maior conteúdo de fruta seriam mais facilmente confundidos. No entanto, os dados não apresentaram qualquer evidência de que este fosse um fator de confusão.

Ao comparar os resultados obtidos da dieta das diferentes espécies de acordo com os diferentes métodos de identificação não foram identificadas diferenças estatisticamente significativas entre ambos. A falta de evidência estatística pode estar relacionada com o facto de as espécies em estudo apresentarem grandes semelhanças nas suas dietas, pelo que o efeito dos dejetos mal identificados pode não ser detetado. Contudo, ao visualizar os dados, é possível identificar várias situações em que a utilização exclusiva do método de identificação morfológica das amostras teria induzido em conclusões erróneas. De notar que como resultado da identificação genética dos dejetos, não foi possível realizar qualquer tipo de conclusão em relação à dieta de *M. nivalis*. Além disso, algumas irregularidades foram encontradas em relação ao consumo de répteis, onde seria concluído que esta categoria alimentar teria uma maior importância para outras espécies do que para *H. ichneumon*. Tendo isto em conta, torna-se possível identificar a importância da utilização de métodos cada vez mais precisos de forma a ser possível obter conhecimento mais fidedigno em relação a estas espécies. De outra forma, poderão ser retiradas conclusões erradas que poderão levar à criação de medidas de conservação ineficientes.

Em relação à avaliação do nicho trófico de cada espécie, houve alguns fatores que impediram uma comparação mais abrangente das possíveis diferenças nas dietas destes mesocarnívoros entre estações e locais devido ao número reduzido de amostras de *H. ichneumon*, *G. genetta* e *M. meles* provenientes da Serra de Grândola. Contudo, as diferenças encontradas foram superiores entre estações do ano do que entre locais, indicando uma maior influência da sazonalidade dos recursos na dieta destas espécies por as mesmas serem generalistas. Os recursos que demonstraram ser consumidos com maior frequência foram insetos, fruta e mamíferos, por esta ordem. Era esperado uma maior frequência de mamíferos na dieta destas espécies, mas tal não foi verificado.

Todas as espécies apresentaram dietas com um nível de diversidade intermédio e amplitudes relativamente reduzidas, o que não seria esperado devido ao carácter generalista destas espécies. No entanto, estes resultados podem estar relacionados com o facto de as categorias alimentares definidas serem demasiado gerais e a diferenciação ocorrer a níveis mais específicos. A sobreposição de nichos foi bastante elevada entre os diferentes pares de espécies podendo igualmente estar relacionada com a amplitude das categorias alimentares. Os mecanismos que permitem a coexistência destas cinco espécies de mesocarnívoros podem, contudo, não se verificar a nível trófico, devendo ser também avaliados a nível espacial e temporal. Porém, o par de espécies que demonstrou uma maior diferenciação alimentar, e que se demonstrou consistente entre estações, foi *G. genetta* e *M. foina*. Sendo ambas as espécies arborícolas e de tamanhos semelhantes, seria esperado uma maior sobreposição dos seus nichos tróficos. Isto indica que estas espécies aparentam de facto ter desenvolvido especializações a nível trófico que lhes permitem coabitar um mesmo local.

Os resultados obtidos neste estudo permitem perceber a importância do uso de métodos de identificação genética de amostras fecais em vez da utilização do método tradicional de identificação morfológica. Devido ao aumento das pressões antropogénicas e das ameaças que estas representam para a biodiversidade, é necessário obter informação rigorosa de modo que seja possível definir medidas de conservação que permitam garantir a preservação das espécies e a estabilidade das comunidades. O método de identificação genética de amostras fecais demonstra ser um método não invasivo que permite obter um diverso leque de informações e que contribuiu de forma significativa para o aumento do conhecimento sobre os mesocarnívoros.

Palavras-chave: Coexistência, dejetos, identificação genética, dieta, montado

Abstract

Trophic niche studies are of great importance as they facilitate the acquisition of information regarding the mechanisms that enable the coexistence of biologically similar species. One of the most employed methods in the study of mesocarnivores is the collection of faeces. However, misidentification can result in erroneous conclusions. The development of genetic identification methods enables investigators to obtain more accurate results. This study aims to demonstrate the importance of utilising scats' genetic identification to evaluate the trophic niche of different mesocarnivore species, in order to ascertain how they vary between seasons and between two *Montado* landscapes. Of the 442 samples collected, morphologically assigned to *Vulpes vulpes*, *Mustela nivalis*, *Martes foina*, *Meles meles*, *Genetta genetta*, and *Herpestes ichneumon*, only ≈75% were successfully genetically identified using PCR techniques and specific *primers*. Approximately 65% of all samples were correctly identified. The most significant discrepancy identified in the morphological identification of the scats was between *M. foina* and *M. nivalis,* two taxonomically related species. A comparison of the dietary results, obtained while comparing the two different identification methods, revealed no statistically significant differences. However, some of the data could lead to erroneous conclusions. The trophic niche evaluations were constrained by the limited number of samples from *M. meles*, *G. genetta*, and *H. ichneumon*. All species exhibited narrower trophic niches with intermediate levels of diversity in the consumed resources, which is likely attributable to the broad categorisation of food resources. All pairs of species exhibited considerable trophic niche overlap, with the exception of *M. foina* and *G. genetta*. This study effectively demonstrated the value of employing genetic techniques to identify scats, establishing a foundation for future diet studies of these species. However, additional research may be necessary to gain a more comprehensive understanding of the coexistence mechanisms.

Keywords: Coexistence, scats, diet, genetic species identification, *Montado*.

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List of abbreviations

CL – Companhia das Lezírias

DNA – Deoxyribonucleic acid

FO – Frequence of occurrence

FS – *Felis sp.*

GG – *Genetta genetta*

HI – *Herpestes ichneumon*

MF – *Martes foina*

MN – *Martes nivalis*

PCR – Polymerase chain reaction

SG – Serra de Grândola

VV – *Vulpes vulpes*

1. Introduction

1.1. Interspecific interactions and coexistence between mammalian carnivores

Interspecific interactions are known to play a significant role in shaping communities. These interactions can have various origins, benefiting some species while negatively impacting others, benefiting both, negatively impacting both, or having no effect on one of the parties. Competition among predators is one of the most studied interspecific interactions and a key driver in the ecological and demographic dynamics within communities (Grassel et al., 2015; Satgé et al., 2017).

Competition can be classified as either exploitative (indirect competition) or interference (direct competition) (Schoener, 1983; Linnell & Strand, 2000). According to Schoener (1983), exploitative competition occurs when some individuals deprive others of the benefits of some resources that they both consume. Interference competition usually occurs when individuals physically restrict the access to resources by aggressive behaviors (Case & Gilpin, 1974). This can lead to intraguild predation and result in the death of one of the parties involved (Linnell & Strand, 2000).

The *Competitive Exclusion Principle* states that "complete competitors cannot coexist" (Hardin, 1960). However, coexistence between sympatric and phylogenetic related and/or morphological/ecological similar species is often observed (Ferreiro-Arias et al., 2021). To better understand which factors drive this coexistence to occur, it is necessary to examine possible differences in spatial, temporal, and/or trophic niches (Pauli et al. 2022).

Carnivores (i.e. mammalian carnivores) play a crucial role in community maintenance given their status as apex predators. The removal or extinction of these predators can trigger cascading events, affecting mesopredators (mid-ranking predators) of the food web, herbivores, their competitors, and plants (Prugh et al., 2009; Dickman et al., 2019; Müller et al., 2022).

The density of one competitor species can increase when the density of another competitor species decrease due to the reduction of exploitative and interference competition effects (Dickman et al., 2019), relieving some of the competitive pressure and allowing a greater forage of the resources. The *Mesopredator Release Hypothesis* (Soulé et al., 1988) states that the decline or absence of an apex predator can cause an expansion in the density of a mesopredator due to the lack of predation (Prugh et al. 2009). Ultimately, the combination of these two factors has a negative impact on prey populations in the form of "trophic cascades" (Dickman et al., 2019). Additionally, the absence of apex predators can modify the behaviour and distribution of both competitors and prey species, creating, in this case, an "ecological cascade" (Dickman et al., 2019).

These predators also exert a direct influence on herbivore species, which in turn gives rise to an indirect influence on plant species (Schmitz et al., 2000). The potential for an increase in the number of herbivores, as a consequence of the absence of control exerted by the top predators, results in an increase in plant damage and a decrease in plant biomass and reproduction (Schmitz et al., 2000). This can ultimately lead to an increase in change in the ecosystem.

Considering all these factors, the conservation and equilibrium of ecosystems are contingent upon the preservation of mammalian carnivore species. In order to facilitate the enhancement of the efficacy of conservation initiatives for these species, it is imperative to increase the existing knowledge and continuous monitoring.

1.2. Difficulties in the study of ecology and trophic interactions of carnivores

Studying and monitoring a carnivore population presents several challenges. Carnivore mammals are characterized by low population densities, cryptic and mostly nocturnal activity patterns, and large home ranges (Gese, 2001; Wilson & Delahay, 2001). They often are also difficult to locate due to their habitat, which can comprise dense vegetation, and/or because inhabit remote places difficult to access (Gese, 2001; Wilson & Delahay, 2001). Furthermore, the use of invasive methods, such as those requiring the catching or handling of individuals, can result in undue stress and may also result in a small sample size due to the necessity of allocating significant resources and overcoming logistical challenges (Kelly et al., 2012). In light of these considerations, it is clear the necessity of investing in non-invasive methodologies.

There are several non-invasive methods that can be employed to study mesocarnivore (i.e. small to midsized carnivore species) populations (Kelly et al., 2012). The most commonly used is the collection and analysis of the faecal matter (Klare et al., 2011), which allows the investigator to obtain information about diet, parasites, population size, sexual structure, habitat use, and home range, among others (Putman, 1984; Gompper et al., 2003). Scats are composed of non-digested parts of consumed resources, such as bones, hair, feathers, scales, seeds, etc. This allows for the reconstruction of the dietary niche of the animal in question. Furthermore, scat analysis does not require the utilization of significant resources and represents a highly cost-effective approach (Sadlier et al., 2004). Although being an advantageous technique, it does however present important limitations.

1.3. Scats morphological and genetic identification

There is a high error rate associated with the morphological identification of scats in communities comprising sympatric and biologically similar mesocarnivores (Kelly et al., 2012; Monterroso et al., 2012; Alexandre et al., 2020; Descalzo et al., 2021). Faeces are typically identified based on their size, shape, and placement patterns, which can be different from species to species (Chame, 2003; Descalzo et al., 2021). When different species exhibit similar morphology, habitat preferences and behaviour, scats may be difficult to distinguish, as they may appear similar to each other (Monterroso et al., 2012).

As previously stated, the disappearance of apex predators can result in the release of mesopredators (Prugh et al., 2009). The Iberian Peninsula is an example of this situation, which led to the formation of carnivore communities composed by several ecologically similar mesocarnivores (Jiménez et al., 2019). This factor raises the question of the reliability of the success of the morphological identification of faeces. There is also some evidence that seasonality can also influence the identification success, as it can alter the shape and quality of the scat (Sadlier et al., 2004; Monterroso et al., 2012). The Iberian Peninsula, under the influence of a Mediterranean climate, is characterised by seasonal shifts in the availability of food resources. During the dry season, there is a greater availability of resources, while during the wet season, these resources are more limited, which can lead to a higher trophic niche overlap and, consequently, an increased error rate in the morphological identification of faeces (Monterroso et al., 2012; Alexandre et al., 2020).

In addition to morphological identification, genetic identification techniques offer an alternative approach to obtain more accurate results for researchers. As these molecular techniques become more prevalent, several studies indicate that misidentifications may occur during fieldwork (Davison et al., 2002; Monterroso et al., 2012; Alexandre et al., 2020; Descalzo et al., 2021). Although molecular analysis are becoming increasingly accessible and cost-effective, these are still employed in a relatively limited number of studies (Monterroso et al., 2019).

The surface of scats contains epithelial cells derived from the intestinal lining of the individual from which the scat originated (Kelly et al., 2012). The DNA from these cells can be extracted and then amplified, through polymerase chain reaction (PCR) techniques, allowing the species from which the scat belongs to be identified. The identification in question can be achieved through the use of either mitochondrial DNA or nuclear DNA. However, mitochondrial DNA does not undergo recombination and is present in larger copies within cells than the nuclear genome, which only presents one copy per cell (Kohn et al., 1995; Reed et al., 1997). This makes mitochondrial DNA a more suitable choice for achieving a higher resolution (Barbosa et al., 2013).

This methodology enables researchers to obtain different types of data pertaining to the individual responsible for the faecal matter. It is possible to identify the individual, their species, their sex, their kinship, and even any pathologies that may be present (Kohn et al., 1995; Reed et al., 1997). Also, the feeding habits of an organism can be identified by genetically analyzing the components of its faeces (Monterroso et al., 2019). The identification results obtained through this methodology can be considered as absolutes (Foran et al., 1997).

Nevertheless, it is important to acknowledge that molecular identification also has its own inherent limitations. One of the major challenges that this technique can present is a low DNA extraction success (Reed et al., 1997). Due to the ease with which faecal samples can be degraded, the quantity and quality of the DNA present is often insufficient for analysis and older samples can be particularly challenging to amplify (Kohn et al., 1995; Reed et al., 1997). Additionally, the epithelial cells from which the DNA is extracted are not evenly distributed along the scat, which may necessitate the repetition of extractions until a sufficient number of cells can be collected (Kohn et al., 1995). Meteorological conditions can influence the success of identifications, particularly during wet seasons. Rain can wash away DNA from the scat, and older scats may appear fresher due to the effects of precipitation (Foran et al., 1997).

1.4. Mesocarnivore populations in the *Montado* landscapes in Portugal

Montado areas in Portugal represent a distinctive habitat, comprising a diverse array of 10 mesocarnivore species (*Vulpes vulpes, Mustela nivalis, Mustela putorius, Martes foina, Meles meles, Lutra lutra, Genetta genetta, Herpestes ichneumon, Felis silvestris,* and *Lynx pardinus*).

This community is composed of species that exhibit differences in habitat preferences, food habits, and social organization (Rosalino et al., 2005b). Two of these species, *Genetta genetta* and *Herpestes ichneumon*, are African-originated species that were historically introduced and naturalized in Portugal (Gaubert, 2016). With regard to activity patterns, the majority of these species exhibit nocturnal or crepuscular activity; only *H. ichneumon* presents diurnal activity patterns (Curveira-Santos et al., 2017). In terms of habitat preferences, the Eurasian otter (*Lutra lutra*) is typically found associated to water bodies, and its diet consists primarily of fish, crustaceans, and amphibians (Mathias et al., 2023). The remaining species are distributed throughout the *Montado* area, although *Martes foina* and *Genetta genetta* are known to be arboreal species that tend to utilize the higher vegetation strata (Mathias et al., 2023). Most of the mesocarnivores are solitary, with the exception being *Meles meles* that can form social groups, building large dens where they live (Kruuk, 1978).

The abundance and distribution of mesocarnivores in the *Montado* landscapes can be influenced by several factors. Mesocarnivores require dense shrubs and understory vegetation that can provide shelter from predation and higher food availability, as well as assisting with predation activities (Mangas et al., 2008; Marques, 2017). Furthermore, prey availability has been demonstrated to exert a positive influence on the abundance and distribution of mesocarnivores (Marques, 2017). Human activities associated with land use and management practices, such as livestock raising, can result in a decline of mesocarnivore abundance due to a reduction in vegetation coverage and higher disturbance (Marques, 2017; Barros et al., 2024). However, more generalists mesocarnivores species can benefit from heterogeneous landscapes and agricultural practices, as it allows for a greater variety of food items and a larger foraging area (Curveira-Santos et al., 2017).

Although these species belong to the Carnivora order, the majority has a high degree of plasticity and besides animal prey can also eat other types of food, such as fruits produced in agricultural areas. In addition, seasonal variations can influence the distribution and diet of mesocarnivores. In wet seasons, mesocarnivores may exhibit a greater consumption of small mammals, while in the dry season they exhibit a more generalist food spectrum (Rosalino & Santos-Reis, 2002; Rosalino et al., 2005a). This is due to the fact that during the wet season, fruits are not as available, whereas, during the dry season, there is a greater abundance of fruits, which allows carnivores to have a more diverse diet.

1.5. Study aims

In order to investigate the mechanisms underlying the coexistence of mesocarnivores, it is essential to conduct an accurate analysis of the trophic niche overlap at the community level. Such an analysis can provide insight into the resource partitioning between different species and the potential for competition, and guide the scientific community towards the implementation of better and more efficient conservation measures, namely at the level of habitat management.

The objective of this study is therefore to gain a more comprehensive understanding of the trophic niche of different mesocarnivore species in *Montado* landscapes, including how it varies between locations and seasons. Furthermore, it is our intention to contribute to demonstrate the significance of employing genetic techniques to identify faecal samples.

In order to achieve this, we will first determine the error associated with the identification of the scats using solely phenotypic criteria. Secondly, we will evaluate whether the consumed resources have an influence on the success of the morphological identification of the scats. Finally, we will evaluate seasonal and spatial variations of the trophic niche of mesocarnivore communities in two *Montado* landscapes. We hypothesized that (1) species with greater taxonomic relatedness or similar food preferences would exhibit a higher error rate of scat classification, (2) scats with a higher content of vegetal matter would be more susceptible to morphological misidentification, (3) there would be a significant difference between the data retrieved using morphological and genetic methods, and (4) the trophic niche segregation would be higher seasonally than spatially, with greater overlap between related species.

2. Methods

2.1. Study areas

This study was conducted in two *Montado* locations in Portugal (Figure 2.1), Charneca do Infantado, part of Companhia das Lezírias, S.A. (CL), and Serra de Grândola (SG). Although they are both *Montado* landscapes, they differ in orography, tree density, and management options, allowing us to investigate how these factors are affecting the existing mesocarnivore communities.

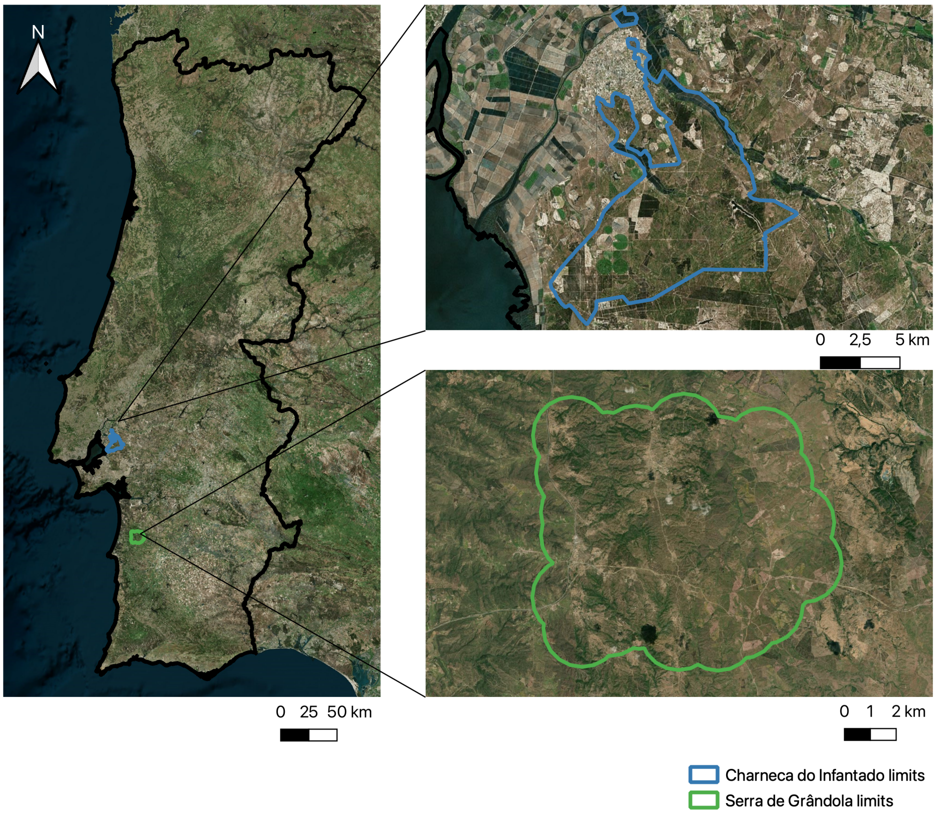

Figure 2.1 - Location of both study areas. On the top-right side are the limits of Charneca do Infantado and on the bottom-right side are the limits of Serra de Grândola.

The *Montado* is an agro-sylvo-pastoral ecosystem characterized by having sparsely distributed trees with an open canopy, pastures (composed of herbaceous plants and shrubs), and grazing. Cork oak (*Quercus suber)* and holm oak (*Quercus ilex*) are the main species of trees characterizing the Montado system, and its maintenance is very dependent on grazing activities (Aronson et al., 2012; Pinto-Correia et al., 2013). *Montado* landscapes are also characterized by a Mediterranean climate, which means having dry summers and irregular precipitation periods (Aronson et al., 2012; Pinto-Correia et al., 2013). It has a marked seasonality and drought periods (Aronson et al., 2012; Pinto-Correia et al., 2013).

Montado areas represent 33% of all of Portugal's forested area (Paço et al., 2009) and have a high biodiversity value (Pinto-Correia et al., 2013). Due to their importance for several threatened species, including carnivores, *Montado* is a habitat classified and protected by the Directive Habitats of the European Union (habitat 6310 – Dehesas with evergreen *Quercus* spp), which promotes conservation measures to protect this important habitat with elements of high cultural and natural value (Pereira et al., 2004). In this habitat, it is possible to find more than 130 vertebrate species, including 28 mammal species, corresponding to more than half of all terrestrial mammal species found in Portugal (Belo et al., 2009).

2.1.1. Companhia das Lezírias

Companhia das Lezírias, S.A. is located in Santarém district (mid-west region) and is the largest farmstead in the country, occupying 17 952 hectares. It is divided into two main regions, Lezíria, in Vila Franca de Xira, and Charneca, where the samples were collected, in Samora Correia. Charneca do Infantado corresponds to 10 983 hectares of its total area (Companhia das Lezírias, 2016). It represents a heterogeneous landscape composed of different types of forested areas (8 848 hectares), being the main tree type the cork oak (*Quercus suber*) which corresponds to 75% of the total area, followed by the maritime pine (*Pinus pinaster* - 11%), the stone pine (*Pinus pinea* - 8%) and the eucalyptus (*Eucalyptus globulus* - 6%) (Companhia das Lezírias, 2016). It also includes 1 330 hectares of agricultural areas and 6 047 hectares of pastures. Companhia das Lezírias represents one of the research and monitoring stations of the LTSER *Montado* platform (https://deims.org/ef87d551-9bae-467f-9c5d-b538b8206b0b).

More than half (55%) of the Charneca do Infantado area is included in the Special Protection Area of *Estuário do Tejo*, making it an area of conservation interest due to its high biodiversity value (Companhia das Lezírias, 2016). Although this area is subject to several use restrictions, it is still under a strong anthropogenic influence due to agricultural activities, grazing, and cork extraction.

Charneca do Infantado is crossed by several water lines which are part of the Tejo River basin and have an influence on the high level of biodiversity found in the area (Simões, 2009). Charneca do Infantado is characterized by a Mediterranean climate, presenting a very marked seasonality with hot, dry summers followed by moderate, wet winters, and a mean annual temperature of 16,3 ºC (Companhia das Lezírias, 2016).

Nine different mesocarnivore species are known to occur in this study area: red fox (*Vulpes vulpes*), weasel (*Mustela nivalis*), European polecat (*Mustela putorius*), European stone marten (*Martes foina*), European badger (*Meles meles*), Eurasian otter (*Lutra lutra*), common genet (*Genetta genetta*), Egyptian mongoose (*Herpestes ichneumon*), and cat (*Felis sp.*) (Gonçalves et al. 2013). However, recent studies have indicated that *M. putorius* has exhibited markedly low capture rates (Curveira-Santos et al., 2017; Gonçalves et al., 2013). A recent study conducted to evaluate the biotic interactions as structuring factors of this mesocarnivore community (Barros, 2024) revealed that the most abundant species in the area are the red fox, the European badger, and the Egyptian mongoose.

2.1.2. Serra de Grândola

Serra de Grândola is located in the district of Setúbal (SW region) and its landscape is also dominated by *Montado* (more than 90% of its cover). The other 10% comprises small patches of olive groves, riparian vegetation, and small agricultural areas. It is also possible to find some patches of *Quercus ilex*, *Eucalyptus globulus,* and *Pinus pinaster* (Rebelo et al., 2009). Given the soil's low fertility, many agricultural fields were abandoned, giving place to the formation of shrubland and woodlands and the subsequent area homogenization (Rebelo et al., 2009).

Although this region presents a very low human population density, it suffers the influence of anthropogenic activities, being the main economic activity the cork exploitation and increasing livestock density (Rosalino et al*.,* 2005a).

Serra de Grândola is included in a Mediterranean climate region showing marked seasonality and a mean annual temperature of 15,6 ºC (Rebelo et al., 2009). This study area includes the field station of the Centre for Ecology, Evolution and Environmental Changes (CE3C), Herdade da Ribeira Abaixo, a property covering 220 ha that also represents one of the long-term ecological research and monitoring stations of the LTSER *Montado* platform (https://deims.org/ecfcc7e2-82e5-4ec1-adee-a3f9f815fc0d).

The composition of the mesocarnivore community inhabiting Serra de Grândola includes the same nine mesocarnivore species also present in Companhia das Lezírias. However, *M. putorius* has presented low detection rates in recent studies (Costa et al., 2014; Gomes, 2015). Given the data obtained in the study of Barros (2024), the most abundant species found in this area were the red fox and the stone marten.

2.2. Field and laboratory methods

2.2.1. Scats collection

Given the results of Barros (2024) for the same study areas, the selected target species for this study were *V. vulpes*, *M. nivalis, M. foina, M. meles*, *G. genetta,* and *H. ichneumon*, due to the rare occurrence of the remaining species.

Mesocarnivore scats were collected between October 2021 and February 2023, while walking transects of 100m randomly selected on dirt roads surrounding the camera trap locations defined in Barros et al. (2024). The scats were identified in the field by two observers with different levels of expertise, on the basis of size, shape, and location. To allow genetic identification and prevent possible contaminations, none of the samples were touched by hand and all were stored in sterile tubes and subsequently preserved at -20ºC. To ensure the DNA quality, only recent and intact scats were collected. Data such as location, date, and potential carnivore species were recorded for each scat.

Given the initial low number of scats collected in Companhia das Lezírias, 38 preserved scats previously collected in 2020 at the same location, were also used to increase sample size.

For analytical purposes, samples were divided into wet (October to April) and dry season (May to September) on the basis of climate data retrieved from local meteorological stations at the time of the study (Annexes 1 & 2).

2.2.2. Genetic identification

The scats' DNA extraction was made using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek), following the manufacturer's protocol. The extracted DNA was eluted and stored in a final volume of 150 μL of elution buffer at -20ºC. The material selected for DNA extraction were parts of the scats that were expected to have a higher concentration of intestinal epithelial cells, such as the outer layer and the extremities. All the used material was sterile and disinfected using UV light and some with alcohol and flame. Possible contaminations were monitored using a negative control (extraction blank) in each extraction round of eleven samples.

Through PCR (Polymerase Chain Reaction), the extracted DNA was amplified using specific primers (Table 2.1), developed for each species, capable of amplifying a specific portion of the mitochondrial cytochrome b gene (Fernandes et al., 2008). The amplifications were made in a final volume of 15 μL: 7.5 μL of Multiplex NZYTaq (NZYtech, Portugal), 0.15 μL of primer F (0.5 μM), 0.15 μL of primer R (0.5 μM), 1.2 μL of H_2O and 6 μL of extracted DNA. The temperature profile for the thermal cycling consisted of an initial denaturation of 5 min at 95ºC, followed by 55 cycles of 30 s at 94ºC, 30 s at 50ºC, and 30 s at 72ºC. The final extension was 10 min at 72ºC. In each round of PCR, we used a negative control.

We used primers for every species described earlier and for *Felis silvestris* and *Canis lupus*. This was made in an attempt to test if the scats that were not possible to genetically identify would possibly be from domestic cats or dogs. None of these two primer pairs were developed to exclude those domestic forms (Fernandes et al., 2008). First, the samples were tested for the potential species identified in the field. If the result was negative, they were tested for the second most probable species and this process was repeated until it was possible to identify the species to which the scat belonged. Every sample was tested twice for each of the study species before being considered unidentifiable.

The results of each PCR were evaluated by electrophoresis with a gel of 1,5% agarose/TBE containing RedSafe™ (iNtRON Biotechnology, South Korea). Each sample was mixed with Green GoTaq® Flexi Reaction Buffer (2 µL of sample and 2.4 µL of green buffer). In each line of PCR wells, it was added 2 µL of NZYDNA Ladder I (NZYTech, Portugal), at the first and last well, to facilitate the reading of the results.

Table 2.1 - Used primers in the genetic identification of the collected samples (Fernandes et al., 2008).

Species	Primer	Sequence (5'-3')	Fragment extension (bp)
Vulpes vulpes	Vvulpes F2	AGGGTTTTCCCAGTCACGACGTTATAATCCTAGCCCTAGTG	227
	Vvulpes R2	GTTTCTTGCGGTCAATAAGATAGCA	
Mustela nivalis	Mnivalis F1	AGGGTTTTCCCAGTCACGACGTTTAGTCCGCTATTCCGTAT	182
	Mnivalis R1	GTTTCTTTATGAGGGGTTGTTAGAC	
Martes foina	Mfoina F2b	AGGGTTTTCCCAGTCACGACGTTATATTAGAACCAGCCTCG	242
	Mfoina R2b	GTTTCTTCAGAAGTAGGGCACCTAA	
Meles meles	Mmeles F1	AGGGTTTTCCCAGTCACGACGTTCGCCTAATAYTAATGCTC	192
	Mmeles R1	GTTTCTTAGGAAGATTAGGGCTAAC	
Genetta genetta	Ggenetta F2	AGGGTTTTCCCAGTCACGACGTTCCGTGATTACAAACCTCT	216
	Ggenetta R2	GTTTCTTCTGAGTCAGATATTACGC	
Herpestes ichneumon	Hichneumon F2	AGGGTTTTCCCAGTCACGACGTTATGCTTGTAGCACTCATG	224
	Hichneumon R2	GTTTCTTATATGGAGTAGTGGTACG	
Felis silvestris	Fsilvestris F2	AGGGTTTTCCCAGTCACGACGTTATTATGGCTCCTACACCT	181
	Fsilvestris R2	GTTTCTTCGTTCTACTAGTTCAGTC	
Canis lupus	Clupus F1	AGGGTTTTCCCAGTCACGACGTTCCCACTAGCCAAAATTGT	252
	Clupus R1	GTTTCTTATGAAGAATATGGAAGCG	

2.2.3. Identification of consumed resources

For evaluating the trophic niche of each mesocarnivore species, an analysis of the non-digested contents of each genetically identified scat was made. The scats were put individually in water for a minimum of 24 hours and passed through a thin mesh sieve, to eliminate all the organic matter that dissolved in the water facilitated the observation of the rest of the contents and its triage.

The identifiable food remains (bones, fur, feathers, scales, seeds, etc.) were separated, in a Petri dish, relating to the following categories: "Mammals", "Birds", "Reptiles", "Insects", "Crayfish", "Organic Matter" and "Fruit". For "Organic Matter" all the vegetable materials that were not fruits or seeds, such as leaves and sticks, were considered. The different components were subsequently put in a stove for 48h under 50ºC and stored. For each scat, the presence/absence data of each category were collected to calculate the frequency of occurrence of each food category for each species. Due to the lack of nutritional value, the contents that were categorized as organic matter were discarded and not taken into consideration in the diet analysis. Data pertaining to volume proportion of each food category was also collected to understand the possible influence of the presence of fruit and organic matter in the success rate of morphological identification.

2.3. Data analyses

Data analyses were performed based on the genetic identification of the scats but the comparison with the scats' morphological identification in the field allowed us to estimate the ID error and discuss the implications of misidentification in studies without genetic identification testing.

For the diet analysis of each mesocarnivore, the frequency of occurrence (FO) of each food category was calculated, using the number of occurrences of each food category divided by the total number of occurrences of all the food categories from all the scats of each species (Klare et al., 2011). In order to ascertain whether there were any statistically significant differences between some data sets under examination, the Chi-squared and the Wilcoxon Rank Sum tests were employed.

The standardized Levin's index (B_A) was used to estimate niche breadth (Krebs, 1989). This index varies between 0 and 1. A species that presents fewer food categories in its diet will present lower values (lower niche breadth) and be considered a species with a more specialist regime. With higher values, it would be considered a species with a generalist regime.

$$B_A = \frac{B - 1}{n - 1} \qquad B = \frac{1}{\sum p_j^2}$$

Equation 2.1 - Standardized Levin's index formula. *B* is the Levins index; *pj* is the proportion of individuals using one of the resources; *n* is the number of resource categories.

The Shannon-Wiener index (*H'*) was used to estimate the consumed resource diversity. These results range between 0 and ln (total number of food categories), where low values represent a low diversity and high values represent a higher diversity (Krebs, 1989).

$$H' = -\sum p_j \log p_j$$

Equation 2.2 - Shannon-Wiener index formula. *pj* is the number of individuals using *j* resource.

The trophic niche overlap was evaluated by calculating Pianka's niche overlap index (O_{jk}) (Pianka, 1974), where the values vary between 0 and 1, with higher values meaning a higher overlap, being 1 considered a total overlap.

$$O_{jk} = \frac{\sum_{i}^{n} p_{ij} p_{ik}}{\sqrt{\sum_{i}^{n} p_{ij}^2 \sum_{i}^{n} p_{ik}^2}}$$

Equation 2.3 - Pianka’s niche overlap index formula. *n* is the total number of food categories; *i* is the number of occurrences; *j* and *k* correspond to the two different samples under evaluation; *pij* and *pik* are the percentages of occurrence of each category in the respective samples.

In order to obtain a 95% confidence interval for each of the indices used, the bootstrapping method was used, with 1000 replicates, using the R package *pgirmess* (Pla, 2004; Giraudaux et al., 2018).

3. Results

A total of 442 scat samples were collected (Table 3.1) with differences between sites and seasons not significant ($\chi2 = 0.1323$, df = 1, $p = 0.7161$). Of the total number of samples, 331 (≈75%) were genetically identified. The data indicates a statistically significant difference in the number of unidentified scats between seasons ($\chi2 = 51.103$, df = 1, $p < 0.001$) and locations ($\chi2 = 34.067$, df = 1, $p < 0.001$), showing a lower success rate at the dry season and in Serra de Grândola.

Table 3.1 - Number of scats collected and number of scats that could not be identified genetically per season and location.

	Total scats			Total n/ identified		
	Wet season	Dry season	Total	Wet season	Dry season	Total
CL	89	110	199	2	21	23
SG	114	129	243	16	72	88
Total	203	239	442	18	93	111

3.1. Morphological vs. genetic identification success of faecal samples

According to morphological identification, the collected scats belonged to *V. vulpes*, *M. foina*, *M. meles*, *G. genetta*, *H. ichneumon,* and *M. nivalis* (Table 3.2). However, following genetic identification, some scats also belonged to *Felis sp.* (Table 3.3). The final number of scats collected and identified as *M. nivalis* and *Felis sp.* was considered insufficient to be included in the data analyses for the dietary niche estimation.

Table 3.2 - Number of collected scats, morphologically identified, per species, season, and location.

	CL		SG		Total
	Wet season	Dry season	Wet season	Dry season	
Vulpes vulpes	34	60	57	67	218
Mustela nivalis	5	8	3	0	16
Martes foina	13	15	40	53	121
Meles meles	31	17	11	2	61
Genetta genetta	4	6	0	3	13
Herpestes ichneumon	2	4	3	4	13

Table 3.3 - Number of genetically identified scats per species, season, and location.

	CL		SG		Total
	Wet season	Dry season	Wet season	Dry season	
Vulpes vulpes	30	37	52	34	153
Mustela nivalis	0	1	0	0	1
Martes foina	11	15	29	13	68
Meles meles	21	14	6	1	42
Genetta genetta	8	9	3	1	21
Herpestes ichneumon	16	13	5	8	42
Felis sp.	1	0	3	0	4

The proportion of scats that were morphologically correctly identified as belonging to a particular species varied between 6% to 82%, presenting an overall success of 65%; the weasel exhibited the higher identification error (only ≈6% of success), followed by the stone marten (≈54% of success) (Table 3.4). The species with the higher identification success was the Egyptian mongoose (≈82% of success).

Table 3.4 - Error matrix for the morphological identification of the scats. The number of scats morphologically identified as a certain species is in each line distributed by the result of the genetic identification in each column. In green, it is possible to see the number of scats that were correctly identified. VV – *Vulpes vulpes*, MF – *Martes foina*, HI – *Herpestes ichneumon*, GG – *Genetta genetta*, MM – *Meles meles*, MN – *Mustela nivalis*, FS – *Felis sp.*.

	VV	MN	MF	MM	GG	HI	FS	**Total (Morph.)**
VV	112	0	5	1	8	25	2	**153**
MN	1	1	14	0	0	0	0	**16**
MF	30	0	46	2	3	4	1	**86**
MM	3	0	2	40	2	7	1	**55**
GG	1	0	1	0	8	0	0	**10**
HI	2	0	0	0	0	9	0	**11**
Total (Gen.)	**149**	**1**	**68**	**43**	**21**	**45**	**4**	**331**

Data demonstrate a tendency for a misleading morphological identification of scats from certain pairs of species. The closest taxonomic relatives are the stone marten, the weasel and the European badger, as these three species are mustelids. The Egyptian mongoose and the common genet can also be considered as taxonomically close species. However, within these five species, the stone marten and the weasel are the closest (Binida-Emonds et al., 1999).

In the case of the stone marten, approximately 35% of the scats that were morphologically assigned were genetically identified as red fox, while approximately 88% of the weasel's scats were identified as stone marten. The majority of Egyptian mongoose scats (≈56%) were initially identified as red fox. This was the only species, along with the common genet (≈62%), where the majority of the scats were initially identified as belonging to another species. The European badger showed the lowest error rate, when comparing the proportions of the scats that where morphologically and genetically identified as belonging to European badger and the proportions of scats that were morphologically identified as belonging to another species and were genetically identified as belonging to this species. In this case, ≈73% of all morphologically identified scats were correctly identified and only ≈7% were initially identified as another species.

3.2. Impact of identification methods on dietary insights

Figure 3.1 illustrates the diet of six different mesocarnivore species according to the employed identification method and of the potential outcomes that were achieved through the utilization of the two identification methodologies for faecal matter.

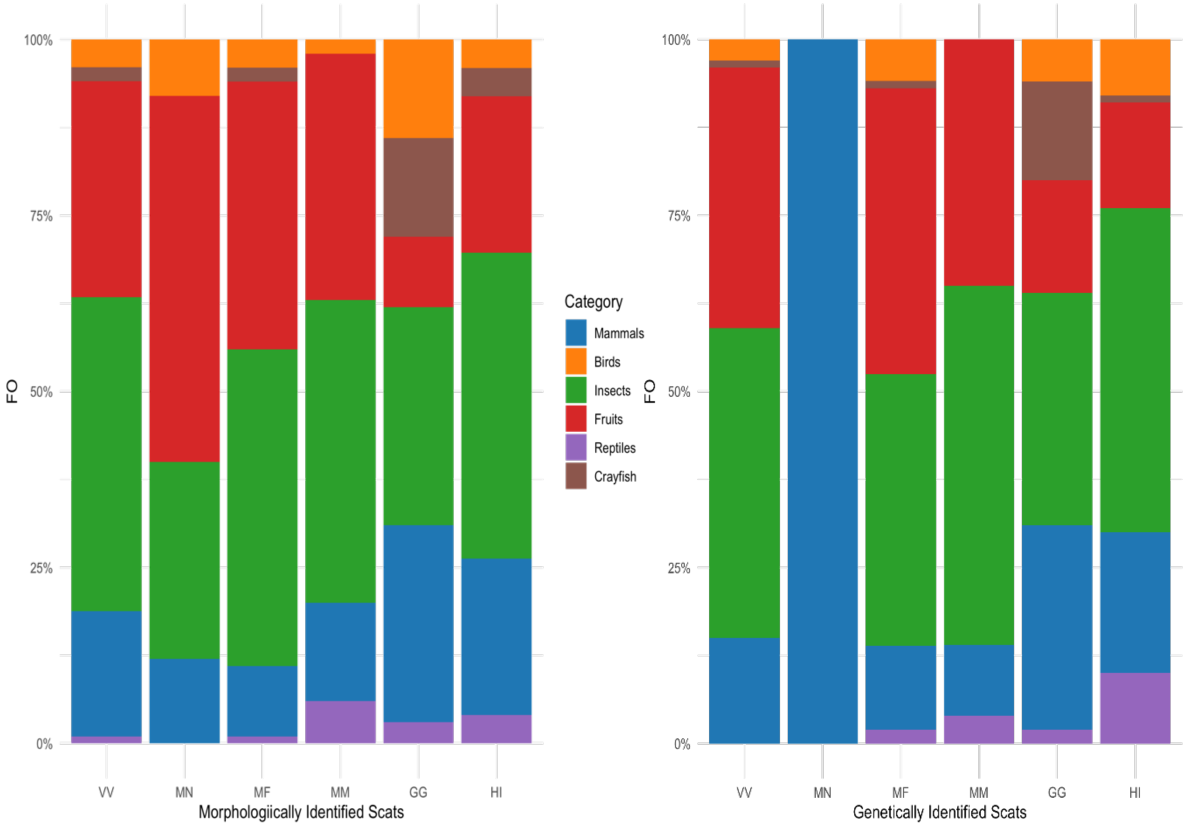

Figure 3.1 - Frequency of occurrence of each food resource present in the diet of the different species. The data represented in the graph on the left were obtained from faecal samples morphologically identified, while the graph on the right presents data obtained from scats genetically identified. The represented species are V. vulpes (VV), M. nivalis (MN), M. foina (MF), M. meles (MM), G. genetta (GG), and H. ichneumon (HI).

Table 3.5 statistically compares differences observed in the diet of the different species, with the exception of the weasel due to sample size, using the different identification methods. Results lead to the conclusion that there are no significant differences. Nevertheless, a visual analysis of the Figure 3.1 reveals the existence of some discrepancies.

Table 3.5 - Comparison of the obtained results by the two different scats' identification methods for each species using a chi-square test. $\chi2$ – chi-square, df – degrees of freedom.

	$\chi2$ test		
	$\chi2$	df	p-value
Vulpes vulpes	7.3953	5	0.1929
Martes foina	2.4880	5	0.7783
Meles meles	3.3638	4	0.4989
Genetta genetta	1.8217	5	0.8732
Herpestes ichneumon	2.6218	5	0.7580

In the case of reptiles' consumption, morphological identification of scats indicates that the majority of species, with the exception of the weasel, include these prey in their diet, being the European badger the species that consumes the greatest proportion of reptiles (≈47% of all consumed reptiles). In the sample of genetically identified scats the Egyptian mongoose is the species that consumes the largest proportion of reptiles (60%), while, besides weasels, the red fox also does not show any consumption of this food category.

Another discrepancy pertains to the consumption of birds by the European badger. In the morphologically identified scat sample, it can be observed that birds constitute approximately 1.8% of their diet, although in the genetic sample they were absent from this species' diet.

The most significant discrepancy is however observed in the case of the weasel, where the consumed food categories show a pronounced shift. Initially, it could be assumed that this species exhibited a diverse diet. However, upon examination of genetic results, it became evident that its diet was exclusively composed of mammals. The data regarding this species in particular may be subject to bias due to the limited number of scats available for analysis. As previously stated, only one of the initial sixteen samples was confirmed as belonging to *M. nivalis* (Table 3.4). This renders the sample size insufficient for drawing any conclusions regarding the dietary niche of this species.

In Figure 3.2 is represented the average volume proportion of each food category of the samples that had the same genetical and morphological identification result and the samples that were morphologically identified as another species.

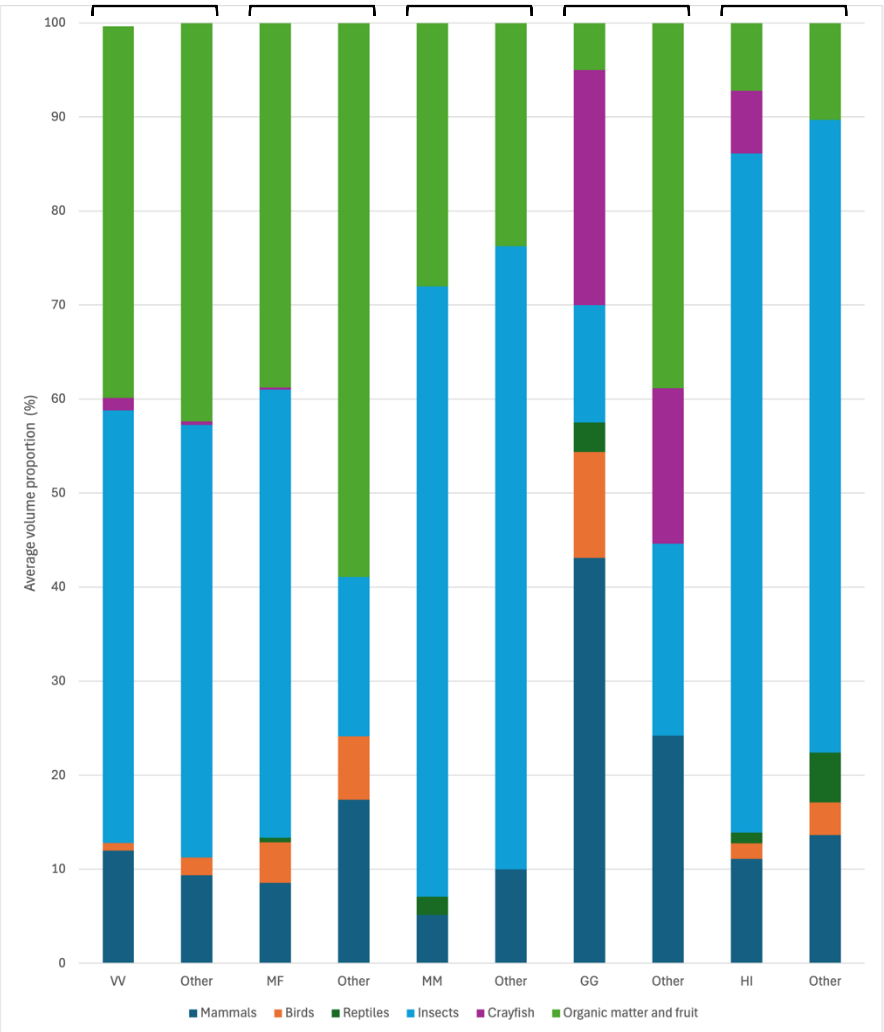

Figure 3.2 – Average volume proportion (%) of each food category present on the faecal samples that were identified as the same species in the morphological and genetical identification ("VV", "MF", "MM", "GG", and "HI") and on the samples that were genetically identified as a certain species that did not correspond to the morphological identification ("Other"). VV – *Vulpes vulpes*, MF – *Martes foina*, MM – *Meles meles,* GG – *Genetta genetta*, HI – *Herpestes ichneumon.*

An analysis of this figure shows that, in the majority of the cases, the composition of fruit and organic matter in the scats that presented the same identification result in both methods appears to be lower than in the samples that were morphologically identified as belonging to another species. However, there is no statistical difference between the two groups ($W = 11664$, $p = 0.6449$), which may be related to the high variability between samples. When comparing the volume proportion of fruit and organic matter of the scats, ≈38% of the scats that presented a percentage inferior (or equal) to 15% of fruit and organic matter in its composition were misidentified, while only ≈33% of the ones that presented a percentage superior to 15% were misidentified.

3.3. Trophic niche assessment[1]

As mentioned previously, in Serra de Grândola (SG) it was not possible to obtain sufficient faecal samples to enable a secure comparison of all the species' diet and to potentially understand how their diet varies with seasonality. As a result, depending on the total number of scats, some comparisons could not be made for all five different species. Although there are no sufficient samples to evaluate the diet of the Egyptian mongoose, the common genet, and the European badger in SG, they are still represented in Figure 3.3.

In general, the least consumed resources were crayfish, birds, and reptiles, while insects and fruits were the most consumed categories (Figure 3.3). The consumption of reptiles, when present, is dominated by *H. ichneumon*. A greater proportion of birds were found in the diet of individuals of CL, with the exception of *M. meles*, which did not show any consumption of this food category. In SG, birds were only present in the diet of *V. vulpes* in a very small percentage. Additionally, crayfish appear to be a more common component of mesocarnivores' diet in CL than in SG.

Table 3.6 demonstrates that there is minimal variation in the diet of each species according to the variables under investigation. When the data are aggregated in a less restrictive manner, *M. foina* exhibits a statistically significant difference in the composition of its diet between CL and SG (p-value < 0.01). The remaining four species do not provide sufficient evidence, or in the case of *G. genetta* and *M. meles*, sufficient data, to support the conclusion of a statistical difference in their diet composition between locations. When only the seasonal separation is considered, the results show that *V. vulpes* (p-value < 0.1), *M. foina* (p-value < 0.05), and *M. meles* (p-value < 0.1) also exhibit significant differences.

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[1] From this point forward, all data presented will pertain to the genetic identification of the scats.

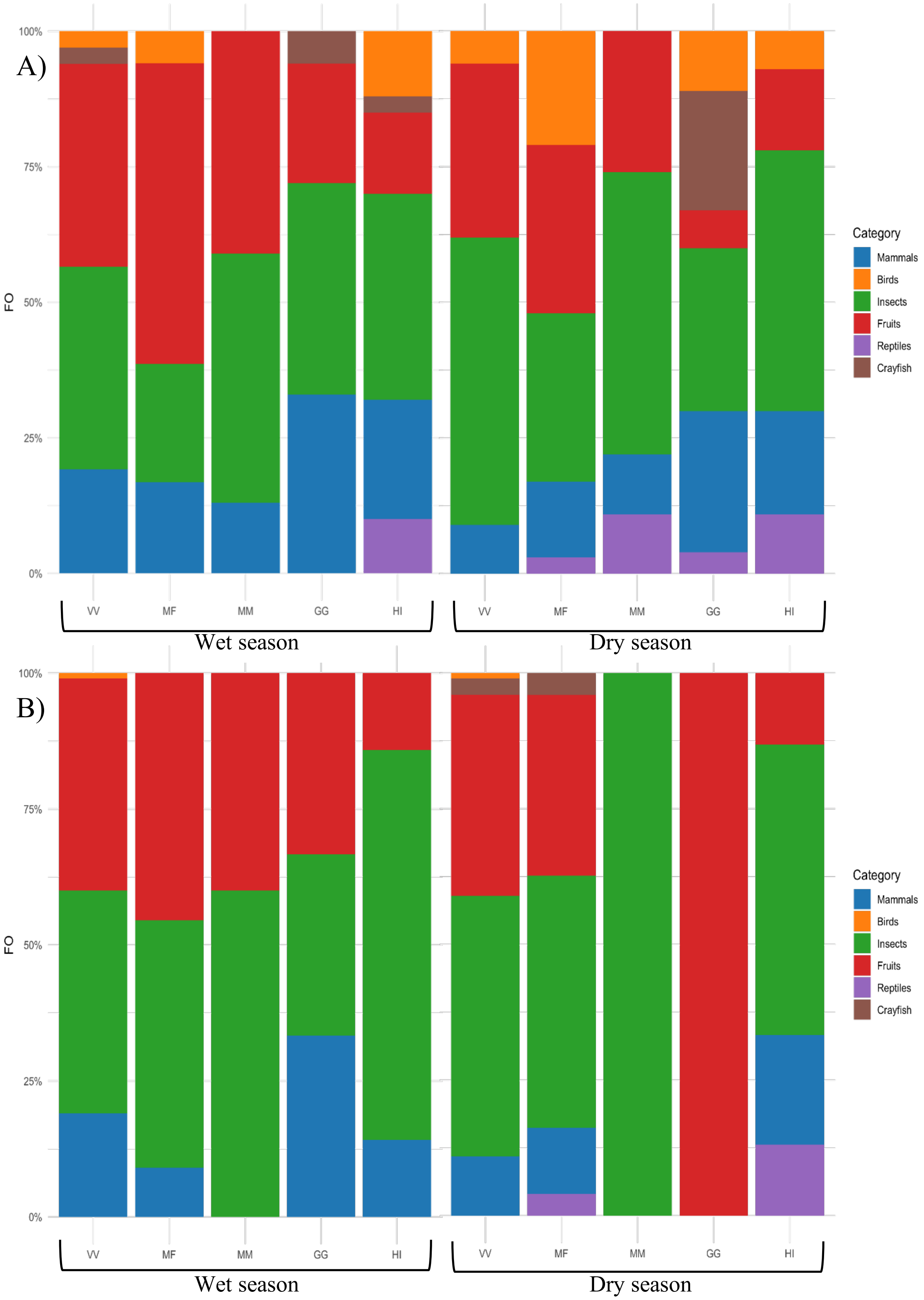

Figure 3.3 - Frequency of occurrence of each food resource present in the diet of the different species. A) corresponds to the samples collected in CL and B) to the samples collected in SG. The data represented on the left corresponds to the faecal samples collected during the wet season while the data on the right corresponds to the faecal samples collected during the dry season. The represented species are *V. vulpes* (VV), *M. foina* (MF), *M. meles* (MM), *G. genetta* (GG), and *H. ichneumon* (HI).

When aggregating the scats by their respective location and season and analysing both variables simultaneously, it is possible to identify some differences in the consumed items (Figure 3.3). However, looking at Table 3.6, it becomes evident that there are no statistically significant differences.

Table 3.6 - Chi-square test results testing the homogeneity of the diet of each species according to its location and according to seasonality. The results correspond to the homogeneity between different seasons in the same location, between different locations during the same season, between locations, and between seasons. * - *p-value* < 0.1; ** - *p-value* < 0.05; *** *p-value* < 0.01.

		V. vulpes	*M. foina*	*M. meles*	*G. genetta*	*H. ichneumon*
CL: Wet vs Dry season	χ2	6.9135	4.3540	5.5225	6.8559	1.6542
	df	4	4	3	5	5
	p-value	0.1405	0.3602	0.1373	0.2316	0.8946
SG: Wet vs Dry season	χ2	5.8796	5.1348	-	-	-
	df	4	4	-	-	-
	p-value	0.2083	0.2737	-	-	-
Wet Season: CL vs SG	χ2	5.6584	5.6763	-	-	-
	df	4	3	-	-	-
	p-value	0.2262	0.1285	-	-	-
Dry season: CL vs SG	χ2	4.4135	6.9922	-	-	-
	df	4	5	-	-	-
	p-value	0.3529	0.2212	-	-	-
CL vs SG	χ2	4.9835	15.0890	-	-	3.8121
	df	4	5	-	-	5
	p-value	0.2890	0.0099***	-	-	0.5768
Wet vs Dry season	χ2	7.8251	11.263	6.5998	7.2188	2.4472
	df	4	5	3	5	5
	p-value	0.0982*	0.04642**	0.0858*	0.2049	0.7844

3.3.1. Niche breadth

In order to evaluate niche breadth, the Standardized Levins' Index for each eligible species was calculated in accordance with the season and location in question (Table 3.7). The results presented all exhibit considerably large confidence intervals, which may be indicative of substantial variability in the data obtained.

Table 3.7 - Standardized Levins' Index (B_A) results for the 5 different species according to their location and season and its 95% confidence interval obtained by the bootstrapping method, with 1000 replicates.

	CL Wet Season		**CL Dry Season**		**SG Wet Season**		**SG Dry Season**	
	B_A	**Δ95%**	B_A	**Δ95%**	B_A	**Δ95%**	B_A	**Δ95%**
V. vulpes	0.435	[0.116; 0.801]	0.305	[0.044; 0.742]	0.363	[0.009; 0.800]	0.330	[0.061; 0.738]
M. foina	0.314	[0.040; 0.780]	0.582	[0.243; 0.936]	0.277	[0.000; 0.676]	0.388	[0.131; 0.793]
M. meles	0.303	[0.000; 0.797]	0.354	[0.082; 0.759]	-	-	-	-
G. genetta	0.435	[0.065; 0.834]	0.694	[0.506; 0.919]	-	-	-	-
H. ichneumon	0.633	[0.397; 0.937]	0.454	[0.230; 0.926]	-	-	-	-

In the context of CL, it is evident that during the wet season, 3 of the 5 species under study exhibit a more specialized dietary pattern, characterized by a narrow niche breadth. Amongst these species, the stone marten and the European badger demonstrate the lowest niche breadth. The Egyptian mongoose is the only species that can be considered to have a generalist diet. However, this is also the only species that exhibits consumption of every six food categories (Figure 3.3). During the dry season, a clear shift is evident in the observed results, with the stone marten and the common genet demonstrating a potential for generalist behaviour, while the Egyptian mongoose exhibits a tendency towards a more specialized diet.

It is not possible to identify the same behavioural patterns in the stone marten in SG as were identified in CL. An increase in niche breadth from the wet season to the dry season is observable, however, this increase is minimal. No significant differences were observed between seasons for the red fox.

As previously stated, the most consumed food categories for all species in both locations and in both seasons were insects, followed by fruits (Figure 3.3). These two food resources, in addition to mammals, are the only food resources that are present in all five species, independently from season and location, which results in a notable reduction in niche breadth. It is only in the cases of the Egyptian mongoose during the wet season in CL and the common genet during the dry season in CL that we observe a higher consumption of other food categories (Figure 3.3), which corresponds to a higher niche breadth (Table 3.7).

3.3.2. Diversity in the consumed resources

The diversity and equitability of the different consumed resources were evaluated for each species using the Shannon-Wiener Index, as detailed in Table 3.8. The considerable disparity in the proportion of fruit and insect consumption in comparison to the remaining four categories results in a substantial reduction in dietary diversity and equitability for most species.

As with the niche breadth, the same pattern is also visible in this case. The European badger during the wet season in CL and the stone marten during the wet season in SG are the ones who present the least diverse diet, while the Egyptian mongoose during the wet season in CL and the common genet during the dry season in CL the ones who show the most diverse diet. The remaining species can be considered to exhibit an intermediate level of diversity.

Table 3.8 - Shannon-Wiener Index (*H'*) results for the 5 different species according to their location and season and its 95% confidence interval obtained by the bootstrapping method, with 1000 replicates.

	CL Wet Season		**CL Dry Season**		**SG Wet Season**		**SG Dry Season**	
	H'	**Δ95%**	***H'***	**Δ95%**	***H'***	**Δ95%**	***H'***	**Δ95%**
V. vulpes	1.262	[0.578; 1.892]	1.087	[0.385; 1.788]	1.094	[0.362; 1.835]	1.114	[0.440; 1.789]
M. foina	1.128	[0.470; 1.777]	1.434	[0.726; 2.055]	0.935	[0.217; 1.797]	1.235	[0.623; 1.924]
M. meles	0.988	[0.265; 1.803]	1.176	[0.486; 1.779]	-	-	-	-
G. genetta	1.235	[0.502; 1.967]	1.602	[1.172; 1.988]	-	-	-	-
H. ichneumon	1.575	[1.168; 1.971]	1.381	[0.744; 1.868]	-	-	-	-

In the context of the CL study, it was observed that during the wet season, there was a slight increase in the dietary diversity of the stone marten and the European badger, from the wet to the dry season. Additionally, a more pronounced increase was noted in the dietary diversity of the common genet. The Egyptian mongoose and red fox exhibited a slight decline. In SG, the dietary diversity of the stone marten exhibited a slight increase, while that of the red fox did not show any significant difference.

3.3.3. Trophic niche overlap

In light of the data presented in Tables 3.9 and 3.10, it is possible to examine the results of the Pianka Index with regard to the trophic niche overlap between the various pairs of species. In general, all species exhibit a high degree of overlap in their trophic niches, with only a few pairs of species demonstrating some degree of segregation.

Table 3.9 reveals that *M. foina* exhibits the highest degree of segregation in comparison to the other species during the wet season, presenting the lowest values of the Pianka Index when testing the overlap between its diet and the diet of *H. ichneumon* and *G. genetta*. In contrast, the highest degree of overlap is observed in *V. vulpes*. However, during the dry season, the overlap between *M. foina* and *H. ichneumon* increases considerably, and the species that presents the lowest overlap values with the other species is *G. genetta*. In general, in the dry season, there is a very slight decrease in the overlap between the different pairs of species. However, this difference does not appear to have statistical significance given the high confidence interval range.

In SG (Table 3.10), the observed overlap between *V. vulpes* and *M. foina* reaches a value that is nearly the maximum possible, but it is once more subject to a high degree of variation.

Table 3.9 - Pianka Index (O_{jk}) results for the interaction between the 5 different species according to wet (A) or dry (B) season in CL and its 95% confidence interval obtained by the bootstrapping method, with 1000 replicates.

A)

	V. vulpes		*M. foina*		*M. meles*		*G. genetta*	
	O_{jk}	**Δ95%**	**O_{jk}**	**Δ95%**	**O_{jk}**	**Δ95%**	**O_{jk}**	**Δ95%**
M. foina	0.923	[0.661; 0.989]						
M. meles	0.986	[0.853; 0.995]	0.895	[0.594; 0.988]				
G. genetta	0.927	[0.633; 0.987]	0.759	[0.334; 0.961]	0.886	[0.564; 0.979]		
H. ichneumon	0.883	[0.663; 0.957]	0.691	[0.357; 0.914]	0.853	[0.611; 0.947]	0.930	[0.632; 0.964]

B)

	V. vulpes		*M. foina*		*M. meles*		*G. genetta*	
	O_{jk}	**Δ95%**	**O_{jk}**	**Δ95%**	**O_{jk}**	**Δ95%**	**O_{jk}**	**Δ95%**
M. foina	0.908	[0.632; 0.985]						
M. meles	0.974	[0.818; 0.991]	0.858	[0.561; 0.957]				
G. genetta	0.706	[0.373; 0.874]	0.732	[0.411; 0.875]	0.724	[0.346; 0.889]		
H. ichneumon	0.929	[0.726; 0.982]	0.859	[0.533; 0.968]	0.966	[0.721; 0.990]	0.818	[0.460; 0.930]

Table 3.10 - Pianka Index (O_{jk}) results for the interaction between Vulpes vulpes and Martes foina according to wet (A) or dry (B) season in SG and its 95% confidence interval obtained by the bootstrapping method, with 1000 replicates.

A)

	V. vulpes	
	O_{jk}	**Δ95%**
M. foina	0.982	[0.892; 0.998]

B)

	V. vulpes	
	O_{jk}	**Δ95%**
M. foina	0.996	[0.826; 0.996]

4. Discussion

This study facilitated a more comprehensive understanding of the trophic niche of certain mesocarnivore species within *Montado* systems. Additionally, it demonstrated the potential limitations of traditional scat identification methods in discerning the dietary habits of sympatric species and the importance of the use of molecular techniques.

Despite the considerable variation in the data set, the data suggests that (1) species with greater taxonomic relatedness would exhibit a higher error rate in the classification of the faecal samples. Nevertheless, no relation could be identified between the content of the scats and the misidentification (2). A comparison of the dietary results obtained by the two different methods revealed some data that could potentially lead to erroneous conclusions, though not to the extent initially anticipated (3). With regard to trophic niche segregation (4), the results were found to be in accordance with the initial hypothesis pertaining to the trophic niche overlap, with significant overlap between the majority of species and greater seasonal than spatial differentiation.

4.1. Which *Montado* mesocarnivores are more prone to scat misidentification?

The genetic identification success obtained in this study was within the range found in other studies (72-94%; Fernandes et al., 2008; Monterroso et al., 2012; Alexandre et al., 2020; Descalzo et al., 2021). However, this success rate may be influenced by the difficulties encountered in the identification success of scats collected in SG during the dry season, which presented a much higher number of scats that could not be genetically identified than those found in CL or during the wet season. During the dry season, scats are exposed to high temperatures, which can lead to rapid DNA degradation and may explain these results (Santini et al., 2007). On the other hand, rain can also be responsible for washing away the DNA present in the scats (Farrel et al., 2000), which should have led to more similar results between seasons. The differences found between the sites can be explained by the differences in rainfall recorded between them (Annexes 1 & 2). In SG, greater rainfall was recorded than in CL, possibly leading to a greater washout of the low quality and quantity of DNA present in these samples. Other possible factor to have in consideration is the fact that some of the collected scats may belong to species that were not part of the PCR tests (e.g. *Lutra lutra*). *L. lutra* is a mesocarnivore species typically associated with watercourses. The majority of transects were conducted in locations distant from such habitats. Consequentially, the discovery of samples from this species was not initially anticipated.

The success rate obtained in the morphological identification of the scats was comparable to that reported in other studies, such as Alexandre and colleagues (2020), where the success rate was evaluated in relation to the experience of the observers. The greatest discrepancies in our study were observed in the case of scats initially identified as belonging to weasels. In this case, only one of the scats initially identified as belonging to a weasel was correctly assigned. With the exception of one, the remaining scats were predominantly identified as belonging to stone martens. This discrepancy may be attributed to the smaller size of the faeces produced by juvenile stone martens, which could potentially be misidentified as those of weasels. Within the studied community, the stone marten and the weasel are the species most closely related taxonomically (Bininda-Emonds et al., 1999). This finding is consistent with the initial hypothesis that species taxonomically related are more likely to be confused. Additionally, the weasel is the least abundant of the species under study, a factor that has previously been demonstrated to contribute to the potential for misidentification (Davison et al., 2002). The observed error between the scats of the red fox, the stone marten, the Egyptian mongoose, and the common genet can be attributed to their similar diets which can produce more similar scats. While some characteristics of the scats allow for accurate identification, scats are commonly found broken or pieces, which can lead to misidentification. The low error rate associated with the scats of the European badger can be attributed to the fact that this species is the largest, producing the most voluminous scats. Additionally, this species is known to dig small pits in the soil where faeces are deposited in close proximity to the dens or movement paths (Fischer et al., 2003).

Prior research has suggested that the misidentification of faecal samples may be attributed to the presence of plant material, mostly fruit, in their diet (Monterroso et al., 2012). However, the findings revealed no statistically significant discrepancies between the samples that were successfully identified morphologically and those that were not. This suggests that the content of the faecal samples may not be a determining factor in their correct identification. Nevertheless, these findings may also be attributed to the high prevalence of fruits in the diets of all species, except for *M. nivalis*. The presence of a high fruit content in the diet of most species makes it challenging to conclude that this food item has no influence on the potential for accurate identification.

4.2. How much do scat-based molecular approaches contribute to dietary insights on a *Montado* mesocarnivore community?

Despite the absence of a statistically significant differences, the observed discrepancies between the dietary patterns of each species, as determined by the employed identification method for the faecal samples, have the potential to yield erroneous conclusions.

A noticeable significant discrepancy pertains to the consumption of reptiles. As previously stated, the Egyptian mongoose is the sole Portuguese mesocarnivore to exhibit diurnal activity patterns (Palomares & Delibes, 1992; Curveira-Santos et al., 2017). This would indicate that, among all species, the Egyptian mongoose would exhibit a higher consumption of reptiles, as it has been documented on numerous occasions to play a significant role in their diet (Delibes et al., 1984; Descalzo et al., 2023). This pattern, however, would not be visible if we consider the scat's morphological results, in which a much lower consumption of reptiles was found.

The degree of divergence observed between the two scat identification methodologies was not as pronounced as anticipated, which may be attributed to the food generalist behaviour of most studied species, resulting in a considerable overlap in trophic niches. The high degree of overlap permits the misidentification of samples to remain undetected in the results. Nevertheless, it can be stated that a more conventional approach to scat identification is unable to provide an accurate assessment of the diet of mesocarnivores. These results illustrate both the potential for misinterpretation of the data and the value of investing in molecular methods.

Moreover, conclusions regarding the weasel must be treated with caution due to the lack of sufficient data to evaluate its trophic niche. Food consumption data resulting from scats morphologically identified would suggest that this species has a very generalized diet, consisting primarily of fruit. This conclusion would be at odds with the existing literature on the species in question. This species is recognized as a specialist carnivore, with its diet primarily consisting of small mammals (Erlinge, 1975; Santos-Reis, 1990; Lanszki & Heltai, 2007).

4.3. Do *Montado* mesocarnivores respond more to season than space while feeding?

The dietary habits of all species were found to exhibit minimal variation across seasons and locations, with only a few statistical variations. Insects and fruits correspond to more than half of the diet for each species. The significance of fruits in the diet of Mediterranean mesocarnivore species has been previously documented (Rosalino & Santos-Reis, 2009), as has the importance of invertebrates (Virgós et al., 1999; Díaz-Ruiz et al., 2013). Despite mammals being identified as the third most consumed item, higher FO values were anticipated (Santos et al., 2007; Díaz-Ruiz et al., 2013). This result can be explained by the reduction in lagomorph availability over the years caused by habitat deterioration, viral diseases, and hunting pressure (Ferreira, 2012). The European rabbit (*Oryctolagus cuniculus*) is known as a significant prey species for these mesocarnivore species (Delibes-Mateos et al., 2008). Previous studies have demonstrated that the absence of this prey can result in a dietary shift (Ferreras et al., 2011; Díaz-Ruiz et al., 2013). The fact that the majority of remains of mammals found in the faecal samples belonged to small mammals can help to support this conclusion.

Reptiles were more frequent to be found in the diet of these species during the dry season, which can be explained by the higher activity of these prey during this season (Delibes et al., 1984). The higher presence of crayfish in the diet of the community in CL than in SG can suggest a higher density of *Procambarus clarkii* in this location, that could be explained by the high presence of wetlands (eg. rice fields) (Correia, 2001; Companhia das Lezírias, 2016). A higher presence of birds in the diet of the individuals in CL can be related to a possible higher abundance of birds in these location. This area has a more heterogenous landscape and the landscape heterogeneity has been shown to increase birds' diversity (Morelli et al., 2013).

The comparison between locations was constrained by the limited number of samples available for the Egyptian mongoose, common genet and European badger. Nevertheless, it was demonstrated that the diet of the stone marten exhibited notable discrepancies. In SG this species exhibited a markedly higher consumption of insects relative to fruits, whereas in CL, an equilibrium was observed between the consumption of these two food categories. The landscape of CL is more heterogeneous than that of SG, with a greater diversity of crops, including a higher quantity and variety of fruits. Inversely, a slightly higher consumption of mammals was observed in SG. This can be attributed to the fact that CL is a managed landscape, characterised by a lower density of herbaceous plants and shrubs. This frequently results in a reduction in the density of small mammals due to less vegetation cover and increased exposure to predators (Torre et al., 2007), which in turn leads to a decline in prey availability.

The seasonal variations were found to be more pronounced than the spatial variations. In general, the diet of the species exhibited a greater proportion of mammals and fruits during the wet season than during the dry season. These findings regarding the consumption of mammals have been previously documented in previous studies (Rosalino & Santos-Reis, 2002; Rosalino et al., 2005a).

4.3.1. Niche breadth and diet diversity

All species exhibited a narrow range of trophic niche breadth, even though the majority of the species under study are considered generalist feeders (e.g. red fox, stone marten, European badger, and common genet; Rosalino & Santos-Reis, 2002; Rosalino et al., 2005a; Rosalino & Santos-Reis, 2009). Having this in consideration, it was anticipated that a wider range of niche breadths would be observed for each species. The narrow niche breadth observed in this species can be attributed to its dietary reliance on two primary food categories: fruits and insects. In the case of the Egyptian mongoose exhibited a broader niche breadth than the other species during the dry season. A higher consumption of other food categories, such as mammals and reptiles, can be observed in comparison with the other four species.

The diversity and balance of resources consumed followed the same pattern as niche breadth. In general, these mesocarnivores showed a high diversity in the consumption of the six defined food categories and a high balance between them. However, this high diversity and equilibrium may be related to the fact that the food categories initially defined are very broad and do not represent the detailed range of the trophic niche of these species, hiding some possible variations.

In this case, some variation was also observed between seasons, and more pronounced than the variation found between locations. This may be attributed to the differing availability of resources between the two seasons, which can be higher than the differences in resources between the two locations.

4.3.2. Trophic niche overlap

These species demonstrate a significant degree of trophic niche overlap, regardless of season or location. Given the species generalist behaviour and the lack of specificity in food categories consumed, this high degree of overlap was to be expected. One unexpected finding is that the overlap between the stone marten and the common genet is among the lowest observed. These are two arboreal species with similar sizes and weights and both are described as generalist foragers. Therefore, it would be anticipated that they would have a greater dietary overlap. López-Martín (2006) also reported similar results. These findings indicate that these two species have developed behavioural adaptations that enable them to reduce competition. This may be one potential explanation for the coexistence of these two sympatric species.

It is possible that the defined food categories are insufficiently descriptive for the remaining species, thereby preventing an accurate analysis of their dietary niche overlaps. But this result may also suggest that the mechanisms enabling these mesocarnivore species to coexist may not be contingent on their trophic level. It is possible that these mechanisms may be identified at the spatial and/or temporal level, as some studies have already shown (Barrul et al., 2012; Curveira-Santos at al., 2017; Barros, 2024).

4.4. Study limitations

There were a number of limitations in this study that prevented some conclusions from being drawn and a higher representation of some species and sites.

The low success rate in molecular identification of faecal samples collected in SG during the dry season was one of the main challenges of this study. This has limited the evaluation of the trophic niche of some species and prevented the understanding of the influence that different management of the *Montado* areas studied may have on the diet of each mesocarnivore species.

The fact that the scats were mainly collected on dirt roads may have led to the underrepresentation of some species. Some of the species (e.g. European badger and common genet) are known to leave their scats in latrines, in low density regions mostly near the dens (Fischer et al., 2003) and for genets in high places such as tree trunks (Espirito-Santo et al., 2007), making them more difficult to find in other locations such as along foot survey paths.

The defined coarse food categories ultimately showed some limitations when assessing the trophic niche of the species and their overlap. A higher discrimination of the food categories would allowed us to gain a deeper insight into the food resources these species are relying upon. In addition, DNA techniques to identify the food items present in the samples may help to identify items that may not be detected by other means (Lee et al., 2013) as a complement to the technique used.

4.5. Final remarks

Morphological identification of faecal samples has been widely used in the study of mesocarnivores. Although some features can be used to discriminate between species, scats have a high degree of plasticity in shape, and their shape and form can be very similar between ecologically similar species. This can easily lead to misidentification by observers, as has been shown. It is not always possible to find scats in their pristine shape and form. Molecular identification techniques can provide an alternative and more accurate method of scat identification.

In this study, we have successfully demonstrated the importance of implementing this method, but also its limitations. The main limitation found was related to the need to use fresher scats, which may contain higher quality DNA. It is not always possible to find a sufficient number of samples that meet these criteria, and this can limit the amount of data that can be obtained, although the data obtained are more accurate and reliable.

The development of new and more accurate methods is of great importance for the development of conservation measures. There is a greater need to obtain more information in order to properly design conservation measures and to understand the needs of each species, their role in the ecosystems, and how they are affected by anthropogenic pressures. With this study, it is possible to understand some of the misleading conclusions that can be drawn from the traditional method of identifying scat samples.

Given the increase in anthropogenic activities and pressures in the present day, it is also important to understand their influence on the communities composition and population dynamics, as well as on changes in species behaviour. For future studies, it would be of interest to compare different *Montado* areas with different types of anthropogenic activities, such as the ones under study, as an attempt to have a better understanding of this influence. However, the differences found between seasons can be helpful in understanding how the ecological needs of each species can change according to changes in environmental conditions.

Understanding the mechanisms of coexistence between sympatric and ecologically similar species and the resources they consume is also extremely useful for applying efficient conservation measures. Although the trophic niche assessments obtained are very broad, these results can be useful as a baseline for further studies. Although no great differentiation at the trophic level was found between these species, the results reinforce that species coexistence mechanisms may be based on levels other than the trophic level. For future studies, it would be of great interest to evaluate the diet of each species in comparison with the available prey and other food items. This would allow a better understanding of the importance of each food category for these species and also to understand if their diet varies according to the resources available.

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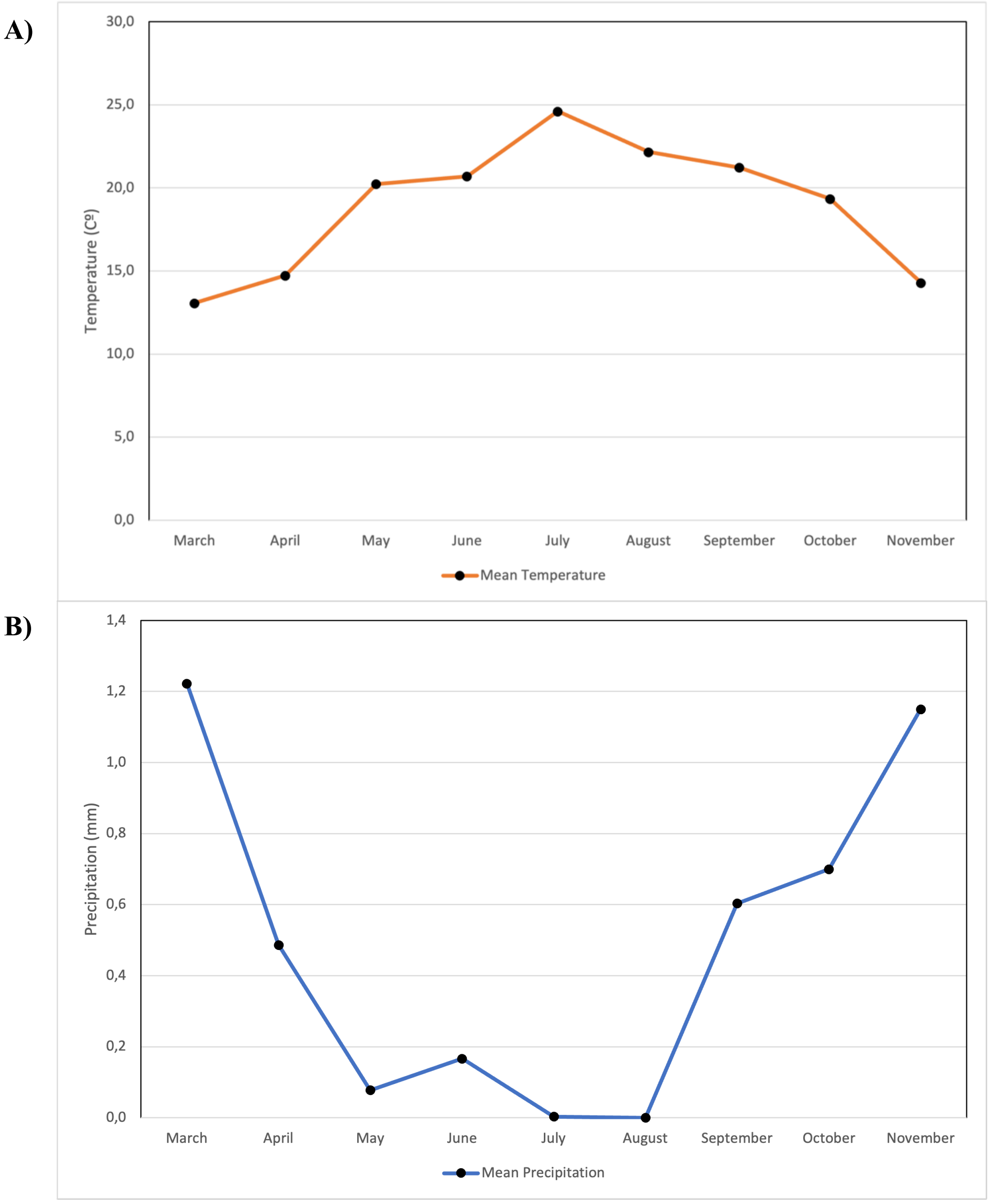
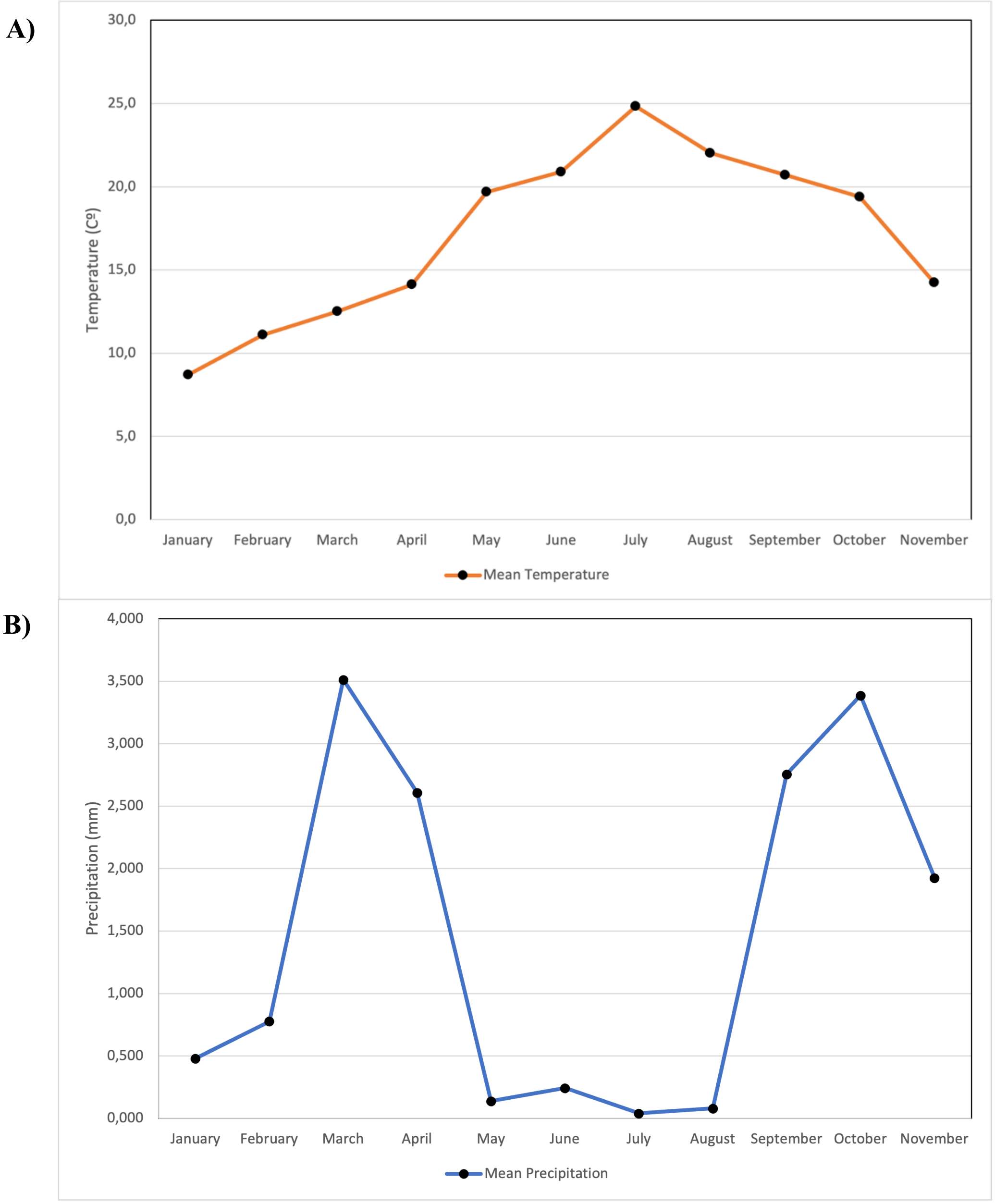
Supplementary material



Annex 1 - Mean temperature (A) and precipitation (B) from CL during 2022.



Annex 2 - Mean temperature (A) and precipitation (B) from SG during 2022.

Annex 3 - Number of presences of each food category observed in the faecal samples collected from Companhia das Lezírias divided by season and species and the total number of samples collected for each species. The food categories are mammals (M), birds (B), reptiles (R), insects (I), fruits (F), and crayfish (C). The collected samples that are represented belong to *V. vulpes* (VV), *M. foina (MF)*, *M. meles* (MM), *G. genetta* (GG), and *H. ichneumon* (HI).

	Companhia das Lezírias													
	Wet season							Dry season						
	M	B	R	I	F	C	**Total**	M	B	R	I	F	C	**Total**
VV	11	2	0	22	22	2	**30**	6	4	0	36	22	0	**37**
MF	3	1	0	4	10	0	**11**	4	6	1	9	9	0	**15**
MM	5	0	0	18	16	0	**21**	3	0	3	14	7	0	**14**
GG	6	0	0	7	4	1	**8**	7	3	1	8	2	6	**9**
HI	9	5	4	15	6	1	**16**	5	2	3	13	4	0	**13**

Annex 4 - Number of presences of each food category observed in the faecal samples collected from Serra de Grândola divided by season and species and the total number of samples collected for each species. The food categories are mammals (M), birds (B), reptiles (R), insects (I), fruits (F), and crayfish (C). The collected samples that are represented belong to *V. vulpes* (VV), *M. foina* (MF), *M. meles* (MM), *G. genetta* (GG), and *H. ichneumon* (HI).

	Serra de Grândola													
	Wet season							Dry season						
	M	B	R	I	F	C	**Total**	M	B	R	I	F	C	**Total**
VV	23	1	0	48	46	0	**52**	8	1	0	34	26	2	**34**
MF	5	0	0	24	24	0	**29**	3	0	1	11	8	1	**13**
HI	1	0	0	5	1	0	**5**	3	0	2	8	2	0	**8**
GG	1	0	0	1	1	0	**3**	0	0	0	0	1	0	**1**
MM	0	0	0	6	4	0	**6**	0	0	0	1	0	0	**1**