Joel Sartore

Feeding ecology of the Pyrenean desman in NW Iberia revealed by DNA metabarcoding: the role of habitat suitability

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Abstract

Freshwater ecosystems are among the most threatened in the world, and downstream stretches of stream networks are often the most affected by multiple anthropogenic stressors, compromising the habitat, and also food availability. The Pyrenean desman, *Galemys pyrenaicus*, is a threatened semiaquatic mammal, endemic to Southwestern Europe and suffering a range contraction into headwaters. All the data collected so far suggests that habitat suitability may influence the desman's diet although the relation isn't fully understood. In fact, although there are some studies focusing on the desman's diet, there are still unknown aspects of its feeding ecology, namely regarding spatial and ecological variation in Northwestern Iberia.

The goal of this work was to understand how habitat suitability shapes the diet of *Galemys pyrenaicus* in Northwestern Iberia, and for that, data was collected in two watersheds in northern Portugal, that encompass a gradient of habitat suitability. A DNA metabarcoding approach, an innovative molecular technique with increasing application in non-invasive diet analyses, was used to determine the diet of the Pyrenean desman, based on a two-marker approach (COI and 18S). A total of 215 faecal samples were collected between 2014 and 2016, within the Sabor and Tua watersheds. Overall, the results indicate a specialization for particular groups of aquatic insects, belonging to four main orders, namely, Ephemeroptera, Trichoptera, Diptera and Plecoptera which is consistent with previous studies. However, there is a wide diversity of taxa ingested within these orders and significant differentiation in the diet composition was found when considering both the two watersheds and habitat suitability categories.

Our findings suggest that, although using the same main type of particular groups of aquatic insects regardless of the habitat, there is some local differentiation in the diet of this species considering both watersheds and habitat suitability categories. Thus, it discloses that *G. pyrenaicus* might feed on the fauna that is present in the microhabitats of streams it can access. Nonetheless, even if a wider dietary niche increases the chance of adaptation to altered environments, food availability remains an important issue for species conservation.

Keywords

Galemys pyrenaicus, freshwater ecosystems, diet, COI & 18S two-marker approach, species conservation

Resumo

Os ecossistemas de água doce estão entre os mais ameaçados do mundo, e as regiões a jusante das redes hidrográficas são normalmente as mais afetadas por múltiplas pressões antropogénicas, comprometendo a disponibilidade de habitat e consequentemente a disponibilidade de alimento. A toupeira-de-água, *Galemys pyrenaicus*, é um mamífero semiaquático ameaçado, endémico do sudoeste da Europa e que tem sofrido uma contração para montante nas linhas de água. A informação recolhida até agora sugere que a adequabilidade do habitat pode influenciar a dieta desta espécie apesar da relação existente não ser ainda totalmente compreendida. De facto, apesar de existirem alguns estudos sobre a dieta da toupeira-de-água, ainda existem aspetos por esclarecer sobre a sua ecologia trófica, nomeadamente no que se refere à variação espacial e ecológica no noroeste Ibérico.

O objetivo principal deste trabalho consiste em compreender como é que a adequabilidade do habitat influencia a dieta da toupeira-de-água no noroeste Ibérico. Para tal, foram analisadas 215 amostras de excrementos, recolhidas entre 2014 e 2016, em duas bacias hidrográficas no norte de Portugal (Sabor e Tua), área esta que abrange um gradiente de adequabilidade do habitat. Para a determinação da dieta foi utilizada uma abordagem de *DNA metabarcoding*, uma técnica inovadora com aplicação crescente em análises de dieta a partir de amostras não invasivas, baseada na utilização de dois marcadores (COI e 18S). No geral, os resultados indicam a especialização para um conjunto de insetos aquáticos, pertencentes a quatro ordens, nomeadamente, Ephemeroptera, Trichoptera, Diptera e Plecoptera, o que é consistente com estudos anteriores. No entanto, observou-se que esta espécie ingere uma grande diversidade de taxa dentro destas ordens e foi detetado um efeito significativo tanto da localização (bacias hidrográficas) como da adequabilidade do habitat na composição da dieta desta espécie.

Os nossos resultados sugerem que embora, a um nível geral, a toupeira-de-água utilize o mesmo tipo de grupos de insetos aquáticos independentemente do habitat, esta espécie parece alimentar-se especificamente de taxa existentes nos micro-habitats das linhas de água a que consegue aceder. Mesmo que um nicho alimentar mais amplo aumente a possibilidade de adaptação para ambientes alterados, a disponibilidade de alimento continua a ser uma questão importante para a conservação das espécies.

Palavras-chave

Galemys pyrenaicus, ecossistemas de água doce, dieta, dois marcadores (COI e 18S), conservação das espécies

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List of abbreviations

API - Application Programming Interface

BOLD - Barcode of Life Data System

COI - Cytochrome *c* oxidase I

Cyt*b* - Cytochrome *b*

DNA - Deoxyribonucleic Acid

ESV - Exact Sequence Variant

FO - Frequency of occurrence

GBIF - Global Biodiversity Information Facility

GPS - Global Positioning System

IUCN - International Union for Conservation of Nature

LTER - Long-Term Ecological Research

MOTUs - Molecular Operational Taxonomic Units

NCBI - National Center of Biotechnology Information

NGS - Next Generation Sequencing

PCR - Polymerase Chain Reaction

PERMDISP - Permutational analysis of multivariate dispersions

PERMANOVA - Permutational Multivariate Analysis of Variance

Chapter 1 – Introduction

1.1. Global environmental change and freshwater ecosystems

Biodiversity is severely impacted by global environmental changes such as land use and climate warming, which are mainly promoted by the increase on human population size, posing new challenges to the conservation of natural resources (Taylor-Brown *et al.,* 2019). Human related activities are dominating earth's ecosystems, causing severe habitat fragmentation and biodiversity loss. They are a threat to the maintenance of ecosystem functions and services (Vitousek et al., 1997; Cardinale et al., 2012), leading towards extinctions at unprecedented high rates. This is especially important when it comes to freshwater ecosystems (Vörösmarty *et al.,* 2010; Quaglietta *et al.,* 2018; Flitcroft *et al.,* 2019).

Freshwater ecosystems occupy less than 1% of the Earth's surface, containing roughly 12% of all known species (Garcia-Moreno *et al.,* 2014). These ecosystems are of extreme importance since they host approximately 40% of global fish diversity and are either a shelter for amphibians, aquatic reptiles and mammals, being one third of all vertebrate species confined to freshwater (Dudgeon *et al.,* 2006). Moreover, freshwater ecosystems in unglaciated regions can be hotspots of biodiversity, where the insular nature of freshwater habitats has led to the evolution of many species with small geographic ranges, resulting in biotas with high endemism and high species turnover between basins (Dudgeon *et al.,* 2006; Strayer and Dudgeon, 2010).

However, freshwater ecosystems are endangered due to human freshwater exploitation (i.e., flow alteration, pollution, habitat degradation and loss, overexploitation of species, and invasive non-native species; Dudgeon *et al.,* 2006). Of the 29,500 freshwater dependent species so far evaluated for the IUCN Red List, 27% are threatened with extinction (Tickner *et al.,* 2020). Also, the current rate of wetland loss is three times that of forest loss (Gardner and Finlayson, 2018), and the decline rate of populations of freshwater vertebrate species more than doubles those in the terrestrial or marine systems (Grooten and Almond, 2018). Thus, with the predicted increase in human population, freshwater extinction risks will remain high over the next few decades (Strayer and Dudgeon, 2010; Vörösmarty *et al.,* 2010; Flitcroft *et al.,* 2019).

The simultaneous loss of freshwater biodiversity and increase in human pressures on water resources demands action be taken (as wildlife conservation) to stop the progressive deterioration of freshwater ecosystems and sustain the valuable services they provide (Nel *et al.,* 2009).

The multiple anthropogenic drivers do not act homogeneously across the stream network, often concentrating in downstream reaches where there is an accumulation of impacts generated upstream. Furthermore, there are higher human population densities in downstream areas, and, as such, more intensive water and land use and infrastructures (Vörösmarty *et al.,* 2010; Filipe *et al.,* 2017; Quaglietta *et al.,* 2018). This indicates that species associated with downstream reaches may become extinct or limited to more preserved upstream reaches (Meyer *et al.,* 2007; Grant *et al.,* 2010; Isaak *et al.,* 2016; Quaglietta *et al.,* 2018), being at risk due to small population sizes and loss of connectivity (Labonne *et al.,* 2008; Williams *et al.,* 2009; Quaglietta *et al.,* 2018). Yet, there is a lack of historical surveys that could have helped us understand whether the species is confined to headwaters due to its ecological requirements or headwater populations are just remnants of once larger distributions. This question is particularly harder to answer in the case of small, rare or otherwise elusive species such as the Pyrenean Desman (Quaglietta *et al.,* 2018). These uncertainties on species ecology have major consequences for conservation biology, as it is very important to understand species ability to respond to threatening processes (Scheele *et al.,* 2017). For instance, changes in macroinvertebrates community composition, structure and distribution in headwater ecosystems or across the river continuum, is often associated with habitat reduction, and long-term warming or extreme hydroclimatic events (Daufresne *et al.,* 2007; Durance and Ormerod, 2007; Chessman, 2012; Domisch *et al.,* 2013). Moreover, Domisch *et al.* (2013), in a study modelling the climatic suitability of stream macroinvertebrate species across Europe under climate change scenarios, found that cold-adapted headwater macroinvertebrate species were more vulnerable to warming climates and would lose more climatically suitable areas than warm-adapted species distributed along the mid- and lower-reaches of the river continuum. Thus, direct and indirect actions on the stream morphology, hydraulic regime and water quality compromise habitat availability and specially food availability, particularly for macroinvertebrates, which are important bioindicators of stream water quality (Herman and Nejadhashemi, 2015). Moreover, these effects on macroinvertebrate communities, will subsequently have major impacts on the presence and resilience of freshwater species that depend upon this community as main prey (e.g. Lodge *et al.,* 2012; Biffi *et al.,* 2017; Quaglietta *et al.,* 2018).

1.2. Trophic interactions and DNA Metabarcoding

Biotic interactions such as competition, predation, and trophic resource availability play a central role in the distribution of species and their abundance (Boulangeat *et al.,* 2012; Kissling *et al.,* 2012) and influence their responses to changing environments (Pearson and Dawson, 2003; Araújo and Luoto, 2007; Belmaker *et al.,* 2015).

Therefore, the analyses of trophic interactions provide key data for understanding not only animal ecology, but also evolution and conservation (Shehzad *et al.,* 2012). DNA metabarcoding has been revolutionizing this knowledge since the classical methods of diet analyses via faecal samples have been unable to identify soft and well-digested components (Pompanon *et al.,* 2012; Soininen *et al.,* 2014). For instance, stomach and faecal content analysis using microscopy is time-consuming, requires a considerable amount of training to correctly identify the prey of different taxonomic groups and often has a low taxonomic resolution, where DNA barcoding provides taxonomically more detailed results (Soininen *et al.,* 2009; Valentini *et al.,* 2009; Brown *et al.,* 2014). Likewise, direct observations can be difficult to achieve when studying elusive species (Pompanon *et al.,* 2012). Moreover, the analysis of naturally occurring stable isotope ratios (a non-invasive method), as carbon and nitrogen, has also been used to determine the diets and trophic levels of each prey assimilated during tissue development. Nevertheless, characterization of diets using stable isotopes are complicated by the variation in tissue turnover rates among organs (Peterson and Fry, 1987; Lajtha and Michener, 1994; Shiels *et al.,* 2013), the need to obtain a broad knowledge of the prey isotopic signatures (Corse *et al.,* 2010) by the difficulty to infer upon herbivore diet composition due to the large number of potential food items and the overlap between their stable isotope ratios (e.g. Soininen *et al.,* 2014).

Henceforth, DNA-based methods are being recently used in dietary studies. DNA metabarcoding uses recent techniques such as Next Generation Sequencing (NGS) and is based on the mass-amplification of DNA fragments using general or group-specific primers, followed by the cloning and sequencing of amplicons to identify individual taxa. This technique allows to study the diet of species that are difficult to determine using direct methods or whose prey are visually hard to identify (Kartzinel and Pringle, 2015), as it can be applied to non-invasive samples. It provides precise taxonomic identification of food items within highly diverse diets, for different types of samples and including non-invasive samples, without taxonomic expertise (Pompanon *et al.,* 2012).

Although DNA metabarcoding can virtually identify all species consumed by a predator, including rare food items (Soininen *et al.,* 2009; Hope *et al.,* 2014; Nielsen *et*

al., 2018), this depends on DNA quality and the availability of DNA reference databases (Deagle *et al.,* 2006; Elbrecht *et al.,* 2016). Likewise, there are still some uncertainties regarding metabarcoding potential biases and pitfalls, and how to address them, which may significantly impact the results of the dietary analysis (Nielsen *et al.,* 2018; da Silva *et al.,* 2019). For instance, DNA quality is affected by its transition time across the gut, as well as the exposure of scats to environmental conditions (Oehm *et al.,* 2011; Nielsen *et al.,* 2018). Also, amplification bias can occur due to unequal primer binding and the use of "universal" barcoding markers can bias the amplification efficiency of different species when using DNA metabarcoding (Deagle *et al.,* 2014; Piñol *et al.*, 2015). Furthermore, the proportional biomass of each species consumed (that reflects the proportions of the recovered DNA and not the number of preys consumed), may also be a constraint once it is hard to establish the contribution of each prey item to the overall diet. Therefore, all these factors should be accounted for in dietary studies (Pompanon *et al.,* 2012; Piñol *et al.*, 2015; Mata *et al.,* 2019). Thus, bias may appear during DNA extraction, DNA pooling, sequencing and during bioinformatic sorting, which might lead to misleading results. Moreover, the use of such techniques does not indicate at which life stages prey could be eaten and such a lack of information hinders our comprehension of stream food webs and species interactions (Quaglietta and Beja, 2019). However, the continual improvement of NGS technologies, on-going decreases in costs and current-massive expansion of reference databases make this approach promising in facilitating the analysis of prey taxa.

Metabarcoding using NGS technique is becoming an increasingly important tool for conservation since it delivers rapid and holistic results on species composition, diversity, ecological networks and ecosystem health, at relatively low costs (Taylor and Gemmell, 2016).

In conclusion, DNA metabarcoding facilitates high-resolution dietary analyses further disclosing trophic and habitat requirements of consumers and providing an invaluable tool to unravel food web structures (Pompanon *et al.,* 2012), being particularly useful for elusive and endangered species such as the Pyrenean desman.

1.3. *Galemys pyrenaicus*

The Pyrenean desman, *Galemys pyrenaicus* (É. Geoffroy Saint-Hilaire, 1811), is an evolutionarily unique semiaquatic mammal, being one of the two surviving species of the Tribe Desmanini (Mammalia, Soricomorpha, Talpidae; He *et al.,* 2017). This species

is characterized by large webbed hindfeet, double layered fur, a longtail and a mobile prehensile snout, which makes it a specialist in finding and feeding on the larvae of benthonic macroinvertebrates (Palmeirim and Hoffmann, 1983; Richard, 1986). This species is often associated with fast-flowing, shallow and well-oxygenated waters (e.g. Queiroz *et al.,* 1998; Charbonnel *et al.,* 2015). The Pyrenean desman is endemic to South-western Europe, where it is restricted to the Pyrenees, northern and central Spain, and northern Portugal, being listed as vulnerable by the IUCN (Fernandes *et al.,* 2008). Despite having been suffering a significant range decline across Portugal (Quaresma 2001, Quaglietta *et al.,* 2018), Spain (Gisbert and García-Perea, 2014), and France (Charbonnel *et al.,* 2016), this species is one of the less-studied European mammals (Charbonnel *et al.,* 2015).

Over the last two decades, the Pyrenean desman largely retreated to cooler, and mountainous headwater streams (Quaglietta *et al.,* 2018). Its regression was apparent in several river basins, nowadays occurring punctually in the hydrographic basins north of the Douro river (Minho, Âncora, Lima, Neiva, Cávado, Ave and Leça rivers), in the main sub-basins of Douro river, in the basins of the Vouga and Mondego rivers (middle and upper sections) and in in the headwaters of the river Zêzere (Queiroz *et al.*, 1998; Fernandes *et al.*, 2008). Local extinctions occurred in higher order streams and streams with lower slope steepness, being the species currently confined to lower order streams with high slope steepness and lower maximum temperature of the warmest month (Quaglietta *et al.,* 2018). Although there is no certainty about the reasons for these declines, they can be related to water pollution, reduced stream flows, habitat loss and fragmentation, predation by the invasive American mink *Neovison vison*, and climate change (Queiroz *et al.,* 1996; Fernandes *et al.,* 2008; Morueta-Holme *et al.,* 2010; Charbonnel *et al.,* 2015, 2016). Despite recent studies about this species (e.g. Melero *et al.,* 2012; Igea *et al.,* 2013; Charbonnel *et al.,* 2016; Biffi *et al.,* 2017; Quaglietta *et al.,* 2018), its biology and ecology remain poorly known (Melero *et al.,* 2012; Quaglietta *et al.,* 2018) and the general knowledge gap in the Pyrenean desman's natural history includes its feeding behaviour (Quaglietta and Beja, 2019).

1.3.1. What is known so far on the desman's diet

Initial studies performed on the diet of the Pyrenean desman, based on the morphologic analyses of prey content in faeces, detected that this species feeds mainly of macroinvertebrates of four orders: Ephemeroptera, Trichoptera, Diptera and Plecoptera (Santamarina and Guitian, 1988; Castién and Gosálbez, 1995). Recent

studies, using high throughput technology, namely, DNA metabarcoding of a fragment of the COI gene, also detected these four orders as the main components in the desman's diet (Gillet *et al.,* 2015; Biffi *et al.,* 2017; Esnaola *et al.* 2018a; and Hawlitschek *et al.* 2018). In particular, Biffi *et al.* (2017) reported Ephemeroptera as the most frequent order on desman's summer diet, pointing out Heptageniidae as the most frequent family, and *Baetis* as the most frequent genus. Likewise, Esnaola *et al.* (2018a) and Hawlitschek *et al.* (2018) identified Ephemeroptera and Diptera as the most abundant prey groups. Furthermore, Esnaola *et al.* (2018a) also found that the most common prey taxa were *Baetis sp*., *Hydropsyche sp*., *Odontocerum sp*. and *Psychoda sp*. on the desman winter diet. All these metabarcoding studies revealed in general a higher resolution in the taxonomic identification of food items, than it would be obtained using traditional methods, without the need for taxonomy expertise and in a cost-effective way.

Some of the recent studies, such as in Biffi *et al.* (2017) and Quaglietta and Beja (2019), suggest a higher flexibility in the desman's trophic niche. For instance, Biffi *et al.* (2017) documented the consumption of Lissamphibia by the Pyrenean desman. Likewise, Quaglietta and Beja (2019) observations illustrate the ability of Pyrenean desmans to attack, kill, handle, and eat vertebrates, reporting as well, that this species (in semi-captivity conditions) may feed on amphibians (Iberian frog – *Rana iberica),* but also fish (trout – *Salmo trutta fario*). Although Castién and Gosálbez (1995), in a work based on morphology, already described trout eggs in the intestinal tract of some individuals, these recent findings reinforces *G. pyrenaicus* as a species with a flexible feeding habit and foraging behaviour.

Although all these studies agree in the main groups eaten by this species, there are uncertainties in whether the trophic needs of this species are regulating its regression into headwaters. For instance, even though Richard (1986) mentioned that high water velocity could help the animal to counteract its natural buoyancy, Charbonnel *et al.* (2015) hypothesized that the preference of this species for fast-flowing waters was caused by a higher abundance and richness of invertebrates in fast-flowing reaches. Moreover, Esnaola *et al.* (2018b) found that desmans prefer riffles, which are more productive in terms of prey availability (Dewson *et al.,* 2007) and are easier for foraging by a buoyant predator. However, Biffi *et al.* (2017) reported that, when clustering analysed sites, based on prey assemblage, those with a more diverse diet were associated with intermediate local environmental conditions and with lower slopes, less heterogeneous riverbeds, and wooded riverbanks, while others with a less diverse diet, corresponded to sites with low human impact along the near floodplain, with higher slopes in higher altitude. They hypothesized that the difference in prey composition found in the diets between sites, could be explained by the local availability of prey, which is

dependent on fine-scale environmental conditions, and that the abundance and richness of aquatic macroinvertebrates would be directly dependent on local habitat conditions (as human-induced pollution, the different types of microhabitat in streams, water current and oxygenation, and small-scale climate variables).

Hence, all the data collected so far suggests that habitat suitability may influence the desman's diet although the relation isn't fully understood. Moreover, there are still other questions remaining regarding the Pyrenean desman's feeding ecology, particularly in Northwestern Iberia and how this varies between different adjacent watersheds. Therefore, answers for these uncertainties are of major concern since this is fundamental for this species conservation success and to help developing accurate conservation plans.

1.4. General objectives

The main objective of this study was to understand how habitat suitability shapes the feeding ecology of *G. pyrenaicus* in Northwestern Iberia. In order to achieve this main goal, this study specifically aims to: i) characterize the diet of this endangered species in Northwestern Iberia using a DNA metabarcoding approach; ii) analyse local variation in the diet, by comparing the diet between two different watersheds (Tua and Sabor), and finally iii) understand how habitat suitability may influence the species diet, by considering the probability of occurrence of the Pyrenean desman as a proxy for habitat suitability.

The accomplishment of these goals will provide relevant data concerning *G. pyrenaicus* trophic relations and its vulnerability to changes in habitat quality. Therefore, it will be of value for the establishment of appropriate conservation measures for this species.

This study was performed in the scope of a wide study focusing in assessing the distribution of the species in the region (see Quaglietta *et al.,* 2018 and Quaglietta and Beja, 2019). These works were funded by the Portuguese Science and Technology Foundation (FCT) under a Long-Term Ecological Research (LTER) project (grant LTER/BIA-BEC/0004/2009), by Energias de Portugal (EDP) Biodiversity Chair and by the EnvMetaGen project (European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 668981).

Chapter 2 – Methodology

2.1. Study area

The study was performed in Northeastern (NE) Portugal, specifically within the Sabor and Tua watersheds (N 41°09′–42°06′, W 7°37′–6°16′), encompassing the Long-Term Ecological Research (LTER) site of Baixo Sabor (Figure 2.1). The area comprises a wide range of environmental conditions regarding elevation (99–1676 m), total annual precipitation (568–1266 mm) and mean annual temperature (6.3–15.5°C). The climate is typically Mediterranean, with precipitation mostly occurring between October and March and being virtually absent during the hot summer months (June-August). Water flow is highly seasonal, with smaller streams frequently drying out or being reduced to several disconnected pools in summer, although the main watercourses and some tributaries are permanent (Ferreira *et al.,* 2016). Although the desman was widespread in the Sabor and Tua watersheds in the 1990s, (Queiroz *et al.,* 1998), currently, it occurs only in a minor portion of the streams of the study area, mainly being confined to mountainous headwater streams (Quaglietta *et al.,* 2018). In the scope of this previous work, the information on the presence of this species was used to estimate the probabilities of occurrence in the region, which was further used in the current study as a proxy of habit suitability. The probability of occurrence was predicted for each stream segment and computed considering the geostatistical model, the values of the environmental variables (i.e., stream order, slope, slope steepness, flow accumulation, distance to source and temperature), and the spatial dependencies (for more details see Quaglietta *et al.*, 2018).

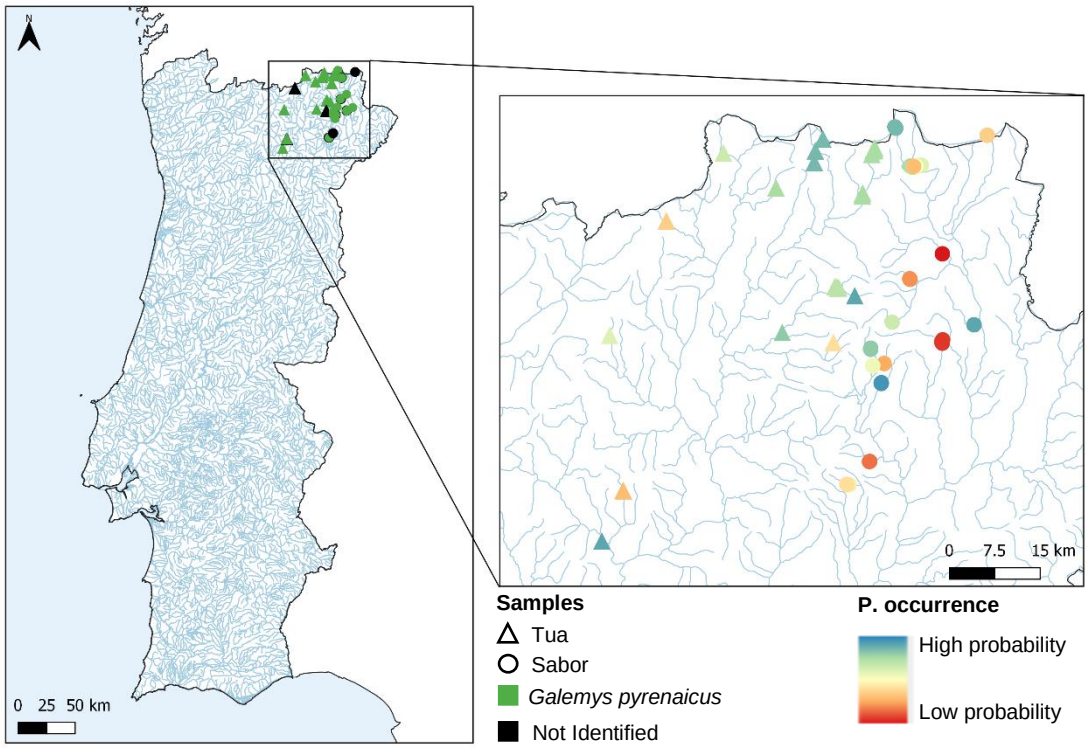

Figure 2.1: Distribution of the analysed samples within the study area. The map on the right shows only *G. pyrenaicus* samples that are coloured according to the probability of occurrence (P. occurrence) estimated for the collection site in Quaglietta *et al.* (2018).

2.2. Sampling

Pyrenean desman faecal samples were collected between 2014 and 2016 in the Sabor and Tua watersheds, in the scope of the wider study described above, that focused in assessing the distribution of the species in the area (see Quaglietta *et al.*, 2018 for details). Surveys were performed from May to September 2014, May to November 2015 and June to October 2016, and samples were collected from 39 sites following the procedures described in Quaglietta *et al.* (2018). For the collection of faeces, sterilized tweezers were used, and samples were kept in 96% ethanol until DNA extraction. All faeces were georeferenced in the field using a GPS receiver and were scored in the field to prioritize molecular identification, based on smell, colour, texture and deposition site (Queiroz *et al.,* 1998; L. Quaglietta, pers. obs.), ranging from those unlikely (1) to those very likely (10) to belong to desman. Samples were selected based on previously available information (Quaglietta *et al.,* 2018) regarding the occurrence of the species, in order to include the whole range of the estimated probability of occurrence (as proxy for habitat suitability) occupied by *G. pyrenaicus*. A total of 256 samples from the two watersheds, with a high probability of being *G. pyrenaicus* according to the field observations, were then selected and carried on to DNA extraction (Table 2.1).

Table 2.1: Detailed information on the 256 faecal samples extracted including site code, watershed, rivers, number of samples (Nr samples), collection date (Date, represented by month/year), number of samples belonging to *G. pyrenaicus* (GP samples) and not identified (NI samples), and the probability of occurrence estimated for each site (P. occurrence), that is used as proxy for habitat suitability.

Site code	Watershed	Rivers	Nr Samples	Date	P. occurrence	GP samples	NI samples
~G-390_A	Sabor	Sabor river	2	10/2016	0.64	2	
~G-390_B	Sabor	Sabor river	1	10/2016	0.90	1	
Chacim-2	Sabor	Balsemão stream	7	06/2014	0.15	2	5
Down G-395	Tua	Baceiro river	8	10/2016	0.91	8	
down S-13	Sabor	Vale de Moinhos stream	12	07/2016	0.12	9	3
Fervença-1	Sabor	Fervença river	3	05/2014	0.03	3	
G-37	Tua	Tinhela river	2	06/2015	1.00	2	
G-384	Sabor	Sabor river	10	08/2016	0.99	10	
G-390_A	Sabor	Sabor river	6	05/2014 07/2016	0.29	4	2
G-390_B	Sabor	Sabor river	5	07/2016	0.64	5	
G-390_C	Sabor	Sabor river	5	07/2016	0.72	5	
G-390_D	Sabor	Sabor river	1	07/2016	0.90	1	
G-395	Tua	Baceiro river	6	08/2016	0.91	6	
G-399 A	Tua	Baceiro river	12	09/2015 08/2016	0.91	12	
G-399 B	Tua	Baceiro river	1	08/2016	0.93	1	
G-403	Tua	Tua river	5	07/2015	0.97	5	
G-426	Tua	Rabaçal river	4	10/2015	0.88	4	
G-443	Tua	Veigas stream	8	07/2014	0.92	8	
G-451	Tua	Tua river	6	09/2015	0.99	6	
G-453	Tua	Tua river	9	09/2015	0.99	9	
G-46 A	Sabor	Azibo river	2	06/2016	0.67	2	
G-46 B	Sabor	Azibo river	15	05/2014 06/2015 09/2015	0.94	15	
G-47	Sabor	Penacal stream	1	06/2016	0.87	1	
G-49	Sabor	Salselas stream	6	05/2014 06/2015	1.00	6	
G-50	Sabor	Viveiros stream	8	07/2016	1.00	8	
G-75	Tua	Torto river	2	07/2014	0.85	2	
G-79	Tua	Macedo river	19	09/2015 07/2016	0.89	19	
G-81	Tua	Macedo river	1	06/2015	0.95	1	
Gebelim-1	Sabor	Gebelim stream	12	06/2014 06/2016	0.61	8	4
Olga-1	Sabor	Vila Franca stream	7	06/2014 06/2016	0.27	4	3
PT72	Tua	Macedo river	3	10/2016	1.00	3	
S18	Sabor	Onor river	7	06/2015	0.38	6	1
S22	Sabor	Azibo river	9	06/2015	0.67	9	
Serzeda-1_A	Sabor	Sarzeda stream	1	05/2014	0.11		1
Serzeda-1_B	Sabor	Sarzeda stream	6	05/2014 07/2016	0.20	5	1
T14	Tua	Mousse river	12	07/2015	0.36	5	7
T16	Tua	Aila stream	14	07/2015	0.30	8	6
T5	Tua	Fisão stream	15	06/2015	0.52	7	8
Up G-390	Sabor	Sabor river	3	10/2016	0.92	3	

2.3. Molecular analyses

DNA extraction was performed from faecal samples using only one pellet per sample, with the EZNA® Tissue DNA Kit (Omega Bio-tek, Norcross, USA), following the manufacturer's instructions and including an additional lysis step using the buffer described in Maudet et al. (2004) for 30 min at 56 ºC. DNA was eluted in a total volume of 50 µL and frozen at -20 ºC until DNA amplification.

Species identification was performed in all samples using a mitochondrial marker (cyt*b*) with two primer sets, one specific to desman (278bp; GpCytb-F2: TCTTACCATGGGGTCAAATATC; and GpCytb-R2: AGATAATTAGAATGAGGATTAGTG; Igea *et al.,* 2013) and an universal primer set (360bp; CB2-F: TGAGGACAAATATCATTCTGAGGG; and CB3-H: GGCAAATAGGAARTATCATTC; modified Kocher and White, 1989; Palumbi, 1996), that allows the identification of other potential species, as described in Quaglietta *et al.* (2018). The PCR products obtained from both reactions were purified using ExoSAP-IT® PCR Cleanup and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing reactions were carried out with the reverse primer using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and subsequently sequenced on a 3130xl Genetic Analyzer Sequencer (Applied Biosystems, Foster City, Ca USA). Reverse sequences were assembled and edited in Geneious Pro v8.1.7 (http://www.geneious.com/), and sequence similarity searches were performed using GenBank BLAST identification system for species identification.

Then, to study the desman's diet, two different markers were used, the 18S marker with a general set of primers that provide a broader feeding spectrum (118bp; Round1-18Sf-msq: CACCGCCCGTCGCTACTACCG; and Round1-18Sr-msq: GGTTCACCTACGGAAACCTTGTTACG; Jarman *et al.*, 2013), and COI, using a set of primers specific for insects in order to obtain more detail on the macroinvertebrate items of the diet (217bp; ind_BF1: ACWGGWTGRACWGTNTAYC; and ind_BR1: ARYATDGTRATDGCHCCDG; Elbrecht and Leese, 2017). The PCR thermal cycling profile for 18S consisted of a general protocol: 15 min at 95 °C, 20 sec at 95 °C, 20 sec at 67 °C, and 20 sec at 72 °C (a total of 35 cycles was carried out), plus 10 min at 60 °C. Also, PCR reactions were performed with a total of 10 µL using 5 µL of Qiagen© PCR Multiplex Kit Master Mix (Qiagen, Hilden, Germany), 0.3 µL of each primer (10 µM) and 1 µL of genomic DNA. The PCR thermal cycling profile for COI consisted of: 15 min at 95 °C, 30 sec at 94 °C, 20 sec at 45 °C, and 30 sec at 72 °C (a total of 41 cycles was carried out), plus 10 min at 60 °C. PCR reactions were performed with a total of 10 µL

using 5 µL of Qiagen© PCR Multiplex Kit Master Mix (Qiagen, Hilden, Germany), 0.4 µL of each primer (10 µM) and 1 µL of genomic DNA. Three PCR replicates were performed for both markers to assure reliability of the data for diet assessment.

All extraction and amplification protocols were performed in physically separated and isolated rooms, with conditions that avoided risk of DNA contamination (Gilbert *et al.,* 2005). Negative controls were performed within every extraction and PCR amplification to detect possible contaminations.

Library preparation was carried out for 18S and COI following the Illumina MiSeq protocol. Before sequencing, an indexing PCR was performed for the incorporation of the Illumina-compatible indexing primers (P5 and P7 indexes, based on Meyer and Kircher, 2010), after an initial dilution of 1:4 in order to reduce the amount of initial template and guarantee the complete incorporation of indexes in the library. Each index contained a unique 7 bp long barcode that differed at least 3 bp from any other index, allowing for the multiplex of several hundred samples in a single run (P5: 5′-AATGATACGGCGACCACCGAGATCTACACxxxxxxxTCGTCGGCAGCGTC-3′, P7: 5′-CAAGCAGAAGACGGCATACGAGATxxxxxxxGTCTCGTGGGCTCGG-3′). The indexing PCR thermal cycling profile consisted of a general protocol: 3 min at 95 °C, 30 sec at 95 °C, 30 sec at 55 °C, and 30 sec at 72 °C (a total of 10 cycles was carried out), plus 5 min at 72 °C. The indexed PCR products were cleaned, using AMPure XP beads (Beckman Coulter, Brea, CA, USA) to remove free primers and primer dimers, with two following cleaning steps with ethanol. For COI, PCR products were cleaned before indexing. The cleaned and indexed PCR products were subsequently quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted to 15 nM. Purified and normalized PCR products were pooled per marker. These two libraries were then individually quantified using qPCR (KAPA Library Quant Kit qPCR Mix; Bio-Rad iCycler) and diluted to 4 nM. Finally, libraries were sequenced separately in two runs, using approximately 90% of a 600 cycles V3 MiSeq run (Illumina) for each marker, with 20% of PhiX, for an expected average of 60,000 paired-end reads per sample-marker combination.

2.4. Bioinformatics

The software package OBITools (https://git.metabarcoding.org/obitools/obitools) was used for general sequence processing. Forward and reverse sequences were aligned (command illuminapairedend) and discarded if the overlapping quality was lower than 50. Reads were then assigned to samples, and primers and barcodes were

removed (command ngsfilter). Afterwards, unaligned sequence records were removed (command obigrep) and reads were collapsed into exact sequence variants (ESVs) (command obiuniq). Lastly, reads with a count lower than 5 were discarded and remaining sequences filtered by length size according to each marker expected size to eliminate sequences with a length outside the expected (command obigrep). The command 'obiclean' was then used to denoise the data by removing potentially spurious sequences with an 'r' level of 0.5.

All ESVs from each PCR product that had a read count <1% of the total number of reads of that PCR were removed (Mata *et al.,* 2019). This should allow the removal of most PCR and sequencing errors that still passed the 'obiclean' denoising step.

ESVs were identified by comparing the final set against the GenBank online database (https://www.ncbi.nlm.nih.gov/) and for COI also against the BOLD online database (https://www.boldsystems.org/) and the InBIO Barcoding Initiative Database (a reference database for invertebrates developed within CIBIO). Those presenting more than 98% of similarity were classified to the species level, and those above 95% were classified to the genus level. The ones with similarity above 92% were classified to the family level, and sequences presenting more than 80% of similarity were classified to the remaining levels, above family. For this assignment to each classification level, all blast results within a threshold of 2% below the highest score were considered (for instance, when there was one hit with 99% of similarity, the script looked for all the other hits between 99% and 97%), and if there was discordance the highest concordant classification was used. The results from this step were reviewed, taking into account the phylogenetic relationship inside each order. Furthermore, all taxa identified (below the order level), were verified to occur in the Iberian Peninsula, using a script that used GBIF and BOLD APIs. Those that were not assigned were verified by hand, excluding fungus and bacteria. Also, incongruences between taxa from NCBI and BOLD for COI were verified, keeping the lower taxa level, and the information obtained for each marker was merged with the final output table from the OBITools analyses.

In order to define molecular operational taxonomic units (MOTUs), Vsearch (Rognes *et al.,* 2016) was used to cluster ESVs of the phyla Arthropoda, Annelida and Mollusca identified above the genus level, using a threshold of 0.98 (id = 0.98). Afterwards, ESVs corresponding to the same taxa/cluster were merged and corresponding reads summed. The taxa detected in the extraction and PCR blanks were removed from the samples.

The final dataset for analyses included, for each marker, all samples with a minimum of 500 reads per replica and sample, and a total of 2000 reads per sample.

MOTUs were considered present in a sample if these were detected in at least two replicates. Moreover, as only potential prey items were considered for analyses, MOTUs with a high probability of arising from contaminations (e.g. chordates, microalgae, protists, plathelminths and amoebas) and from possible secondary ingestion (e.g. plants, insect and fish parasites, and copepods), were discarded. Finally, the data collected for both markers were merged by taxa using the python script developed by da Silva *et al.* (2019).

2.5. Data analysis

Frequencies of occurrence (FO) of each taxon detected were estimated in two ways: FO1 (number of samples where the taxa were detected per total number of samples); and FO2 (number of MOTUs of that taxa per total number of MOTUs). Furthermore, they were also calculated for certain groupings such as EPTs (Ephemeroptera, Plecoptera and Trichoptera orders), once it includes the main orders consumed by the Pyrenean desman that are key bioindicators of water quality, and aquatic and terrestrial taxa. Aquatic taxa included all taxa with at least 1 aquatic stage in their life cycle and terrestrial taxa those with exclusively terrestrial stages.

For analysing local variation in the diet, we have considered the two watersheds sampled, Tua and Sabor, Moreover, to understand the effect of habitat suitability in the diet of the species we have considered the probability of occurrence, estimated by Quaglietta *et al.* (2018), as a proxy for habitat suitability. Therefore, we defined 2 different categories: low habitat suitability (L: probability of occurrence [0.0, 0.84[) and high habitat suitability (H: probability of occurrence ≥0.84), using a cut-off between the highest values of probability of occurrence, in which the higher number of presences was detected (above 0.84), and the lowest values, with sporadic presences.

Overlap on the number of MOTUs between watersheds (Tua and Sabor) and habitat suitability categories (L and H) was visualized with Euler proportional elliptic diagrams, using the Euler command from the package eulerr (Larsson *et al.,* 2020) of the statistical environment R 4.0.2.

In order to assess if there were differences in prey species richness between the two watersheds and habitat suitability categories, measured in MOTUs, we calculated asymptotic MOTU richness and 95% confidence intervals with an endpoint of 1000 samples, using command iNEXT from the INEXT package (Hsieh *et al.,* 2020), using R 4.0.2.

Then, a matrix of the presence of each MOTU in all samples was made. To compare diet composition between the two watersheds and habitat suitability categories, a permutational multivariate analysis of variance (PERMANOVA) was carried out using the vegan package (function ADONIS). Once samples were collected over 3 years, in the summer season, which includes 6 months (May-October), the effect of the factors Year and Month on the diet composition was assessed in an exploratory data analysis. Both variables were shown to have a significant effect on the diet composition, however the significance observed for the variable Month could be caused by data dispersion (Supplementary Table S1 and S2). Therefore, in the comparison between watersheds, the variable Year was used as random effect, while in the comparison between habitat suitability categories, both variable Year and Watershed, were used as random effect. A dissimilarity matrix was calculated using the Jaccard measure, due to the binary (presence/absence) nature of our data. A homogeneity of dispersion test (PERMDISP) was also carried out in order to assure the significance of the PERMANOVA test, as it assumes an equal dispersion of values across the different groups. Afterwards, a similarity percentage analysis was performed, also using the vegan package (function simper), to infer the contribution of each prey (MOTUs) to the differentiation between diets, considering the analysed variables (Watershed and Habitat suitability categories).

Finally, the Czekanowski niche overlap index was calculated to understand the niche overlap in the diet of the Pyrenean desman between the two watersheds and habitat suitability categories using command czekanowski from EcoSimR package (Gotelli *et al.,* 2015), using R 4.0.2.

Chapter 3 – Results

From the 256 faecal samples, 215 were confirmed as *G. pyrenaicus* and the remaining 41 did not amplify during the species ID or were contaminated (Table 2.1). With the 215 samples, we obtained a total of 3699514 reads for COI and 4349314 reads for 18S, being the medium number of reads per replicate of 5736 for COI and 6743 for 18S. After all the bioinformatics and analytical procedures, we obtained a final data set comprising 204 samples from 38 sites, with a medium of 5 samples per site [1 – 19]. Regarding the categories considered, the samples were distributed as follows: 102 samples from Tua and 102 samples from Sabor; 79 from low habitat suitability (L) and 125 from high habitat suitability (H) categories. Overall, we identified 209 prey items of three phyla and five taxonomic classes (Supplementary Table S3). The Arthropoda is the phylum with higher representation in the diet (92.5% of the detected occurrences – FO2), being composed by 3 classes, 17 orders, and 49 families. The phylum Annelida (5.8% of the detected occurrences – FO2) is composed by 1 class, 3 orders, and 4 families. The phylum Mollusca (1.7% of the detected occurrences – FO2) is composed by 1 class, 2 orders and 2 families. In total, 22 orders were identified, being Ephemeroptera, followed by Trichoptera, Diptera and Plecoptera the ones with higher representation in the diet, occurring in 52.9%, 50.5%, 45.1% and 21.1%, respectively, of the faecal samples (calculated with FO1) and are responsible for 22.2%, 20.9%, 17.9% and 7.1%, respectively, of the detected occurrences (calculated with FO2 - Figure 3.1, Supplementary Table S3).

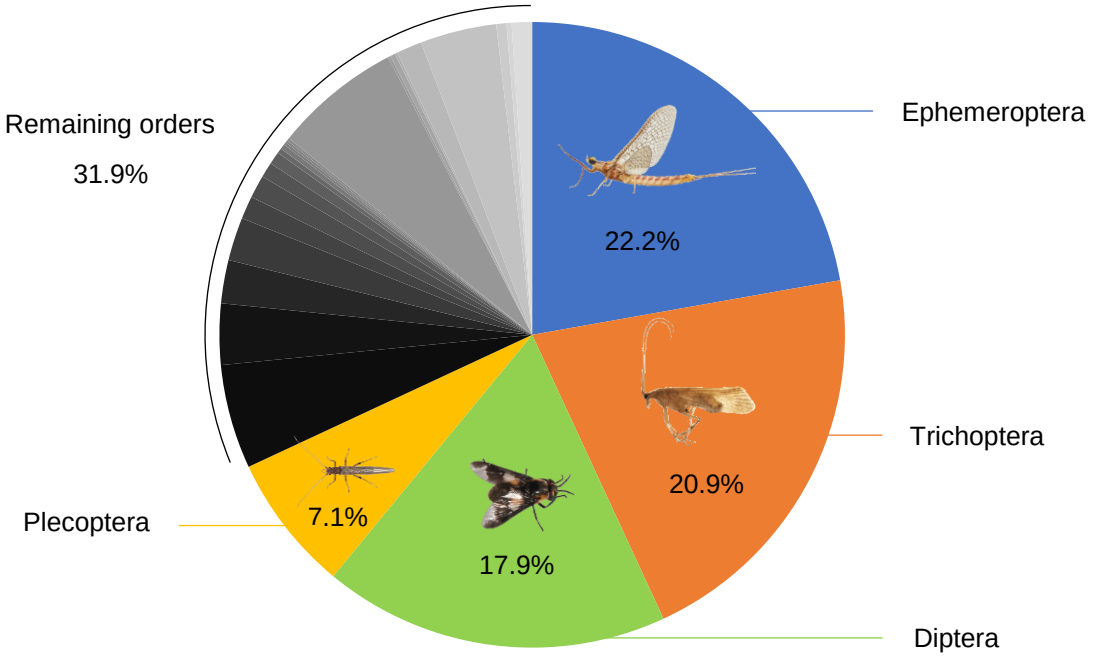

Figure 3.1: Frequency of occurrence (FO2) of the four main orders detected in *G. pyrenaicus* diet.

EPT taxa occur in 73.5% of the faecal samples and are responsible for 50.2% of the detected occurrences. Also, when taking into account aquatic and terrestrial taxa, the aquatic occurs in 95.7% of the faecal samples, while the terrestrial occurs only in 26.6%. Likewise, aquatic taxa are responsible for 89.4% of the detected occurrences, while terrestrial taxa are only responsible for 10.6%.

When comparing the diet of the Pyrenean desman between Sabor and Tua watersheds, we observe that the most consumed orders are the same, namely, Ephemeroptera, Trichoptera and Diptera, followed by Plecoptera. Nevertheless, there is some variation in the FO2 between watersheds, with a higher difference between both in the consumption of Ephemeroptera (with 4.6% higher consumption regarding detected occurrences in Sabor), followed by Diptera (4.2% higher consumption in Sabor), Trichoptera (3.8% higher consumption in Tua) and Plecoptera (3.6% higher consumption in Sabor) (Figure 3.2).

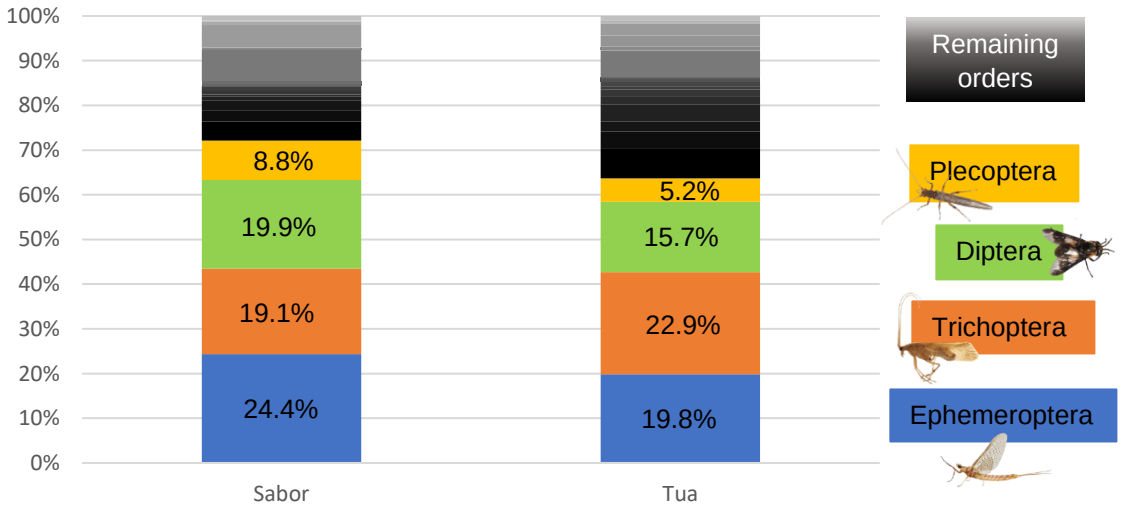

Figure 3.2: Frequency of occurrence (FO2) of the four main orders detected in the diet of *G. pyrenaicus*, in Sabor and Tua watersheds.

When considering all taxa, the number of MOTUs ingested in each of the two watersheds (species richness) is the same (132 MOTUs), and the extrapolated species richness, shows an overlap on both lower and higher 95% confidence limits (Sabor = 249 ± 52.5 (213 – 428); Tua = 256 ± 56.2 (220 - 450)). However, only 55 of those MOTUs was detected on both watersheds, having a considerable number of unique MOTUs (77) in each site (Figure 3.3).

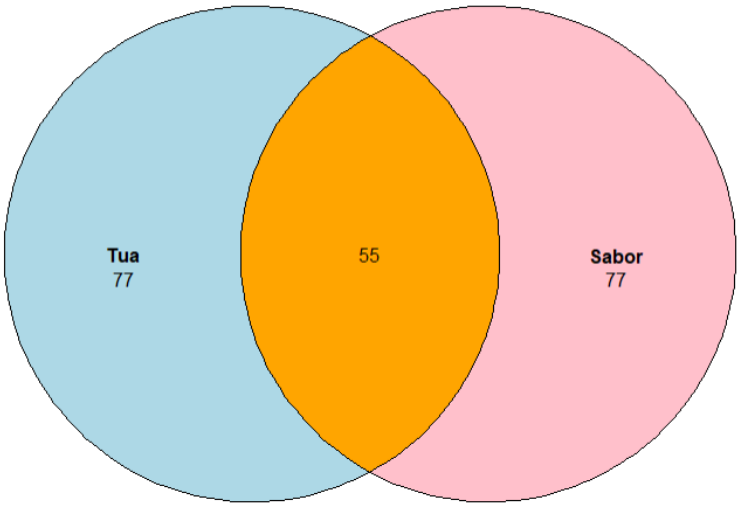

Figure 3.3: Euler diagram showing the number of MOTUs ingested in the Tua and Sabor watersheds.

PERMANOVA revealed significant differences in the MOTU composition of the diet between the two watersheds (p=0.008; Supplementary Table S4), and there was no effect of data dispersion on the results (p=0.4526; Supplementary Table S5). However, there is a medium overlap in the MOTUs ingested between the two watersheds (Czekanowski index = 0.50). The MOTUs that contributed the most for the differences in the diet between watersheds, belong to the Plecoptera (*Isoperla grammatica*) and Ephemeroptera (*Ecdyonurus* sp) orders, followed by Trichoptera (*Allogamus mortoni* and *Limnephilus guadarramicus*) and Diptera (*Psychoda* sp), all of those with a higher frequency of occurrence in Sabor (Figure 3.4).

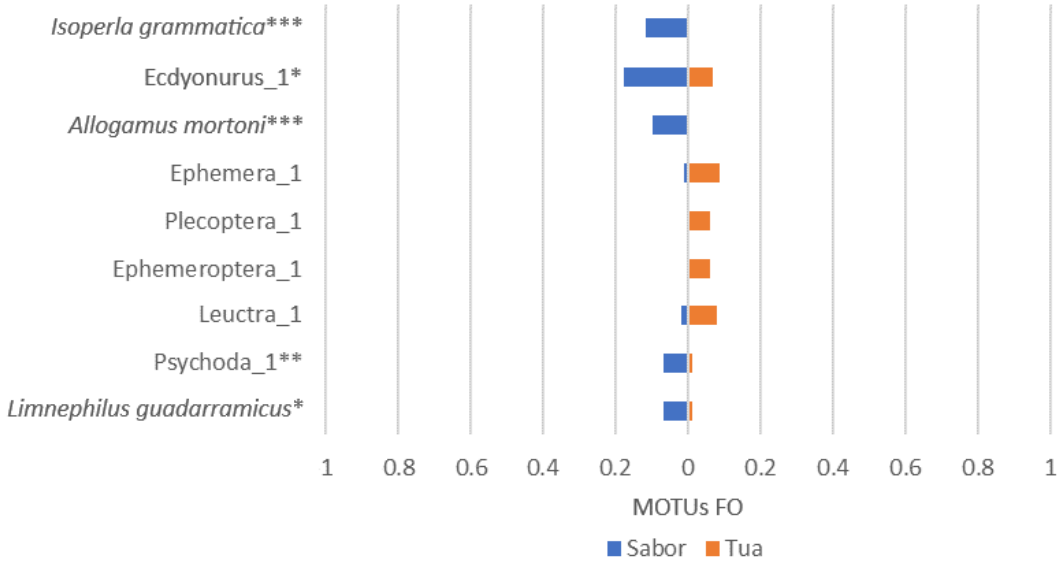

Figure 3.4: Results of the similarity percentage analysis. Frequency of occurrence (FO1) of Molecular Operational Taxonomic Units (MOTUs) with the highest contribution to differences in the diet of *G. pyrenaicus* between Sabor and Tua watersheds. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05.

When looking into the variation in the diet of the Pyrenean desman considering habitat suitability (L=Low and H=High habitat suitability), we observe that the most consumed orders are the same in both categories, namely, Ephemeroptera, Trichoptera and Diptera, followed by Plecoptera, Coleoptera and Hirudinida. Nevertheless, there is some variation in the FO between categories, with a higher difference in the consumption of Coleoptera (with 3.5% higher consumption in the sites with H habitat suitability), followed by Ephemeroptera (2.3% higher consumption in the sites with L habitat suitability) and Hirudinida (2.2% higher consumption in the sites with L habitat suitability) (Figure 3.5).

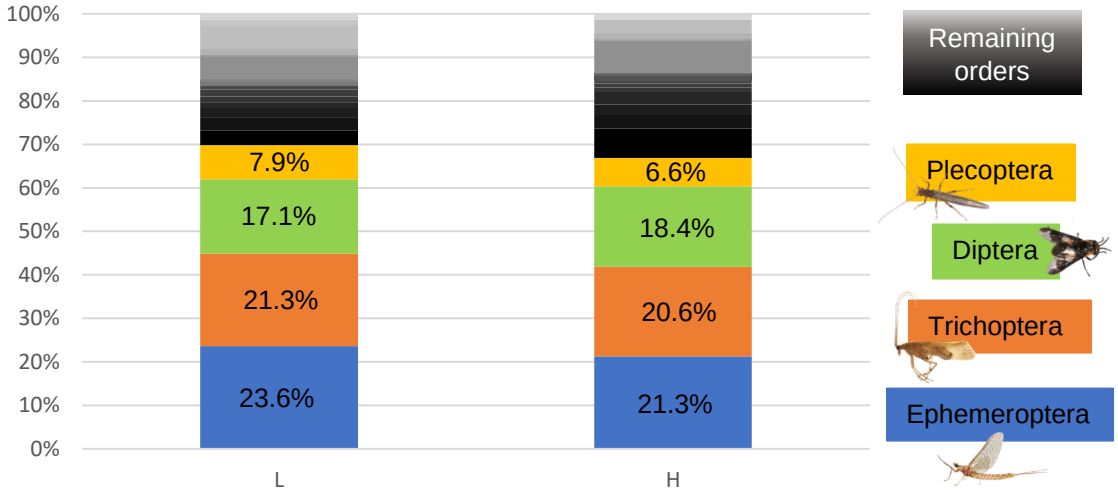

Figure 3.5: Frequency of occurrence (FO2) of the four main orders found in the diet of *G. pyrenaicus* in both habitat suitability categories (L=low habitat suitability; H=high habitat suitability).

The number of MOTUs ingested (species richness) in the two categories of habitat suitability is lower on the L category (L=112 MOTUs; H=156 MOTUs), though the extrapolated species richness is similar, with an overlap on the lower limit of the 95% confidence interval (L = 209 ± 39.5 (166 – 328); H = 237 ± 28.2 (210 – 324)). However, only 59 of those MOTUs were detected on both categories, having a considerable number of unique MOTUs in each category (L=53 MOTUs; H=97 MOTUs; Figure 3.6).

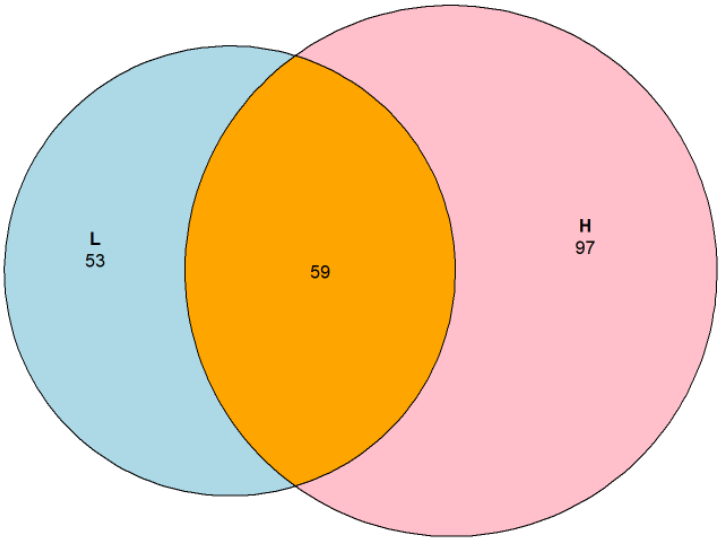

Figure 3.6: Euler diagram showing the number of MOTUs in the two categories of habitat suitability (L=low habitat suitability; H=high habitat suitability).

PERMANOVA revealed significant differences in the MOTU composition of the diet between the two categories, considering the variable year (p=0.017; Supplementary Table S6) and watershed as random effect (p=0.026; Supplementary Table S7) and no effect of data dispersion on the results was observed (p=0.3868; Supplementary Table S8). However, there is a medium overlap between diet MOTUs (Czekanowski index = 0.57).

The MOTUs that contributed the most for the differences in the diet between the two categories belong to the Ephemeroptera (Leptophlebiidae and Baetidae), Trichoptera (*Limnephilus guadarramicus*) and Diptera (*Psychoda* sp) orders, all having a higher frequency of occurrence in the lowest (L) habitat suitability category (Figure 3.7).

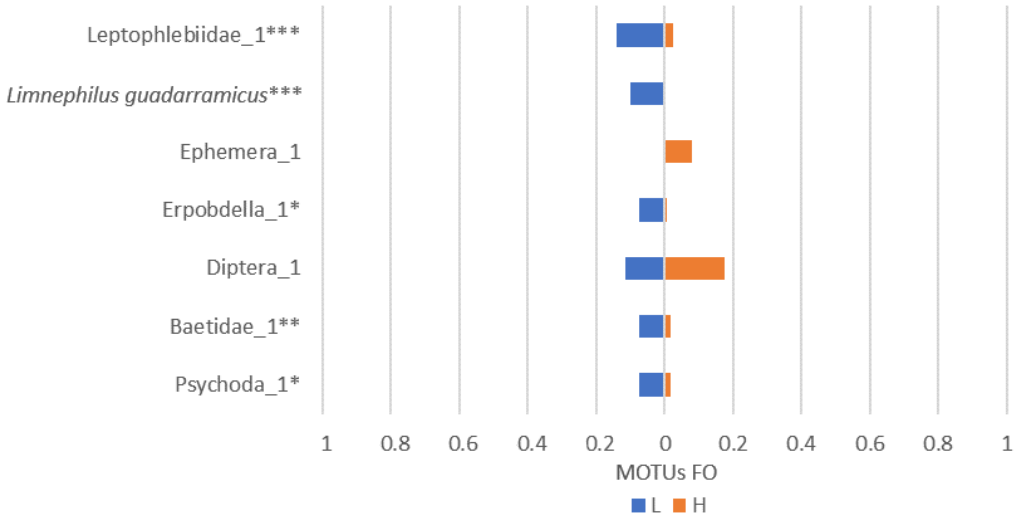

Figure 3.7: Results of the similarity percentage analysis. Frequency of occurrence (FO1) of Molecular Operational Taxonomic Units (MOTUs) with the highest contribution to differences in the diet of *G. pyrenaicus* between the lowest (L) and highest (H) habitat suitability categories. Magnitude of significance levels shown with asterisks: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Chapter 4 – Discussion

4.1. Diet of the Pyrenean desman in Northwestern Iberia

This study unveils the feeding ecology of *G. pyrenaicus* in Northwestern Iberia using DNA-based methods. Overall, our results are consistent with information collected in previous studies of the diet of this species in Iberia, considering the main groups of macroinvertebrates ingested (Ephemeroptera, Trichoptera, Diptera, and Plecoptera).

Despite the ecological importance of Pyrenean desman, previous information regarding its feeding ecology in the Northwestern Iberia was scarce, with only one other DNA-based study performed recently (Hawlitschek *et al.,* 2018). Nevertheless, this previous work was mainly focused on the technical aspects of diet analyses in this species, and just included few samples from our study region (from a Tua affluent). Here, we have extended the analysis by using a larger amount of samples, collected throughout three years, during the summer months, and therefore obtaining a more complete view of the desman's feeding ecology. Furthermore, we considered a two-marker approach, integrating information from two different molecular markers, with different resolution, in order to obtain a general view of the diet, with the 18S marker, and also detail into the macroinvertebrates, with the COI marker, which comprehend the main prey of this endangered species. Also, the specific approach used for data analyses, allowed us to reduce the problems of marker bias and taxonomic resolution, by eliminating the duplicates resulting from the same individual prey being detected, by different markers, at different taxonomic resolutions (da Silva *et al.,* 2019).

Our results show that the Pyrenean desman ingests both aquatic and terrestrial taxa, although it is evident that terrestrial taxa (10.6%) are consumed in lower frequencies in contrast to aquatic taxa (89.4%). This is consistent with previous studies and confirms that *G. pyrenaicus* predates mostly on aquatic prey (Santamarina and Guitian, 1988; Castién and Gosálbez, 1995; Gillet *et al.,* 2015; Biffi *et al.,* 2017; Esnaola *et al.,* 2018a; Hawlitschek *et al.,* 2018). In a more extensive study in the Pyrenees, Biffi *et al.* (2017), that also used a DNA-based approach with a COI gene fragment, reports similar results, as they found that exclusively aquatic items represented 4.1% of prey and taxa with at least 1 aquatic stage and 1 terrestrial stage represented 88.3% of prey, whereas exclusively terrestrial prey represented only 7.7% of prey. Other authors, such as Castién and Gosálbez (1995), also mention the ingestion of terrestrial taxa (e.g. Opiliones), although the lower taxonomic resolution of this study (mostly because it is a

morphology-based study) does not allow the distinction of terrestrial and aquatic taxa within the same order.

Within the aquatic prey, EPTs include the main orders consumed by the Pyrenean desman (that are also key bioindicators of water quality) and occur in 73.5% of our faecal samples being responsible for 50.2% of the detected occurrences. Our results show a higher frequency in the detected occurrences of EPT when comparing to those from Biffi *et al.* (2017), in which EPT corresponded to 28.2% of the identified prey, which can be related to the use of a two-marker approach.

We found Insecta as the class that contributes the most for the diet of *G. pyrenaicus*, which is consistent with Biffi *et al.* (2017). However, in our study, this class is followed by Clitellata (16.2%), Arachnida (11.8%), Gastropoda (6.4%), and Malacostraca (0.5%), the latter having little representation in the diet, while in Biffi *et al.* (2017) Malacostraca was shown to have a higher contribution (18%) (Table 4.1). These differences can be explained by the additional information obtained with the 18S marker, as most of the taxa of the classes Clitellata and Gastropoda were detected by this general marker, highlighting the advantages of the use of a two-marker approach.

Regarding the overall composition of the diet in Northwestern Iberia, our results corroborate previous studies, both morphology-based as well as recent DNA-based studies (using a COI marker), as the desman's diet was found to be mainly composed of aquatic prey such as insects of the orders Ephemeroptera, Trichoptera, Diptera and Plecoptera (Santamarina and Guitian, 1988; Castién and Gosálbez, 1995; Gillet *et al.,* 2015; Biffi *et al.,* 2017; Esnaola *et al.,* 2018a; Hawlitschek *et al.,* 2018). Although the main components of the diet (consumed orders) are the same, there is some variation regarding the ingested families (Table 4.1). As in Biffi *et al.* (2017), the most frequent order in the diet was Ephemeroptera, however, contrarily to these authors that reports Heptageniidae as the most frequent family (and Castién and Gosálbez, 1995) and Baetis (Baetidae, 56.4%) as the most frequent genus included within this order, in our study, the most frequent family was Leptophelebiidae, followed by Heptageniidae, and the most frequent genus Ecdyonurus (12.3%; which belongs to the Heptageniidae family; Table 4.1).

Table 4.1: Summary table with the main taxa consumed by *G. pyrenaicus* (represented by FO1) in the different studies, highlighting the more frequent orders and families inside the Insecta class. n stands for number of samples analysed in each study.

Taxa				Morphological studies		DNA metabarcoding studies	
				Santamarina and Guitian (1988)	Castién and Gosálbez (1995)	Biffi *et al*. (2017)	This study
			n	8	46	383	204
Class	**Order**	**Family**					
Arachnida						2.4%	11.8%
Insecta						99.7%	96.6%
	Diptera			24.0%	13.3%	45.7%	45.1%
	Ephemeroptera			35.8%	46.6%	86.7%	52.9%
		Baetidae				56.4%	6.9%
		Heptageniidae			95.6%	59.0%	22.1%
		Leptophelebiidae					23.0%
	Plecoptera			4.3%	9.0%	84.9%	21.1%
	Trichoptera			32.0%	23.1%	64.0%	50.5%
Malacostraca						18.0%	0.5%
Clitellata						0.5%	16.2%
Gastropoda					<4.0%	0.3%	6.4%

Recent findings suggest higher flexibility in the feeding habits and foraging behaviour of the Pyrenean desman, since it was observed that it could feed on Lissamphibia (Biffi *et al.*, 2017; Quaglietta and Beja, 2019) and fish (Quaglietta and Beja, 2019). Castién and Gosálbez (1995), on a work based on morphology, also described the presence of trout eggs in the intestinal tract of some individuals. However, the detections of vertebrates within the diet were always with relatively low frequencies of occurrence. In the current study, we did not detect fish nor Lissamphibia in the preyed items.

Overall, our results indicate a specialization for particular groups of aquatic insects, belonging to four main orders, namely, Ephemeroptera, Trichoptera, Diptera and Plecoptera which is consistent with previous studies. However, there is a wide diversity of taxa ingested within these orders.

4.2. Effect of location and habitat suitability on the desman's diet

The results of this study revealed that, despite the stability in the main groups ingested, significant differences were detected in the diet of the Pyrenean desman,

considering MOTUs, both between watersheds and the two habitat suitability categories. We found that although the number of taxa ingested in each of the two watersheds is the same, there are some differences in the taxa composition between them. Also, a lower number of taxa was ingested in the L habitat suitability category, although with a similar extrapolated species richness to the H category.

These differences between watersheds and habitat suitability categories could be explained by the local availability of prey, which is dependent on fine-scale environmental conditions, and by the abundance and richness of aquatic macroinvertebrates, that are directly dependent on local habitat conditions (as human-induced pollution, the different types of microhabitat in streams, and water current and oxygenation) (Biffi *et al.*, 2017). Furthermore, our results indicate that there may be some consistent differences between watersheds and habitat suitability categories, as there are taxa, *Limnephilus guadarramicus* and *Psychoda* sp, that contribute significantly to the differences between both factors considered in the analyses (Figure 3.4 and 3.7). These taxa are more frequent in the lowest habitat suitability category and in the Sabor watershed, which may be explained by the fact that Sabor has an overall lower habitat suitability than Tua watershed (Quaglietta *et al.,* 2018). This possibility is considered because, although in 1993–96 desmans were widely distributed across the study area, the distribution of the Pyrenean desman appeared more continuous in the Tua than in the Sabor watershed. In contrast, in 2014–15 the species was largely confined to headwater streams, yet with a larger distribution still found in the Tua than in the Sabor watershed. These distribution shifts are also captured by the map of predicted extinctions across time periods in Quaglietta *et al.* (2018), which showed that the species disappeared from vast areas, particularly in downstream reaches (which are considered to hold low habitat suitability) and in the Sabor watershed.

Taking this into account, it would be expected a higher prey availability upstream once there is less human-induced pollution in those areas (Charbonnel *et al.,* 2015; Quaglietta *et al.,* 2018). Biffi *et al.* (2017) found that the Pyrenean desman had a more diverse diet in areas exhibiting lower slopes, less heterogeneous riverbeds, and wooded riverbanks (i.e., with lower habitat suitability), but also in intermediate local environmental conditions, being less diverse in sites with low human impact along the near floodplain, with higher slopes in higher altitude (i.e., with higher habitat suitability). Although we observed a higher number of MOTUs in areas with higher habitat suitability (Figure 3.6),

we did not find significant differences at the species richness level neither between watersheds nor considering the habitat suitability categories.

Considering the overall obtained data, we can observe that, despite some differences detected between watersheds and related with the habitat suitability categories considered, the most consumed prey belong always to only four orders (Ephemeroptera, Trichoptera, Diptera and Plecoptera) indicating a specialization for these groups. However, there is a wide diversity of taxa ingested within these orders, as indicated by the high number of taxa ingested with low FO (Supplementary Table S3), and some variability concerning spatial and habitat factors, which supports Biffi *et al.* (2017) statement that *G. pyrenaicus* may adopt a more generalist foraging strategy. As these authors suggest, it seems that the availability of foraging habitats for Pyrenean desmans could be constrained by local physical features, thus feeding on the fauna that is present in the microhabitats of streams it can access. This could additionally justify the differences found between watersheds and habitat suitability categories. Nevertheless, even if a wider dietary niche increases the chance of adaptation to altered environments (Murgatroyd *et al.,* 2016), food availability remains an important issue for this species conservation, especially because the main preyed orders are associated to water quality, being susceptible to perturbations. Disturbances to freshwater environments, such as those mentioned above, might result in a decline in the abundance and richness in the aquatic invertebrate communities, having harmful consequences for *G. pyrenaicus*. Since there isn't barely any published information about the status and distribution of invertebrates on these watersheds, it would be important to improve reference collections and catalogues of species of macroinvertebrates that inhabits both watersheds, to ascertain food availability for the species. Moreover, even though two large hydroelectric dams have recently been built near the mouth of both Tua and Sabor rivers, they were still under construction when the study was carried out, and thus potentially only affected a very small portion of the study area. Nevertheless, their presence might be considered as one of the main threats to the habitats and aquatic populations of the area, because it caused the submersion of an important stretch of the river. Additionally, there are records of some punctual pollution in the Tua watershed, mainly of industrial source and from pig farms, being this further compromised due to the lack of Industrial Water Treatment in the area (Carneiro, 2016). Another factor that might affect this species survival and impact its feeding ecology, is that the larger streams of the Tua and Sabor watersheds have been increasingly invaded by two exotic crayfish *Procambarus clarkii* and *Pacifastacus leniusculus* (Bernardo *et al.,* 2011; Filipe *et al.,* 2017). These species have high abundances in some sites, and may contribute to the

decrease in habitat suitability for desmans, as they can affect macroinvertebrate communities and compete with *G. pyrenaicus* for the same resources (Lodge *et al.,* 2012). Furthermore, the increase in extreme summer temperatures due to climate change may reduce water availability (Allan *et al.,* 2005; Brooks, 2009), affecting aquatic prey abundance (Durance and Ormerod, 2007; Domisch *et al.,* 2013) and, thus, the resilience of the Pyrenean desman.

Altogether, we found variation in the diet of *G. pyrenaicus* between watersheds and habitat suitability, although more detailed information is needed to better understand the impact that water quality, hydroelectric dams, invasive species, climate change, the destruction of the riparian galleries and habitat heterogeneity have on the feeding ecology of this elusive species and how it contributes to its decline, which is fundamental to Pyrenean desman conservation success.

Chapter 5 – Conclusion

This study revealed some local differentiation, considering both location and habitat suitability (the last one being determined by the probability of occurrence) in the diet of *G. pyrenaicus* in Northeastern Portugal using DNA-based methods. Our results indicate a specialization for particular groups of aquatic insects, belonging to four main orders, namely, Ephemeroptera, Trichoptera, Diptera and Plecoptera which is consistent with previous studies. However, they also disclose that this species may adopt a more generalist foraging strategy than previously thought. Hence, our findings suggest that, although using the same main type of resources regardless of the habitat, this species might feed on the fauna that is present in the microhabitats of streams it can access.

Nevertheless, it would be important to have additional information regarding the availability of macroinvertebrates that could be related with the habitat suitability for the desman in each watershed in order to allow for a better understanding of the variation detected in our study. Furthermore, several other factors, such as the impact of water pollution, the construction of hydroelectric dams, and the spreading of invasive species should be further explored. Along with these, the effect of temperature shifts due to climate change, the influence of microhabitats, the destruction of the riparian galleries and habitat heterogeneity, should be also analysed. This way, it would be possible to better understand these impacts on the feeding ecology of such an elusive and unique species as the Pyrenean desman, which is fundamental to this species conservation success.

References

Allan, J. D., Pallmer, M., & Poff, N. L. (2005). Climate Change and Freshwater Ecosystems. In *Climate Change and Biodiversity*:274–290. Lovejoy, T.E. and Hannah, L. (Eds.). New Haven: Yale University Press.

Araújo, M. B., & Luoto, M. (2007). The importance of biotic interactions for modelling species distributions under climate change. *Global Ecology and Biogeography*, **16**: 743–753.

Belmaker, J., Zarnetske, P., Tuanmu, M. N., Zonneveld, S., Record, S., Strecker, A., & Beaudrot, L. (2015). Empirical evidence for the scale dependence of biotic interactions. *Global Ecology and Biogeography*, **24**: 750–761.

Bernardo, J. M., Costa, A. M., Bruxelas, S., & Teixeira, A. (2011). Dispersion et coexistence de deux écrevisses non-natives (*Pacifastacus leniusculus* et *Procambarus clarkii*) au NE du Portugal sur une période de 10 ans. *Knowledge and Management of Aquatic Ecosystems*, **401**.

Biffi, M., Gillet, F., Laffaille, P., Colas, F., Aulagnier, S., Blanc, F., Galan, M., Tiouchichine, M. L., Némoz, M., Buisson, L., & Michaux, J. . R. (2017). Novel insights into the diet of the Pyrenean desman (*Galemys pyrenaicus*) using next-generation sequencing molecular analyses. *Journal of Mammalogy*, **98**: 1497–1507.

Boulangeat, I., Gravel, D., & Thuiller, W. (2012). Accounting for dispersal and biotic interactions to disentangle the drivers of species distributions and their abundances. *Ecology Letters*, **15**: 584–593.

Brooks, R. T. (2009). Potential impacts of global climate change on the hydrology and ecology of ephemeral freshwater systems of the forests of the northeastern United States. *Climatic Change*, **95**: 469–483.

Brown, D. S., Ebenezer, K. L., & Symondson, W. O. C. (2014). Molecular analysis of the diets of snakes: Changes in prey exploitation during development of the rare smooth snake *Coronella austriaca*. *Molecular Ecology*, **23**: 3734–3743.

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., Narwani, A., MacE, G. M., Tilman, D., Wardle, D. A., Kinzig, A. P., Daily, G. C., Loreau, M., Grace, J. B., Larigauderie, A., Srivastava, D. S., & Naeem, S. (2012). Biodiversity loss and its impact on humanity. *Nature*, **486**: 59–67.

Carneiro, I. S. M. (2016). *Territorial and Social Behaviour of the Pyrenean desman (Galemys pyrenaicus) assessed from Scat Deposition*. Master Thesis in Ecology. Faculty of Sciences and Technology - University of Coimbra, Coimbra. 133pp.

Castién, E., & Gosálbez, J. (1995). Diet of Galemys Pyrenaicus (Geoffroy, 1811) in the

North of the Iberian Peninsula. *Netherlands Journal of Zoology*, **45**: 422–430.

Charbonnel, A., Buisson, L., Biffi, M., D'Amico, F., Besnard, A., Aulagnier, S., Blanc, F., Gillet, F., Lacaze, V., Michaux, J. R., Némoz, M., Pagé, C., Sanchez-Perez, J. M., Sauvage, S., & Laffaille, P. (2015). Integrating hydrological features and genetically validated occurrence data in occupancy modelling of an endemic and endangered semi-aquatic mammal, *Galemys pyrenaicus*, in a Pyrenean catchment. *Biological Conservation*, **184**: 182–192.

Charbonnel, A., Laffaille, P., Biffi, M., Blanc, F., Maire, A., Némoz, M., Sanchez-Perez, J. M., Sauvage, S., & Buisson, L. (2016). Can recent global changes explain the dramatic range contraction of an endangered semi-aquatic mammal species in the French pyrenees? *PLoS ONE*, **11**: e0159941.

Chessman, B. C. (2012). Biological traits predict shifts in geographical ranges of freshwater invertebrates during climatic warming and drying. *Journal of Biogeography*, **39**: 957–969.

Corse, E., Costedoat, C., Chappaz, R., Pech, N., Martin, J.-F., & Gilles, A. (2010). A PCR-based method for diet analysis in freshwater organisms using 18S rDNA barcoding on faeces. *Molecular Ecology Resources*, **10**: 96–108.

da Silva, L. P., Mata, V. A., Lopes, P. B., Pereira, P., Jarman, S. N., Lopes, R. J., & Beja, P. (2019). Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists. *Molecular Ecology Resources*, **19**: 1420–1432.

Daufresne, M., Bady, P., & Fruget, J. F. (2007). Impacts of global changes and extreme hydroclimatic events on macroinvertebrate community structures in the French Rhône River. *Oecologia*, **151**: 544–559.

Deagle, B. E., Eveson, J. P., & Jarman, S. N. (2006). Quantification of damage in DNA recovered from highly degraded samples - A case study on DNA in faeces. *Frontiers in Zoology*, **3**.

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome *c* oxidase subunit I marker : not a perfect match. *Biology Letters*, **10**: 20140562.

Dewson, Z. S., James, A. B. W., & Death, R. G. (2007). A review of the consequences of decreased flow for instream habitat and macroinvertebrates. *Journal of the North American Benthological Society*, **26**: 401–415.

Domisch, S., Araújo, M. B., Bonada, N., Pauls, S. U., Jähnig, S. C., & Haase, P. (2013). Modelling distribution in European stream macroinvertebrates under future climates. *Global Change Biology*, **19**: 752–762.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., Naiman, R. J., Prieur-Richard, A. H., Soto, D., Stiassny, M. L. J., & Sullivan, C.

A. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews of the Cambridge Philosophical Society*, **81**: 163–182.

Durance, I., & Ormerod, S. J. (2007). Climate change effects on upland stream macroinvertebrates over a 25-year period. *Global Change Biology*, **13**: 942–957.

Elbrecht, V., & Leese, F. (2017). Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Frontiers in Environmental Science*, **5**: 1–11.

Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, P., Beisel, J. N., Coissac, E., Boyer, F., & Leese, F. (2016). Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. *PeerJ*, **4**: e1966.

Esnaola, A., Arrizabalaga-Escudero, A., González-Esteban, J., Elosegi, A., & Aihartza, J. (2018a). Determining diet from faeces: Selection of metabarcoding primers for the insectivore Pyrenean desman (*Galemys pyrenaicus*). *PLoS ONE*, **13**: e0208986.

Esnaola, A., González-Esteban, J., Elosegi, A., Arrizabalaga-Escudero, A., & Aihartza, J. (2018b). Need for speed: Preference for fast-flowing water by the endangered semi-aquatic Pyrenean desman (*Galemys pyrenaicus*) in two contrasting streams. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **28**: 600–609.

Fernandes, M., Herrero, J., Aulagnier, S., & Amori, G. (2008). *Galemys pyrenaicus*. The IUCN Red List of Threatened Species 2008: e.T8826A12934876. https://doi.org/10.2305/iucn.uk.2008.rlts.t8826a12934876.en.

Ferreira, M., Filipe, A. F., Bardos, D. C., Magalhães, M. F., & Beja, P. (2016). Modeling stream fish distributions using interval-censored detection times. *Ecology and Evolution*, **6**: 5530–5541.

Filipe, A.F., Quaglietta, L., Ferreira, M., Magalhães, M. F., & Beja, P. (2017). Geostatistical distribution modelling of two invasive crayfish across dendritic stream networks. *Biological Invasions*, **19**: 2899–2912.

Flitcroft, R., Cooperman, M. S., Harrison, I. J., Juffe-Bignoli, D., & Boon, P. J. (2019). Theory and practice to conserve freshwater biodiversity in the Anthropocene. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **29**: 1013–1021.

Garcia-Moreno, J., Harrison, I. J., Dudgeon, D., Clausnitzer, V., Darwall, W., Farrell, T., Savy, C., Tockner, K., & Tubbs, N. (2014). Sustaining Freshwater Biodiversity in the Anthropocene. In *The Global Water System in the Anthropocene: Challenges for Science and Governance*:247–270. Bhaduri, A., Bogardi, J., Leentvaar, J. & Marx, S. (Eds.). Springer International Publishing.

Gardner, R., & Finlayson, C. (2018). *Global Wetland Outlook: State of the World's*

Wetlands and Their Services to People. Ramsar Convention Secretariat.

Gilbert, M. T. P., Bandelt, H. J., Hofreiter, M., & Barnes, I. (2005). Assessing ancient DNA studies. *Trends in Ecology & Evolution*, **20**: 541–544.

Gillet, F., Tiouchichine, M. L., Galan, M., Blanc, F., Némoz, M., Aulagnier, S., & Michaux, J. R. (2015). A new method to identify the endangered Pyrenean desman (*Galemys pyrenaicus*) and to study its diet, using next generation sequencing from faeces. *Mammalian Biology*, **80**: 505–509.

Gisbert, J., & García-Perea, R. (2014). Historia de la regresión del desmán ibérico Galemys pyrenaicus (É . Geoffroy Saint-Hilaire , 1811) en el Sistema Central (Península Ibérica). In *Conservation and management of semi-aquatic mammals of southwestern Europe*: 19–35. Ruiz, A., deLópez Luzuriaga, J. & Rubines, J. (Eds.). Donostia / San Sebastián: San Sebastian Aranzadi Society of Sciences: Munibe Monographs, Nature Series.

Gotelli, N., Hart, E., & Ellison, A. (2015). *EcoSimR: Null Model Analysis for Ecological Data*. Retrieved from https://cran.r-project.org/web/packages/EcoSimR/index.html (accessed on 7 September 2020)

Grant, E. H. C., Nichols, J. D., Lowe, W. H., & Fagan, W. F. (2010). Use of multiple dispersal pathways facilitates amphibian persistence in stream networks. *Proceedings of the National Academy of Sciences of the United States of America*, **107**: 6936–6940.

Grooten, M., & Almond, R. (2018). *Living Planet Report 2018: Aiming higher*. World Wildlife Fund.

Hawlitschek, O., Fernández-González, A., Balmori-de la Puente, A., & Castresana, J. (2018). A pipeline for metabarcoding and diet analysis from fecal samples developed for a small semi-aquatic mammal. *PLoS ONE*, **13**: e0201763.

He, K., Shinohara, A., Helgen, K. M., Springer, M. S., Jiang, X. L., & Campbell, K. L. (2017). Talpid mole phylogeny unites shrew moles and illuminates overlooked cryptic species diversity. *Molecular Biology and Evolution*, **34**: 78–87.

Herman, M. R., & Nejadhashemi, A. P. (2015). A review of macroinvertebrate- and fish based stream health indices. *Ecohydrology & Hydrobiology,* **15**: 53–67.

Hope, P. R., Bohmann, K., Gilbert, M. T. P., Zepeda-Mendoza, M. L., Razgour, O., & Jones, G. (2014). Second generation sequencing and morphological faecal analysis reveal unexpected foraging behaviour by *Myotis nattereri* (Chiroptera, Vespertilionidae) in winter. *Frontiers in Zoology*, **11**: 1–15.

Hsieh, T. C., Ma, K. H., & Chao, A. (2020). *iNEXT: Interpolation and extrapolation for species diversity*. Retrieved from https://cran.r-project.org/web/packages/iNEXT/index.html (accessed on 7 September 2020)

Igea, J., Aymerich, P., Fernández-González, A., González-Esteban, J., Gómez, A., Alonso, R., Gosálbez, J., & Castresana, J. (2013). Phylogeography and postglacial expansion of the endangered semi-aquatic mammal *Galemys pyrenaicus*. *BMC Evolutionary Biology*, **13**: 1–19.

Isaak, D. J., Young, M. K., Luce, C. H., Hostetler, S. W., Wenger, S. J., Peterson, E. E., Ver Hoef, J. M., Groce, M. C., Horan, D. L., & Nagel, D. E. (2016). Slow climate velocities of mountain streams portend their role as refugia for cold-water biodiversity. *Proceedings of the National Academy of Sciences of the United States of America*, **113**: 4374–4379.

Jarman, S. N., McInnes, J. C., Faux, C., Polanowski, A. M., Marthick, J., Deagle, B. E., Southwell, C., & Emmerson, L. (2013). Adélie penguin population diet monitoring by analysis of food DNA in scats. *PLoS ONE*, **8**: e82227.

Ji, Y., Ashton, L., Pedley, S. M., Edwards, D. P., Tang, Y., Nakamura, A., Kitching, R., Dolman, P. M., Woodcock, P., Edwards, F. A., Larsen, T. H., Hsu, W. W., Benedick, S., Hamer, K. C., Wilcove, D. S., Bruce, C., Wang, X., Levi, T., Lott, M., … Yu, D. W. (2013). Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecology Letters*, **16**: 1245–1257.

Kartzinel, T. R., & Pringle, R. M. (2015). Molecular detection of invertebrate prey in vertebrate diets: trophic ecology of caribbean island lizards. *Molecular Ecology Resources*, **15**: 903–914.

Kissling, W. D., Dormann, C. F., Groeneveld, J., Hickler, T., Kühn, I., Mcinerny, G. J., Montoya, J. M., Römermann, C., Schiffers, K., Schurr, F. M., Singer, A., Svenning, J. C., Zimmermann, N. E., & O'Hara, R. B. (2012). Towards novel approaches to modelling biotic interactions in multispecies assemblages at large spatial extents. *Journal of Biogeography*, **39**: 2163–2178.

Kocher, T. D., & White, T. J. (1989). Evolutionary analysis via PCR. In *PCR technology*:137–147. Kocher, T. D. & White, T. J. (Eds.). UK: Palgrave Macmillan.

Labonne, J., Ravigné, V., Parisi, B., & Gaucherel, C. (2008). Linking dendritic network structures to population demogenetics: The downside of connectivity - Labonne - 2008 - Oikos - Wiley Online Library. *Oikos*, **117**: 1479–1490.

Lajtha, K., & Michener, R. H. (1994). *Stable Isotopes in Ecology and Environmental Science*. Oxford: Blackwell Scientific Publications, Oxford.

Larsson, J., Godfrey, A. J. R., Gustafsson, P., Eberly, D. H., Huber, E., Slowikowski, K., & Privé, F. (2020). *Area-Proportional Euler and Venn Diagrams with Ellipses*. Retrieved from https://cran.r-project.org/web/packages/eulerr/index.html (accessed on 16 October 2020)

Lodge, D. M., Deines, A., Gherardi, F., Yeo, D. C. J., Arcella, T., Baldridge, A. K., Barnes,

M. A., Chadderton, W. L., Feder, J. L., Gantz, C. A., Howard, G. W., Jerde, C. L., Peters, B. W., Peters, J. A., Sargent, L. W., Turner, C. R., Wittmann, M. E., & Zeng, Y. W. (2012). Global introductions of crayfishes: evaluating the impact of species invasions on ecosystem services. *Annual Review of Ecology, Evolution, and Systematics*, **43**: 449472.

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

Maudet, C., Luikart, G., Dubray, D., Von Hardenberg, A., & Taberlet, P. (2004). Low genotyping error rates in wild ungulate faeces sampled in winter. *Molecular Ecology Notes*, **4**: 772–775.

Melero, Y., Aymerich, P., Luque-Larena, J. J., & Gosàlbez, J. (2012). New insights into social and space use behaviour of the endangered Pyrenean desman (*Galemys pyrenaicus*). *European Journal of Wildlife Research*, **58**: 185–193.

Meyer, J. L., Strayer, D. L., Wallace, J. B., Eggert, S. L., Helfman, G. S., & Leonard, N. E. (2007). The contribution of headwater streams to biodiversity in river networks. *Journal of the American Water Resources Association*, **43**: 86–103.

Meyer, M., & Kircher, M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols*, **5**: 1–10.

Morueta-Holme, N., Flöjgaard, C., & Svenning, J. C. (2010). Climate change risks and conservation implications for a threatened small-range mammal species. *PLoS ONE*, **5**: e10360.

Murgatroyd, M., Avery, G., Underhill, L. G., & Amar, A. (2016). Adaptability of a specialist predator: the effects of land use on diet diversification and breeding performance of Verreaux's eagles. *Journal of Avian Biology*, **47**: 834–845.

Nielsen, J. M., Clare, E. L., Hayden, B., Brett, M. T., & Kratina, P. (2018). Diet tracing in ecology: method comparison and selection. *Methods in Ecology and Evolution*, **9**: 278–291.

Oehm, J., Juen, A., Nagiller, K., Neuhauser, S., & Traugott, M. (2011). Molecular scatology: How to improve prey DNA detection success in avian faeces? *Molecular Ecology Resources*, **11**: 620–628.

Palmeirim, J. M., & Hoffmann, S. H. (1983). *Galemys pyrenaicus*. *Mammalian Species*, **207**: 1–5.

Palumbi, S. R. (1996). Nucleic acids II: the polymerase chain reaction. In *Molecular systematics*:205–247. Hillis, D. M., Moritz, C. & Mable, B. K. (Eds.). Sunderland, Massachusetts, USA: Sinauer Associates.

Pearson, R. G., & Dawson, T. P. (2003). Predicting the impacts of climate change on the

distribution of species: Are bioclimate envelope models useful? *Global Ecology and Biogeography*, **12**: 361–371.

Peterson, B. J., & Fry, B. (1987). Stable isotopes in ecosystem studies. *Annual Review of Ecology, Evolution, and Systematics*, **18**: 293–320.

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, **1**: 819-830.

Pompanon, F., Deagle, B. E., Symondson, W. O. C., Brown, D. S., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: diet assessment using next generation sequencing. *Molecular Ecology*, **21**: 1931–1950.

Quaglietta, L., & Beja, P. (2019). Direct observations of vertebrate killing and consumption by the endangered Pyrenean desman (*Galemys pyrenaicus*). *Mammalia*, **83**: 479–482.

Quaglietta, L., Paupério, J., Martins, F. M. S., Alves, P. C., & Beja, P. (2018). Recent range contractions in the globally threatened Pyrenean desman highlight the importance of stream headwater refugia. *Animal Conservation*, **21**: 515–525.

Quaresma, C. M. (2001). *Galemys pyrenaicus*: monitoring species and habitat conservation in Portugal. 4 Rencontres sur les Desmans. Moulis.

Queiroz, A. I., Bertrand, A., & Khakhin, G. V. (1996). Status and conservation of Desmaninae in Europe76. In Council of Europe.

Queiroz, A. I., Quaresma, C. M., Santos, C. P., & Carvalho, A. B. Y. H. (1998). Bases para a Conservação da Toupeira-de-Água *Galemys pyrenaicus*. Estudos de Biologia e Conservação da Natureza, **27**: 118.

Richard, B. (1986). Le desman des Pyrénées: un mammifère inconnu à découvrir. Science et Découvertes. Monaco.

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, **4**: e2584.

Santamarina, J., & Guitian, J. (1988). données sur le régime alimentaire du desman (*Galemys pyrenaicus*) dans le nord-ouest de l'Espagne. *Mammalia*, **52**: 301–308.

Scheele, B. C., Foster, C. N., Banks, S. C., & Lindenmayer, D. B. (2017). Niche Contractions in Declining Species: Mechanisms and Consequences. *Trends in Ecology and Evolution*, **32**: 346–355.

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., Pompanon, F., Coissac, E., & Taberlet, P. (2012). Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Molecular Ecology*, **21**: 1951–1965.

Shiels, A. B., Flores, C. A., Khamsing, A., Krushelnycky, P. D., Mosher, S. M., & Drake,

D. R. (2013). Dietary niche differentiation among three species of invasive rodents (*Rattus rattus*, *R. exulans*, *Mus musculus*). *Biological Invasions*, **15**: 1037–1048.

Soininen, E. M., Ehrich, D., Lecomte, N., Yoccoz, N. G., Tarroux, A., Berteaux, D., Gauthier, G., Gielly, L., Brochmann, C., Gussarova, G., & Ims, R. A. (2014). Sources of variation in small rodent trophic niche: new insights from DNA metabarcoding and stable isotope analysis. *Isotopes in Environmental and Health Studies*, **50**: 361–381.

Soininen, E. M., Valentini, A., Coissac, E., Miquel, C., Gielly, L., Brochmann, C., Brysting, A. K., Sønstebø, J. H., Ims, R. A., Yoccoz, N. G., & Taberlet, P. (2009). Analysing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. *Frontiers in Zoology*, **6**.

Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: Recent progress and future challenges. *Journal of the North American Benthological Society*, **29**: 344–358.

Taylor-Brown, A., Booth, R., Gillett, A., Mealy, E., Ogbourne, S. M., Polkinghorne, A., & Conroy, G. C. (2019). The impact of human activities on Australian wildlife. *PLoS ONE*, **14**: e0206958.

Taylor, H. R., & Gemmell, N. J. (2016). Emerging Technologies to Conserve Biodiversity: Further Opportunities via Genomics. Response to Pimm et al. *Trends in Ecology and Evolution*, **31**: 171–172.

Tickner, D., Opperman, J. J., Abell, R., Acreman, M., Arthington, A. H., Bunn, S. E., Cooke, S. J., Dalton, J., Darwall, W., Edwards, G., Harrison, I., Hughes, K., Jones, T., Leclère, D., Lynch, A. J., Leonard, P., McClain, M. E., Muruven, D., Olden, J. D., … Young, L. (2020). Bending the Curve of Global Freshwater Biodiversity Loss: An Emergency Recovery Plan. *BioScience*, **70**: 330–342.

Valentini, A., Miquel, C., Nawaz, M. A., Bellemain, E., Coissac, E., Pompanon, F., Gielly, L., Cruaud, C., Nascetti, G., Wincker, P., Swenson, J. E., & Taberlet, P. (2009). New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach. *Molecular Ecology Resources*, **9**: 51–60.

Vitousek, P. M., Mooney, H. A., Lubchenco, J., & Melillo, J. M. (1997). Human domination of Earth's ecosystems. *Science*, **277**: 494–499.

Vörösmarty, C. J., McIntyre, P. B., Gessner, M. O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S. E., Sullivan, C. A., Liermann, C. R., & Davies, P. M. (2010). Global threats to human water security and river biodiversity. *Nature*, **467**: 555–561.

Williams, S. E., Williams, Y. M., VanDerWal, J., Isaac, J. L., Shoo, L. P., & Johnson, C. N. (2009). Ecological specialization and population size in a biodiversity hotspot:

How rare species avoid extinction. *Proceedings of the National Academy of Sciences of the United States of America*, **106**: 19737–19741.

Supplementary material

Table S1: PERMANOVA results of month effect in the diet of *G. pyrenaicus*. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

Variable	d.f.	SS	MS	F.Model	R2	Pr (>F)
Month	5	4.797	0.95942	2.1252	0.05093	0.001***
Residuals	198	89.388	0.45146		0.94907	
Total	203	94.185			1.00000	

Table S2: PERMDISP test results of month effect in the diet of *G. pyrenaicus*. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

	d.f.	SS	MS	F.Model	Pr (>F)
Groups	5	0.09703	0.0194057	6.9241	$5.564e^{-06}$ ***
Residuals	198	0.55492	0.0028026		

Table S3: List of the identified prey items to the maximum resolution obtained, with the respective frequency of occurrence in the samples (FO1) and detected occurrences (FO2), and with the terrestrial or aquatic taxa of *G. pyrenaicus* analysed. "T" stands for terrestrial and "A" for aquatic taxa.

Phylum/Class	Order	Family	Species	nr prey items	T/A	FO1 (%)	FO2 (%)
Arthropoda				**193**		**99.0**	**92.5**
Arachnida				**9**		**11.8**	**3.6**
	Araneae			**7**	**T**	**10.3**	**3.2**
		Araneidae		2	T	0.5	0.3
			Larinioides sp	1	T	0.5	0.1
			Larinioides sclopetarius	1	T	0.5	0.1
	other Araneae			5	T	10.3	2.9
	Opiliones NI			**1**	**T**	**1.0**	**0.3**
other Arachnida				1		0.5	0.1
Insecta				**183**		**96.6**	**88.9**
	Coleoptera			**22**		**18.1**	**5.4**
		Chrysomelidae		3		3.9	1.1
			Galerucella lineola	1	A	0.5	0.1
			other Chrysomelidae	2		3.4	0.9
		Corylophidae		1	T	0.5	0.1
			Orthoperus sp	1	T	0.5	0.1
		Dryopidae		3	A	1.5	0.5
			Dryops luridus	1	A	0.5	0.1
			other Dryopidae	2	A	1.0	0.4
		Dytiscidae		6	A	5.4	1.6
			Hydroporus pubescens	1	A	1.0	0.3
			Ilybius chalconatus	1	A	1.0	0.3
			other Dytiscidae	4	A	3.9	1.1
		Elmidae		1	A	0.5	0.1
			Elmis rioloides	1	A	0.5	0.1
		Staphylinidae		1	T	0.5	0.1

Phylum/Class	Order	Family	Species	nr prey items	T/A	FO1 (%)	FO2 (%)
			Ocalea sp	1	T	0.5	0.1
	other Coleoptera			7		6.9	1.8
	Diptera			**64**		**45.1**	**17.9**
		Ceratopogonidae		1	A	0.5	0.1
			Culicoides scoticus	1	A	0.5	0.1
		Chironomidae		20	A	9.8	3.4
			Eukiefferiella sp	1	A	0.5	0.1
			Limnophyes sp	4	A	2.5	0.8
			Polypedilum sp	2	A	1.0	0.3
			Prodiamesa olivacea	1	A	0.5	0.1
			Tanytarsus sp	2	A	1.0	0.3
			other Chironomidae	10	A	5.4	1.8
		Culicidae		2	A	2.0	0.5
			Culex territans	1	A	0.5	0.1
			other Culicidae	1	A	1.5	0.4
		Dolichopodidae		1	T	0.5	0.1
			Hydrophorus sp	1	T	0.5	0.1
		Empididae		1	A	0.5	0.1
		Limoniidae		1	A	0.5	0.1
		Mycetophilidae		1	A	0.5	0.1
		Psychodidae		7	A	7.4	2.4
			Pericoma pseudexquisita	1	A	0.5	0.1
			Psychoda sp	5	A	6.9	2.1
			other Psychodidae	1	A	0.5	0.1
		Sciaridae		2	T	1.0	0.3
			Bradysia sp	1	T	0.5	0.1
			other Sciaridae	1	T	0.5	0.1
		Simuliidae		9	A	3.4	1.5
			Simulium velutinum	1	A	0.5	0.1
			Simulium sp	6	A	2.0	0.8
			other Simuliidae	2	A	1.5	0.5
		Tabanidae		3	T	0.5	0.3
			Chrysops caecutiens	1	T	0.5	0.1
			other Tabanidae	1	T	0.5	0.1
		Tipulidae		3	A	7.4	2.2
			Tipula helvola	1	A	0.5	0.1
			other Tipulidae	2	A	6.9	2.1
	other Diptera			14		24	6.7
	Ephemeroptera			**19**	**A**	**52.9**	**22.2**
		Baetidae		4	A	6.9	2.0
			Baetis rhodani	1	A	2.0	0.5
			other Baetidae	3	A	4.9	1.5
		Ephemerellidae		1	A	6.9	1.8
			Quatica sp	1	A	6.9	1.8

Phylum/Class	Order	Family	Species	nr prey items	T/A	FO1 (%)	FO2 (%)
		Ephemeridae		1	A	4.9	1.3
			Ephemera sp	1	A	4.9	1.3
		Heptageniidae		3	A	22.1	6.7
			Ecdyonurus sp	1	A	12.3	3.3
			Epeorus assimilis	1	A	5.4	1.5
			other Heptageniidae	1	A	7.4	2.0
		Leptophlebiidae		2	A	23.0	6.7
		Oligoneuriidae		1	A	1.0	0.3
	other Ephemeroptera			7	A	11.8	3.4
	Hemiptera			**10**		**5.9**	**2.2**
		Gerridae		1	A	0.5	0.1
			Aquarius najas	1	A	0.5	0.1
		Notonectidae		2	A	1.5	0.4
			Notonecta sp	1	A	0.5	0.1
			other Notonectidae	1	A	1.0	0.3
	other Hemiptera			7		3.9	1.7
	Hymenoptera			**8**	**T**	**4.4**	**1.2**
		Formicidae		3	T	2.0	0.5
			Camponotus cruentatus	1	T	0.5	0.1
			Crematogaster scutellaris	1	T	1.0	0.3
			Tetramorium sp	1	T	0.5	0.1
		Tenthredinidae		1	T	0.5	0.1
	other Hymenoptera			4	T	2.0	0.5
	Lepidoptera			**1**		**2.9**	**0.8**
	Megaloptera			**2**	**A**	**2.5**	**0.7**
		Sialidae		2	A	2.5	0.7
			Sialis sp	1	A	1.5	0.4
			Sialis fuliginosa	1	A	1.0	0.3
	Neuroptera			**1**	**T**	**1.0**	**0.3**
	Odonata			**6**	**A**	**6.4**	**2.2**
		Aeshnidae		1	A	1.0	0.3
			Boyeria irene	1	A	1.0	0.3
		Calopterygidae		2	A	3.4	0.9
			Calopteryx virgo	1	A	0.5	0.1
			other Calopterygidae	1	A	2.9	0.8
		Cordulegastridae		1	A	2.5	0.7
			Cordulegaster boltoni	1	A	2.5	0.7
		Gomphidae		1	A	1.0	0.3
	other Odonata			1	A	0.5	0.1
	Orthoptera			**1**	**T**	**0.5**	**0.1**
		Tettigoniidae		1	T	0.5	0.1
			Tettigonia viridissima	1	T	0.5	0.1
	Plecoptera			**14**	**A**	**21.1**	**7.1**
		Leuctridae		5	A	6.4	2.0

Phylum/Class	Order	Family	Species	nr prey items	T/A	FO1 (%)	FO2 (%)
			Leuctra sp	4	A	5.9	1.8
			Leuctra geniculata	1	A	0.5	0.1
		Nemouridae		1	A	0.5	0.1
			Protonemura sp	1	A	0.5	0.1
		Perlidae		1	A	4.9	1.3
			Perla madritensis	1	A	4.9	1.3
		Perlodidae		4	A	7.4	2.5
			Guadalgenus franzi	1	A	2.0	0.5
			Hemimelaena flaviventris	1	A	0.5	0.1
			Isoperla grammatica	1	A	5.9	1.6
			other Perlodidae	1	A	1.0	0.3
	other Plecoptera			3	A	4.4	1.2
	Psocodea			**1**	**T**	**0.5**	**0.1**
		Epipsocidae		1	T	0.5	0.1
			Bertkauia lucifuga	1	T	0.5	0.1
	Thysanoptera			**4**	**T**	**4.4**	**1.2**
		Thripidae		1	T	0.5	0.1
	other Thysanoptera			3	T	3.9	1.1
	Trichoptera			**29**	**A**	**50.5**	**20.9**
		Calamoceratidae		1	A	0.5	0.1
			Calamoceras marsupus	1	A	0.5	0.1
		Glossosomatidae		1	A	0.5	0.1
			Catagapetus maclachlani	1	A	0.5	0.1
		Hydropsychidae		5	A	10.8	3.0
			Hydropsyche sp	2	A	7.8	2.1
			Hydropsyche siltalai	1	A	1.5	0.4
			other Hydropsychidae	2	A	1.5	0.5
		Lepidostomatidae		2	A	0.5	0.3
			Lepidostoma sp	1	A	0.5	0.1
			Lepidostoma hirtum	1	A	0.5	0.1
		Limnephilidae		9	A	33.3	10.6
			Allogamus laureatus	1	A	1.5	0.4
			Allogamus ligonifer	1	A	6.9	1.8
			Allogamus mortoni	1	A	4.9	1.3
			Enoicyla pusilla	1	A	0.5	0.1
			Halesus radiatus	1	A	0.5	0.1
			Limnephilus guadarramicus	1	A	3.9	1.1
			other Limnephilidae	3	A	21.1	5.8
		Philopotamidae		3	A	3.9	1.1
			Chimarra marginata	1	A	2.0	0.5
			Philopotamus montanus	1	A	1.5	0.4
			Philopotamus sp	1	A	0.5	0.1
		Sericostomatidae		2	A	1.5	0.4
			Sericostoma sp	2	A	1.5	0.4

Phylum/Class	Order	Family	Species	nr prey items	T/A	FO1 (%)	FO2 (%)
	other Trichoptera			6	A	15.7	5.3
other Insecta				1		24.5	6.6
Malacostraca				**1**		**0.5**	**0.1**
	Isopoda			**1**	**T**	**0.5**	**0.1**
		Oniscidae		1	T	0.5	0.1
			Oniscus sp	1	T	0.5	0.1
Annelida				**13**		**16.2**	**5.8**
Clitellata				**13**		**16.2**	**5.8**
	Haplotaxida			**2**	**T**	**1.0**	**0.3**
		Lumbricidae		2	T	1.0	0.3
			Lumbricus sp	1	T	0.5	0.1
			other Lumbricidae	1	T	0.5	0.1
	Hirudinida			**7**	**A**	**12.3**	**3.9**
		Erpobdellidae		4	A	8.3	2.8
			Erpobdella sp	2	A	3.4	1.3
			other Erpobdellidae	2	A	4.9	1.5
		Glossiphoniidae		3	A	4.4	1.2
	Lumbriculida			**3**	**A**	**2.0**	**0.5**
		Lumbriculidae		3	A	2.0	0.5
			Stylodrilus sp	1	A	0.5	0.1
			Lumbriculus sp	1	A	0.5	0.1
			other Lumbriculidae	1	A	1.0	0.3
other Clitellata				1		3.9	1.1
Mollusca				**3**		**6.4**	**1.7**
Gastropoda				**3**		**6.4**	**1.7**
	Basommatophora			**1**	**A**	**0.5**	**0.1**
		Physidae		1	A	0.5	0.1
			Physella sp	1	A	0.5	0.1
	Stylommatophora			**1**	**T**	**1.0**	**0.3**
		Arionidae		1	T	1.0	0.3
			Arion sp	1	T	1.0	0.3
other Gastropoda				1		4.9	1.3

Table S4: PERMANOVA results of watershed effect in the diet of *G. pyrenaicus*, considering the variable year as random effect. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

Variable	d.f.	SS	MS	F.Model	R2	Pr (>F)
Watershed	1	0.811	0.81136	1.7552	0.00861	0.008 **
Residuals	202	93.374	0.46225		0.99139	
Total	203	94.185			1.00000	

Table S5: PERMDISP test results of watershed effect in the diet of *G. pyrenaicus*. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

	d.f.	SS	MS	F.Model	Pr (>F)
Groups	1	0.000753	0.00075312	0.5663	0.4526
Residuals	202	0.268652	0.00132996		

Table S6: PERMANOVA results of two habitat suitability categories effect in the diet of *G. pyrenaicus*, considering the variable year as random effect. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

Variable	d.f.	SS	MS	F.Model	R2	Pr (>F)
2 categories	1	0.745	0.74475	1.61	0.00791	0.017 *
Residuals	202	93.441	0.46258		0.99209	
Total	203	94.185			1.00000	

Table S7: PERMANOVA results of two habitat suitability categories effect in the diet of *G. pyrenaicus*, considering the variable watershed as random effect. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

Variable	d.f.	SS	MS	F.Model	R2	Pr (>F)
2 categories	1	0.745	0.74475	1.61	0.00791	0.026 *
Residuals	202	93.441	0.46258		0.99209	
Total	203	94.185			1.00000	

Table S8: PERMDISP test results of two habitat suitability categories effect in the diet of *G. pyrenaicus*. Magnitude of significance levels shown with asterisks: *** p < 0.001; ** p < 0.01; * p < 0.05. d.f stands for degrees of freedom, SS for sum of squares, and MS for mean of squares.

	d.f.	SS	MS	F.Model	Pr (>F)
Groups	1	0.001166	0.0011660	0.7523	0.3868
Residuals	202	0.313085	0.0015499		