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Novel insights into the diet of the Pyrenean desman, *Galemys pyrenaicus*, using next-generation sequencing molecular analyses

Running header: Genetic analyses of Pyrenean desman diet

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To date, the Pyrenean desman, a threatened, semi-aquatic mammal, is considered a specialist predator feeding on aquatic benthic invertebrates. This knowledge comes from visual identification of prey in scat or gut contents, often based on a limited number of samples and locations. In this study, diet analyses using next-generation sequencing methods were combined with an extensive survey to explore the Pyrenean desman summer diet across the French Pyrenees. This study thus provides an unprecedented level of detail on Pyrenean desman trophic ecology. Our results highlighted an extremely diverse diet containing a high proportion of rare prey and substantial consumption of terrestrial prey, which suggests a more generalist diet than previously understood. Three diet groups were identified, with significant differences in prey composition. These differences were, however, not related to geographic location, but rather to contrasted local environmental variables. These results suggest that the highlighted spatial dietary variation is likely induced by local abiotic parameters that affect prey availability or foraging habitat use.

Keywords: foraging habitat, scat analysis, semi-aquatic mammal, spatial structure

Enhancing knowledge of how species interact with their environment and with other organisms is required to evaluate species vulnerability (Rodrigues et al. 2006). Biotic interactions such as competition, predation, and trophic resource availability are known to play a central role in the distribution of species and their abundance (Boulangeat et al. 2012; Kissling et al. 2012), and to influence their responses to changing environments at different scales (Pearson & Dawson, 2003; Araújo & Luoto, 2007; Wisz et al. 2013; Belmaker et al. 2015). For instance, the decline of the Iberian lynx, *Lynx pardinus*, the world's most threatened felid, was reported to be caused by the decline of its staple prey, the European rabbit, *Oryctolagus cuniculus* (Real et al. 2009), due to changes in environment and diseases (e.g., myxomatosis). In studies focused on one single species, the biotic component has often been overlooked due to sparse data and the difficulty of quantifying and incorporating complex relationships between several organisms (Soberón & Peterson, 2005). This is particularly true when the species of interest is rare and elusive, and thus difficult to study.

The Pyrenean desman, *Galemys pyrenaicus* (E. Geoffroy Saint-Hilaire, 1811), is a small, semi-aquatic mammal (Talpidae) endemic to the Pyrenees Mountains (France, Spain, and Andorra) and the Iberian Peninsula (northern and central Spain, northern Portugal). The species is listed as vulnerable by the IUCN (Fernandes et al. 2008), and is legally protected in the four countries encompassing its distribution area. For a long time, the Pyrenean desman remained relatively unstudied, while also suffering from a substantial decline across its range (Fernandes et al. 2008; Gisbert & García-Perea, 2014; Charbonnel et al. 2016). Although recent studies have improved general knowledge about this species (e.g., habitat: Charbonnel et al. 2015 and Biffi et al. 2016; space use: Melero et al. 2012, 2014; population genetics: Gillet et al. 2015, 2016), a lack of information on biotic interactions, both with predators and trophic resources, remains to be supplemented.

To study the diet of small mammals, morphological identification of prey remains has been widely used (e.g., Castién & Gosálbez, 1999; Churchfield & Rychlik, 2006). With these methods, the Pyrenean desman was considered a specialist predator that fed on benthic invertebrates in streams (mainly Trichoptera, Ephemeroptera, Plecoptera or Diptera, e.g., Santamarina & Guitian, 1988; Santamarina, 1993; Bertrand, 1994; Castién & Gosálbez, 1995).

Even though traditional methods enable the identification of a wide variety of prey, they are becoming less and less popular because (i) they generally involve the destruction of animals to analyse gut contents, (ii) they provide degraded prey remains that cannot be identified with precision (rarely beyond the family level), (iii) they underestimate the consumption of prey that are thoroughly masticated (e.g., arthropods consumed by bats) and soft-bodied prey (e.g., molluscs and earthworms) that leave no hard remains after digestion, and (iv) they are time-consuming and require expert knowledge of the range of potential prey. The recent development of molecular analyses based on barcoding of faecal DNA fragments and next-generation sequencing has overcome these difficulties (see a review by Pompanon et al. 2012).

In that context, the first aim of this study was to improve the knowledge of the trophic ecology of the Pyrenean desman by combining next-generation molecular analyses and an extensive sampling covering the species range over the whole French Pyrenees. Gillet (2015) recently identified three Pyrenean desman genetic populations using contrasted habitats (Biffi et al., 2016) in the three main hydrographic regions of the area. The second aim of the study was to compare the diet of the Pyrenean desman in those three regions. We hypothesized that the diet would vary according to the three hydrographic regions of the French Pyrenees, which differ in terms of catchment surface, climate, elevation, and land cover.

MATERIALS AND METHODS

Study area.— The Pyrenees Mountains are located in southwestern Europe and are a natural barrier between France and Spain. This study focused on the French part of the Pyrenees (W1°400–E3°100, N43°080–N42°230; Fig. 1), which extends for about 400 km from the Bay of Biscay to the Mediterranean Sea and has a maximum elevation of 3,298 m. The stream network can be divided into three main hydrographic regions: (1) the western streams that flow mostly westward to the Atlantic Ocean (coastal streams and Adour catchment; 9,412 km² in the Pyrenees), (2) the central streams that flow mainly northward and form the upstream part of the Garonne river catchment (7,702 km² in the Pyrenees), and (3) the eastern streams that flow to the Mediterranean Sea (Aude, Tech and Têt catchments; 6,773 km² in the Pyrenees).

Faeces collection.— A national survey was conducted from 2011 to 2013 within the framework of the Conservation Action Plan for the Pyrenean desman (Némoz et al. 2011). Given the elusive behaviour of this species, searches for indirect signs (i.e., faeces) were conducted in 1,330 sites covering the entire French Pyrenees (Fig. 1A). Skilled observers meticulously inspected each emergent item (i.e., rock, tree root, and branch) along 500 m riverbed transects (Castién & Gosálbez, 1992; Bertrand, 1994; Aymerich & Gosálbez, 2002; Charbonnel et al. 2014, 2015; Biffi et al. 2016). From the 1,330 original sites, 989 faeces suspected of being left by a Pyrenean desman, based on their colour, size, smell, and position, were collected in 958 sites and preserved in absolute ethanol for further molecular analyses.

Molecular analyses.— DNA was extracted from faeces using the Stool Mini Kit (Qiagen Inc., Hilden, Germany), following the manufacturer's instructions. PCR amplifications and Ion Torrent PGM sequencing (Life Technologies) were duplicated on the 989 faecal samples following the tagging and multiplexing method developed by Galan et al. (2012). Briefly, a 133 bp mini-barcode of the cytochrome oxydase I gene (COI) was amplified for each sample using a modified forward primer LepF1 (Hebert et al. 2004): 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNATTCHACDAAYCAYAARGAYATYGG-3', and a modified reverse primer EPT-long-univR (Hajibabaeiet al. 2011): 5'-CCTCTCTATGGGCAGTCGGTGATNNNNNNNACTATAAAARAAAATYTDAYAAADGCRTG-3'. The 5' parts of the primers were modified by the addition of individual-specific MIDs (Multiplex IDentifiers NNNNNNN), consisting of a short 7 bp sequence and adaptors required for emPCR and Ion Torrent sequencing. By using a combination of different forward and reverse MIDs sequences, several hundred samples can be multiplexed in the same sequencing run, and the sequences can be recognized after sequencing, when all the PCR products from the different samples are mixed together (Gillet et al., 2015)

PCRs were carried out in a 10 µl reaction volume using 5 µl of 2x QIAGEN Multiplex Kit (Qiagen, Hilden, Germany), 0.5 µM of each primer (LepF1 and EPT-long-univR, concentrated at 10µM), and 1 µl of DNA extract. The PCR conditions consisted of an initial denaturation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min. After pooling all PCR products at 5 ng/µL, the amplicon libraries were sequenced by the company Genes Diffusion (Douai, France) on an Ion Torrent PGM system using an Ion 316 Chip version 2 (LifeTechnologies).

In addition, a customised database of COI sequences was built from 84 typical invertebrate species from the study area collected in some Pyrenean rivers and identified by local entomologist experts from the Conservatoire d'Espaces Naturels Midi-Pyrénées (CEN-MP) and the EcoLab laboratory of Toulouse. A 710 bp fragment of COI was amplified in these samples with universal primers LCO1490 and HCO2198 (Folmer et al. 1994), following the PCR conditions reported in Folmer et al. (1994).

The sequences were sorted using SESAME barcode software (SEquence Sorter & AMplicon Explorer; Piry et al. 2012). These sequences were compared with sequences available in the customized and BOLD databases (Ratnasingham & Hebert, 2007). Sequences that had a unique best-hit with an identity score greater than or equal to 98% were considered to be positive matches and allowed identification of the predator producing the faeces as well as the prey contained in them.

Taxa were validated as possibly occurring in France and in the Pyrenees region using the French National Inventory of Natural Heritage (Muséum national d'Histoire naturelle, 2003-2017), the French Office for Insects and their Environment (OPIE-Benthos, 2017) online databases, and the help of local experts. Taxa identified as endemic to other parts of the world were kept in the analysis and are designated by an asterisk hereafter (*), as they correspond more likely to a genetically similar taxon present in the Pyrenees but not available in the reference databases.

The frequency of occurrence (i.e., the number of faeces containing the taxon divided by the total number of Pyrenean desman faeces) in the Pyrenean desman diet was then calculated for each order, family, and genus, and for the different types of prey's modes of life (i.e., exclusively aquatic, exclusively terrestrial, or with aquatic and terrestrial stages).

Study of the summer Pyrenean desman diet.— To investigate a potential spatial structure in the Pyrenean desman diet, 287 Pyrenean desman faeces collected at 115 sampling sites were kept for statistical analysis. This strong reduction in the number of faeces compared to the initial pool of faeces (n=989) was due to drastic selection following three criteria: (i) the predator producing the faeces has been identified by molecular analyses, (ii) the prey contained in the faeces have been identified by molecular analyses, too, and (iii) the faeces were collected during summer (i.e., June to September) in order to prevent potential strong seasonal variation. The summer season was chosen as most samplings were conducted during this low-flow period between 2011 and 2013. Prey occurrences were not different between the three summers of sampling, as revealed by a non-parametric permutation-based multivariate analysis of variance (PERMANOVA, Anderson, 2001), which was not significant (p = 0.12). To correct for potential sampling bias (i.e., more than one faeces collected per site), all the faeces collected in a single site were pooled. Consequently, a taxon was assumed to be present in the Pyrenean desman diet of a site if it was found in at least 50% of the faeces collected for this site. All prey taxa were kept for subsequent analysis.

Cluster Analysis And Identification Of Summer Diet Groups - The binary matrix (i.e., presence/absence of prey genera at each site) was converted to a distance matrix calculated with the Sørensen similarity coefficient, which is the equivalent of Bray-Curtis distance but for presence-absence instead of abundance data (Borcard et al., 2011). Then, a hierarchical ascendant clustering was computed with Ward's algorithm to build a dendrogram representing the distance between each pair of sites according to their similarity in the summer Pyrenean desman diet (i.e., prey taxa genetically identified in faeces). The number of diet groups was chosen according to the dendrogram, so that it increases the variation between groups and decreases the variation within groups while keeping a relatively balanced number of sites within groups.

The mean composition of prey communities within sites was compared among the three diet groups using a PERMANOVA (Anderson, 2001). As PERMANOVA may be sensitive to within-group effects, PERMDISP (Anderson et al. 2006), a permutation dispersion analysis, was then used to test for differences in within-group dissimilarity (i.e., the variability of diet composition within the groups as the mean distance of diet compositions to their group centroid). When the PERMDISP test was significant, a pairwise Tukey HSD test was run to examine which of the diet groups had higher dispersion. The Sørensen matrix was used as the measure of similarity in both PERMANOVA and PERMDISP.

In order to identify the prey taxa specific to each diet group, an indicator value (IndVal) analysis was performed (Dufrêne & Legendre, 1997; Cáceres & Legendre, 2009) using the occurrence matrix of the summer prey taxa and the classification of sites in diet groups obtained through the hierarchical clustering. The IndVal is the product of the specificity (i.e., the probability that the survey site belongs to the target site group, given the fact that the species has been found) and the fidelity (i.e., the probability of finding the species in sites belonging to the site group) of species for each cluster. A taxon was only able to be a potential indicator of one diet group, as group combinations were not allowed. The IndVal statistical significance was tested using a permutation test (9,999 permutations) for each prey taxon.

Finally, a chi-squared test was performed to test whether the assignment of sites to the three diet groups according to prey community composition found in faeces was dependent on their location in the three hydrographic regions of the French Pyrenees.

Comparison of Environmental Parameters Between Diet Groups - To investigate potential local constraints that could influence the diet of the Pyrenean desman during the summer, a linear discriminant analysis (LDA) was performed on a set of 12 environmental variables that described local habitat conditions at the site (Biffi et al. 2016) and reach scales (i.e. stream reaches of approximately 1 km-long; Charbonnel et al. 2016) in each diet group (see Table 1 for a complete description). These variables were expected to influence most invertebrate communities in mountain streams (Tachet et al. 2000; Usseglio-Polatera et al. 2000). This multivariate statistical method searches for linear combinations of quantitative variables (i.e., environmental variables) that maximise inter-group variance (i.e., diet groups). LDA significance was tested using a Monte-Carlo test (9,999 permutations).

All statistical analyses were conducted in R 3.1.1 (R Development Core Team, 2014) using the *ade4, vegan,* and *indicspecies* packages.

RESULTS

Molecular identification of the predator producing the faeces and contents.— After the two PCR amplifications, a total of 9,489,679 reads were obtained, among which 2,962,289 were correctly assigned to 560 of the 989 analysed samples (57%), after removing singleton sequences that were likely to be PCR or sequencing errors from the dataset. Among them, 390 samples (2,695,260 reads; 90%) belonged to the Pyrenean desman while the remaining 170 samples (267,029 reads) were assigned to 25 other host species, including 7 birds (e.g., *Turdus*) and 14 mammals (e.g., *Neomys sp.*, *Glis glis*). Among the 390 samples assigned to the Pyrenean desman, 383 samples also provided information on prey content (representing 37% of the reads), including 287 desman faeces collected during the summer.

The proportion of items obtained twice in the PCR duplicates for the same DNA extract was computed as the percentage of repeatability. Repeatability of the results reached 99% when only host species were considered, 83% when only prey species were considered, and 87% when all species were considered.

Overall diversity of prey contained in the Pyrenean desman faeces.— The 383 Pyrenean desman faeces collected between 2011 and 2013 contained prey belonging to 11 classes, 30 orders (Table 2), 91 families, and 156 genera (Supplementary Data S1). A mean of 5.8 ± 2.0 genera were present per faeces (5.1 ± 1.9 and 3.7 ± 1.2 for family and order, respectively). Among the 156 genera, 100 were confirmed to be present in the Pyrenees, 31 in France, and 21 were endemic of other parts of the World (e.g., New Zealand, Australia) and were thus misidentified by genetic databases. The presence in the Pyrenees of the four additional genera remains unknown. For one taxon, databases were not able to discriminate between two macroinvertebrate genera and families (designated hereafter as "*Perlodes_Epeorus**" at the genus level).

The diet of the Pyrenean desman was mainly composed of Insecta and Malacostraca, which were present in 99.7% and 18.0% of the 383 faeces respectively (Table 2). Other classes were present in less than 4% of the Pyrenean desman faeces (e.g., Diplopoda, Lissamphibia, Arachnida, Clitellata, Gastropoda). Only 7 orders (23.3%), 14 families (15.4%), and 14 genera (9.0%) had a frequency of occurrence in the faeces above 10%, meaning that the majority of prey taxa were found in less than 10% of the faeces. The most frequent order was Ephemeroptera (86.7%), which included the most frequent family, Heptageniidae (59.0%), and the most frequent genus, *Baetis* (Baetidae; 56.4%; Supplementary Data S1).

Ephemeroptera, Plecoptera, and Trichoptera (EPT) together represented on average 79.1% of the identified genera in each faeces. However, when considering the total number of identified taxa (TIT) in the overall Pyrenean desman diet, EPT orders represented only 28.2% of identified prey. Exclusively aquatic items represented 4.1% of prey *vs.* 7.7% of TIT, while exclusively terrestrial prey represented 7.7% of prey (*vs.* 36.5% TIT). Taxa with at least one aquatic stage and one terrestrial stage represented 88.3% of prey (*vs.* 55.8% TIT).

Variation of summer diet across the French Pyrenees.— When considering the summer months (i.e., June to September), analyses were limited to 287 faeces collected from 115 sites. From these faeces, 91 prey genera were kept to study possible spatial variation in the summer Pyrenean desman diet. Hierarchical ascendant clustering made it possible to identify three distinct diet groups of sites (39, 48, and 28 sites, respectively) based on prey assemblage (Fig. 2). There was no significant difference ($X^2 = 4.05$, d.f. = 2, p = 0.13) between the number of samples collected per site and its classification by cluster analysis (i.e., diet group), excluding potential bias due to diet profiles merging. Prey composition was closer between Groups 1 and 2 than with Group 3. Within-site prey composition differed significantly among groups (PERMANOVA: $F = 6.83$, d.f. = 2, $p < 0.01$; Fig. 3). The dissimilarity of prey assemblages among sites similarly differed by diet group (PERMDISP: $F = 3.28$, d.f. = 2, $p < 0.01$), and was driven by slightly lower among-site variability in Group 3 than in Group 2 (Tukey HSD: $p = 0.045$). There was no significant difference in among-site variability between Groups 1 and 2, and Groups 1 and 3 (Tukey HSD: $p > 0.05$).

Diet Group 3 exhibited the least diverse prey assemblage, with 45 different invertebrate genera consumed by the Pyrenean desman, versus 51 in Group 1 and 57 in Group 2. Only 21 genera were consumed in all three groups. Eleven significant indicator taxa were identified for the three summer diet groups of the Pyrenean desman ($p < 0.05$, Table 3, Fig. 3). Group 2 was characterised by three Ephemeroptera taxa, whereas the indicator taxa of Group 1 and Group 3 were more diverse (Trichoptera, Plecoptera, Diptera and Ephemeroptera for Group 1 and Amphipoda, Plecoptera and Diptera for Group 3, Table 3).

The differences in diet composition were not significantly related to the geographical location of sites within the three hydrographic regions of the French Pyrenees ($X^2 = 5.93$, d.f. = 4, $p = 0.20$; Fig. 1B).

Linear discriminant analysis computed two significant functions, F1 and F2 (Monte-Carlo test: $p = 0.001$), accounting for 60.8% and 39.2% of the variability between the three diet groups, respectively (Fig. 4). The first function, F1, separated Group 3 from the two other groups, whereas the second function, F2, separated Group 2 from Groups 1 and 3. The environmental variables discriminating the three diet groups were (i) SHELTER (i.e., proxy for riverbed heterogeneity) and SLO (i.e., slope of river section), which were positively correlated with F1; (ii) WOOD (i.e., proportion of bankside with shrubby-woody vegetation), which was negatively correlated with F1; (iii) HUM_IMP (i.e., proxy for human impacts), which was positively correlated with F2; and (iv) SHEET (i.e., proportion of the stretch with non-turbulent fast water units of shallow water that flows uniformly over smooth bedrock) and TRI (i.e., number of tributaries), which were negatively correlated with F2 (Fig. 4). This suggests that on average, the sites in Group 3 exhibited lower slopes, less heterogeneous riverbeds, and wooded riverbanks (Table 4). Group 2 clustered sites with low human impact along the near floodplain. The sites in Group 1 showed intermediate local environmental conditions (Table 4).

DISCUSSION

Next-generation sequencing as a powerful tool for studying the diet of the Pyrenean desman.—The amplification of a COI mini-barcode successfully provided the identification of the Pyrenean desman and its prey species, as well as several other host species, in 57% of faecal samples. This confirms the relevance of using such a genetic marker with next-generation sequencing methods in diet assessments (Pompanon et al. 2012; Piñol et al. 2014; Gillet et al. 2015) without the need for predator-specific blocking probes. The remaining 43% of the samples could not be correctly assigned. According to McInnes et al. (2017), results are highly dependent on faeces freshness and size. In addition, small faeces such as those of the Pyrenean desman (10 to 15 mm long) limit the amount of DNA they contain, but also affect the reproducibility of the extraction step, since the totality of the faeces has to be used. Moreover, DNA is rapidly degraded by contact with water and UV radiation (Lindahl, 1993).

In spite of these shortcomings, molecular analysis allowed the identification of 156 different invertebrate genera across the French Pyrenees. These genera belonged to 30 orders and 91 families, of which almost 70 were identified as Pyrenean desman prey for the first time. Using traditional methods of faeces analysis, Bertrand (1994) had identified only 20 families from an extensive sampling of 521 faeces collected in two small French catchments. In Northern Spain, Castién & Gosálbez (1995) trapped 49 desmans throughout the year and were able to identify only 11 orders as the lowest taxonomic level. The present study is therefore the most extensive ever done on the diet of this species in terms of number of samples, geographic coverage, and taxonomic resolution altogether. The high number of newly identified prey emphasizes the efficiency of molecular analysis in detecting taxa that cannot be identified in faeces through morphological analysis, and highlights the Pyrenean desman's capacity to adapt to its trophic environment. Nevertheless, some taxa identified, such as Collembola, Eurotatoria, and Eutardigrada, are unlikely to be direct prey of the Pyrenean desman, as they are part of the soil microfauna or aquatic zooplankton. Other taxa, such as *Sargus* and small Coleoptera, may develop at the larval stage or feed on scat, and may have thus been collected with the faeces. This contributes to the debate about the high sensitivity of next-generation sequencing methods and the detection of secondary predation (Sheppard et al. 2005). Molecular diet analyses are also limited by the deficiencies in the reference databases: a taxon can be identified at the species (or genus) level only if it has already been sequenced (Pompanon et al. 2012). In our study, 21 prey species were endemic of other parts of the world, and were thus misidentified. This issue stresses the importance of encouraging inventories of genetic biodiversity. Recently, the usage of COI markers led to some concerns in metabarcoding studies (see Deagle et al. 2014) using environmental DNA or bulk biodiversity sample. However, in this study, we used only samples which belonged to previously identified species. Some taxa could still not have been discovered but as we specifically designed the primers and, in sight of the number of genera found among prey, we are confident that the number of missing taxa is very low.

A large diversity of prey in the Pyrenean desman diet.—So far, the Pyrenean desman has been described as a specialist predator targeting prey in an aquatic environment (e.g., Bertrand, 1994; Castién & Gosálbez, 1995). In this study, we confirmed that dietary preferences seem to be directed toward Ephemeroptera, Plecoptera, and Trichoptera. However, the wide variety of prey identified suggests a more generalist diet for the Pyrenean desman. First, 91% of taxa were identified in less than 10% of the sites, representing a very high proportion of infrequent prey. Second, the most frequent prey (e.g., *Baetis*, *Protonemura,* and *Rhithrogena*) are among the most abundant aquatic macroinvertebrates in the Pyrenees (e.g., Brown et al. 2006; Finn et al. 2013). Third, strictly terrestrial prey represent about 8% of the prey eaten in each faeces and more than 35% of all identified prey taxa. This substantial percentage of terrestrial invertebrate consumption may result from (i) active hunting of terrestrial prey, (ii) opportunistic feeding on terrestrial prey while moving on the banks, and/or (iii) consumption of drowned terrestrial invertebrates. Other aquatic (e.g., brown trout *Salmo trutta*) and semi-aquatic (e.g., European otter *Lutra lutra*) species are known to rely to some extent on a pool of alternative prey including terrestrial subsidies to fulfil their energetic needs (Clavero et al. 2003; Evangelista et al. 2014; Milardi et al. 2016). This diversification of diet may be linked to aquatic stressors (e.g., pollution) or seasonal effects (e.g., variation of climatic conditions and water flow) that limit or modify *in situ* aquatic communities (Clavero et al. 2003; Kraus et al. 2016; Milardi et al. 2016). The highly diverse summer diet of the Pyrenean desman could thus be considered here, as for the otter, a response to summer drought conditions (Ruiz-Olmo et al. 2001). Indeed, during the summer period, most aquatic insects have already emerged in mountain streams (Füreder et al. 2004) or occur in small-size life stages, especially Ephemeroptera, Plecoptera and Trichoptera, which induces a potential diversification of the Pyrenean desman prey diet towards an increasing number of alternative aquatic and terrestrial food items. Its diet would be less diverse and with less abundant terrestrial prey during the other seasons, but this would require confirmation through a seasonal survey. This temporal survey should be combined with standardized sampling of both terrestrial and aquatic potential prey on river banks and in streams to get a quantitative assessment of the trophic resources available for the Pyrenean desman and therefore better understand its feeding behaviour.

Spatial variation in the Pyrenean desman diet and the influence of the environment.— While accounting only for the most common prey consumed during the summer by the Pyrenean desman, three different diets were identified in the French Pyrenees according to prey composition and indicator prey taxa. There was no major regional influence from the three main hydrographic regions of the French Pyrenees, despite contrasted environmental conditions from the western wet Atlantic area to the eastern dry Mediterranean. This result does not support our assumption that the diet of the Pyrenean desman would be different according to the genetic populations identified by Gillet (2015) or to the different habitat use reported by Biffi et al. (2016).

However, sites grouped within the three Pyrenean desman diets exhibited differences in environmental variables, suggesting some influence at the site scale. Group 2 diet sites exhibited conditions typical of upstream parts of river basins with higher reach slope and lower impact from human activities (i.e., urbanization). At the opposite end, the Group 3 diet clustered lower altitude sites (e.g., low slope of river stretches) with quite high levels of human impact on the near floodplain. These sites also included more homogeneous riverbeds.

First, the difference in prey composition among groups could be explained by the local availability of prey, which is dependent on fine-scale environmental conditions. The abundance and richness of aquatic macroinvertebrates are indeed directly dependent on local habitat conditions, such as (i) human-induced pollution (e.g., near agricultural or urban areas), impacts water quality (e.g., Johnson et al. 2013; Pallottini, et al. 2016), (ii) heterogeneity of substrate types and emerging items, which provides different types of microhabitat in streams (Reid et al. 2010), (iii) water current and oxygenation (Tachet et al*.,* 2000; Usseglio-Polatera et al. 2000), which are influenced by the slope of river reaches, and (iv) small-scale climate variables, which can modify the period of invertebrate emergence and their availability in streams (e.g., Füreder et al. 2004). Such diet adaptation to site-scale differences in resource availability would suggest an adaptive foraging strategy for the Pyrenean desman.

Secondly, the availability of foraging habitats of the Pyrenean desman could be constrained by local physical features. The species would thus feed on the fauna that is present in the microhabitats of streams it can access. For instance, in sites of diet Group 3 where riverbeds are the most homogeneous, the Pyrenean desman may forage on the littoral margins, while it may have access to a more diverse choice of microhabitats and prey in sites of diet Groups 1 and 2, where riverbeds are more heterogeneous. This assumption is corroborated by the indicator species identified for each diet. Indicator species of Group 3, such as *Gammarus* and *Protonemura,* can indeed be found in zones of plant and organic debris accumulation (i.e., litter) and dense root hairs, which are typical of low-slope areas along riverbanks (Tachet et al.2000; Usseglio-Polatera et al. 2000). Indicator species of Group 2 are Ephemeroptera taxa sensitive to water quality. This is consistent with the apparent less disturbed environmental conditions of these sites, which also provide higher water velocity (i.e., higher slopes) in more open areas (i.e., higher altitude). These local conditions make possible the development of biofilms, which are important food resources for scraper taxa such as *Ecdyonurus*. The intermediate environmental conditions of Group 1 likely induced a higher diversity of indicator taxa, which may reflect a more heterogeneous set of habitats.

Conclusion and perspectives.— This study suggests that the Pyrenean desman would adopt a more generalist foraging strategy than previously reported. Even if a wider dietary niche is supposed to increase the chance of adaptation to altered environments (Murgatroyd et al. 2016), food availability remains an important issue for species conservation. Many disturbances to freshwater environments may result in the general decline of abundance and richness of aquatic invertebrate communities (Paul & Meyer, 2001) and have thus detrimental consequences for the Pyrenean desman. In addition, many other threats affecting its nesting (on river banks) and foraging (in river beds) habitats as well as population viability, such as predation and mortality induced by human activities, should be limited as much as possible in order to protect the Pyrenean desman. In that context, further studies will be needed to (i) investigate the extent to which the diet and spatial distribution of the Pyrenean desman are influenced by prey availability and other biotic interactions, and (ii) get a thorough knowledge of its foraging areas within streams with the constraint that real-time monitoring and/or radiotracking of this elusive species are difficult and costly to implement.

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SUPPLEMENTARY DATA

Supplementary Data S1.— Complete list of taxa identified as Pyrenean desman prey by molecular analysis of 383 faeces collected in the French Pyrenees. Frequencies of occurrence of each prey (FO: % of faeces with taxa) are displayed. * indicates misidentified taxa whose distribution areas exclude the Pyrenees and France. Habitat type of taxa is given (aq: exclusively aquatic; aq/te: with aquatic and terrestrial stages; te: exclusively terrestrial).

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FIGURES LEGENDS

Fig. 1.— Location of the study area in France. The three hydrographic regions of interest are shown in light grey (western region: coastal streams and Adour catchment), medium grey (central region: Garonne river catchment) and dark grey (eastern region: Aude, Tech and Têt catchments). A) Location of all sampling sites (dark dots). B) Location of sampling sites according to the three summer diet groups for the Pyrenean desman.

Fig. 2.— Hierarchical clustering dendrogram of Sørensen distances between the sampling sites according to their similarity in the summer Pyrenean desman diet (i.e., 91 prey taxa genetically identified in faeces). Three diet groups are identified.

Fig. 3.— Prey composition of the three groups of sampling sites based on the Pyrenean desman summer diet composition (% of sites with prey). The diet groups were determined from a hierarchical ascendant clustering based on a Sørensen distance matrix of presence-absence of prey genera per site (see Fig. 1). Prey are sorted by decreasing proportion in the first group of sites. * indicates prey taxa which were misidentified by the genetic databases. Indicator taxa for diet groups are identified in bold and underlined characters (see Table 3 for details). Habitat type of taxa is given (black triangles: exclusively aquatic; black rectangles: exclusively terrestrial; no symbol: with aquatic and terrestrial stages).

Fig. 4.— Output of the linear discriminant analysis. The projection onto the first two linear discriminants is displayed: A) 12 environmental variables; B) sites clustered by diet groups. See Table 1 for variables description.

TABLES

Table 1.— Environmental variables (Biffi et al., 2016; Charbonnel et al., 2016) expected to influence the prey composition of the Pyrenean desman diet groups in the French Pyrenees.

Scale	Category	Code	Description
Site	Riverbed	SHELTER	Proportion (%) of the heterogeneity of shelters and substrate (i.e. presence of emerging items and cavities, diversity of substrate types and banks components) as a proxy for riverbed heterogeneity
		CLOG	Proportion (%) of the surface of streambed rocks covered by fine sediment (silt and mud)
		SHEET	Proportion (%) of the stretch with non-turbulent fast water units of shallow water that flows uniformly over smooth bedrock
		RUN	Proportion (%) of the stretch with non-turbulent fast water units of shallow gradient with relatively fine substrata ranging from sand to cobble
		RIFFLE	Proportion (%) of the stream stretch with turbulent fast water units with rapid and shallow flow with steep water surface gradient
	Riverbanks	BANKS	Shannon's diversity index derived from the proportion (%) of banks type components along the stretch (DIRT, ROOTS, ROCKS)
		WOOD	Proportion (%) of bankside with shrubby-woody vegetation
	Floodplain	HUM_IMP	Proportion (%) of urbanized land within the stream stretch floodplain as a proxy for human impacts
Reach	Climate	TEM	Atmospheric mean annual temperature (°C)
	Hydrology	FLO	Mean monthly stream flow (m^3/s)
	Hydrography	SLO	Mean slope of the river section
		TRI	Number of tributaries in the focal section and in its adjacent upstream and downstream sections

Table 2.— Prey taxa identified with positive matches (≥ 98 %) from 383 faeces of Pyrenean desman collected in the French Pyrenees. Frequencies of occurrence (FO: % of faeces containing the prey) are displayed. See **Supplementary Data S1** for the complete list of taxa at a finer taxonomic resolution (family and genus). * indicates misidentified taxa by genetic databases (see Material and Methods).

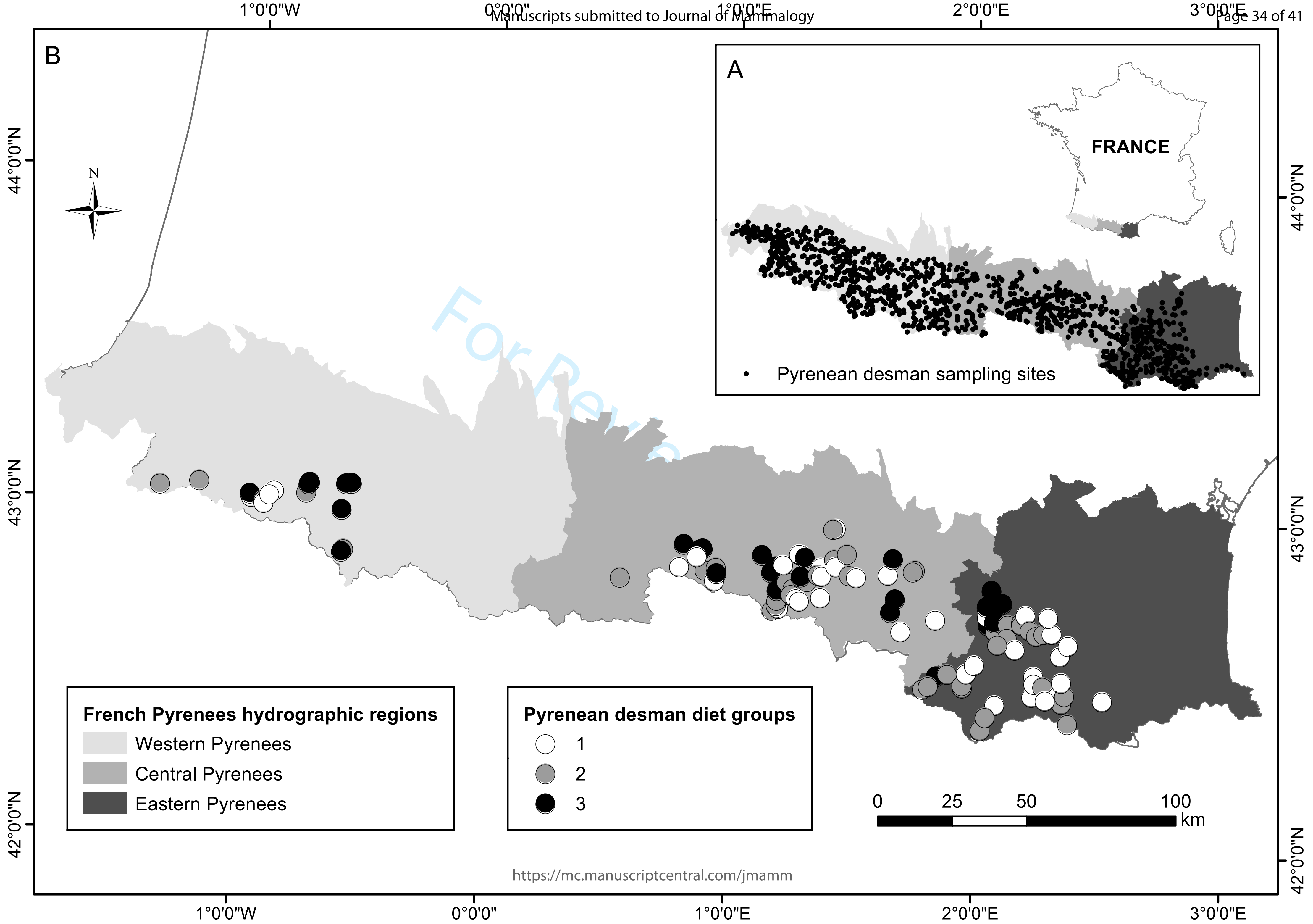
Class	FO (%)	Order	FO (%)
Insecta	99.7	Ephemeroptera	86.7
		Plecoptera	84.9
		Trichoptera	64
		Diptera	45.7
		Plecoptera_Ephemeroptera*	30.8
		Coleoptera	14.1
		Orthoptera	6.5
		Neuroptera	1.8
		Hemiptera	1.3
		Lepidoptera	1.0
		Odonata	0.5
		Hymenoptera	0.3
Malacostraca	18	Amphipoda	16.7
		Isopoda	1.6
		Decapoda	0.3
Diplopoda	3.7	Glomerida	2.4
		Polydesmida	0.8
		Julida	0.5
		Chordeumatida	0.3
Lissamphibia	2.9	Urodeles	2.9
Arachnida	2.4	Araneae	0.8
		Opiliones	0.5
		Sarcoptiformes	0.5
		Trombidiformes	0.5
Clitellata	0.5	Haplotaxida	0.5
Collembola	0.3	Entomobryomorpha	0.3
Eurotatoria	0.3	Bdelloida	0.3
Eutardigrada	0.3	Parachela	0.3
Gastropoda	0.3	Littorinimorpha	0.3
Unidentified fish			0.5

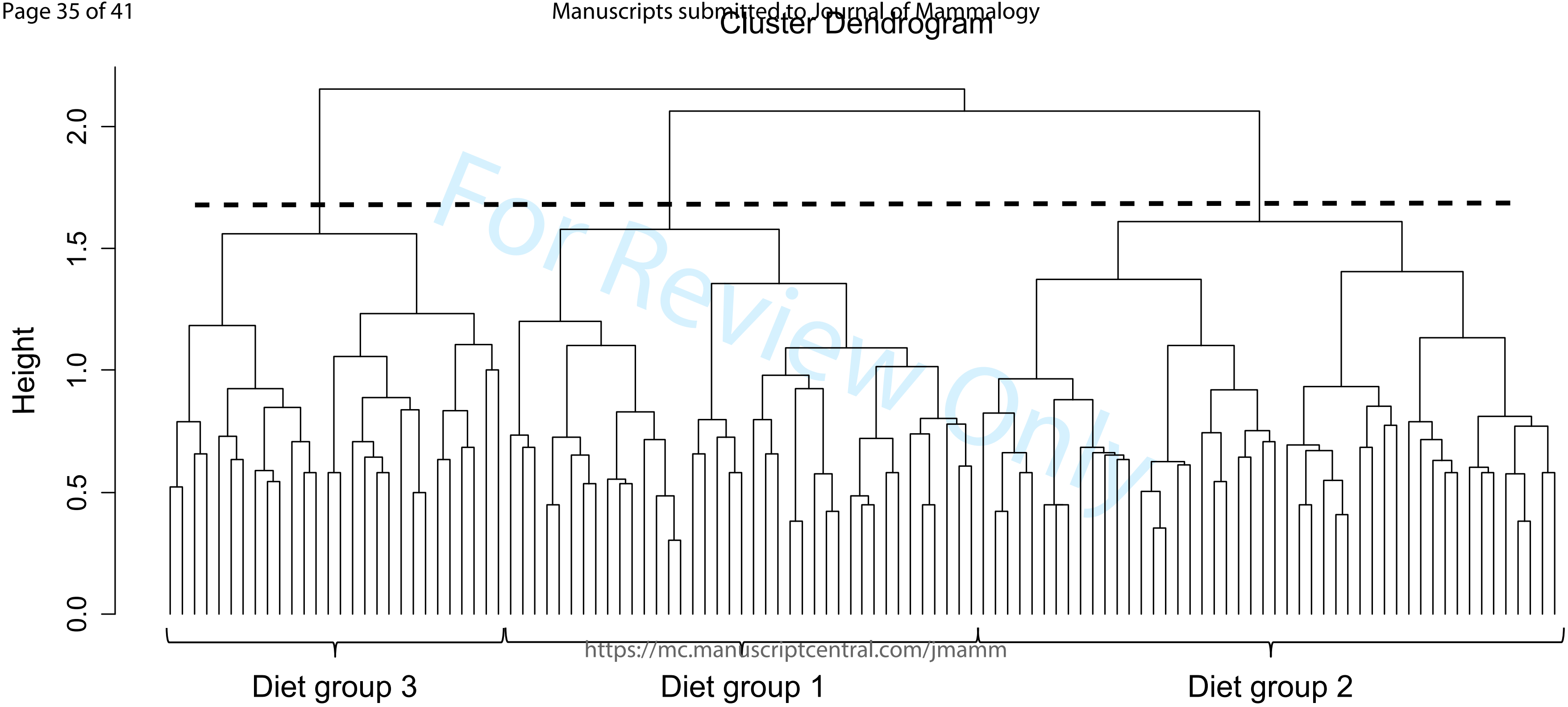
Table 3.— Significant indicator prey taxa ($p < 0.05$) of the three summer diet groups of the Pyrenean desman in the French Pyrenees identified by an IndVal analysis.

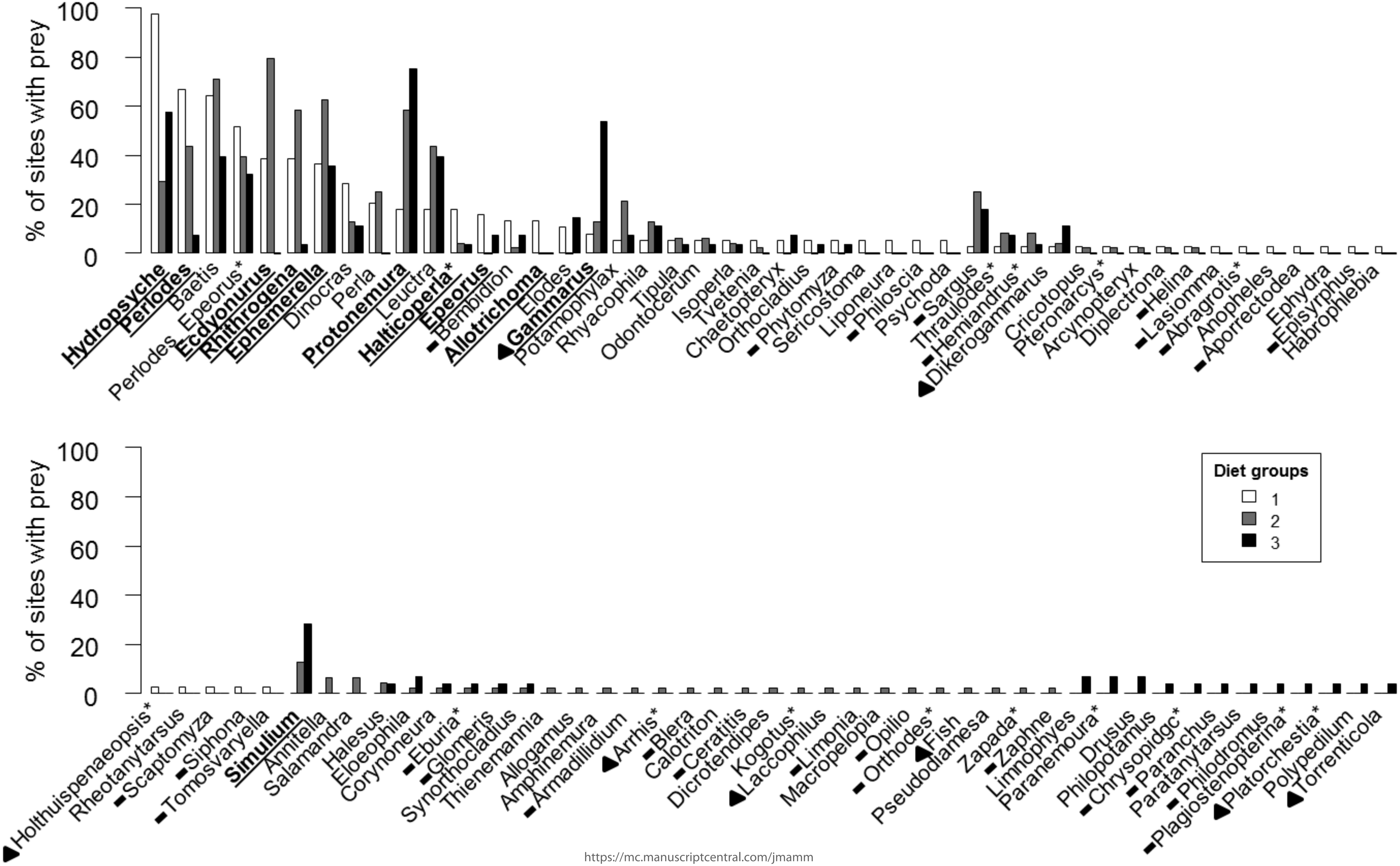
Groups	Indicator taxa		IndVal index	$p < 0.05$
	Order	Genus		
1	Trichoptera	*Hydropsyche*	0.72	0.0001
	Plecoptera	*Perlodes*	0.62	0.0001
	Diptera	*Allotrichoma*	0.36	0.0035
	Plecoptera	*Halticoperla**	0.35	0.0289
	Ephemeroptera	*Epeorus*	0.32	0.0488
2	Ephemeroptera	*Ecdyonurus*	0.73	0.0001
	Ephemeroptera	*Rhithrogena*	0.58	0.0008
	Ephemeroptera	*Ephemerella*	0.54	0.02
3	Amphipoda	*Gammarus*	0.62	0.0001
	Plecoptera	*Protonemura*	0.61	0.0003
	Diptera	*Simulium*	0.45	0.0023

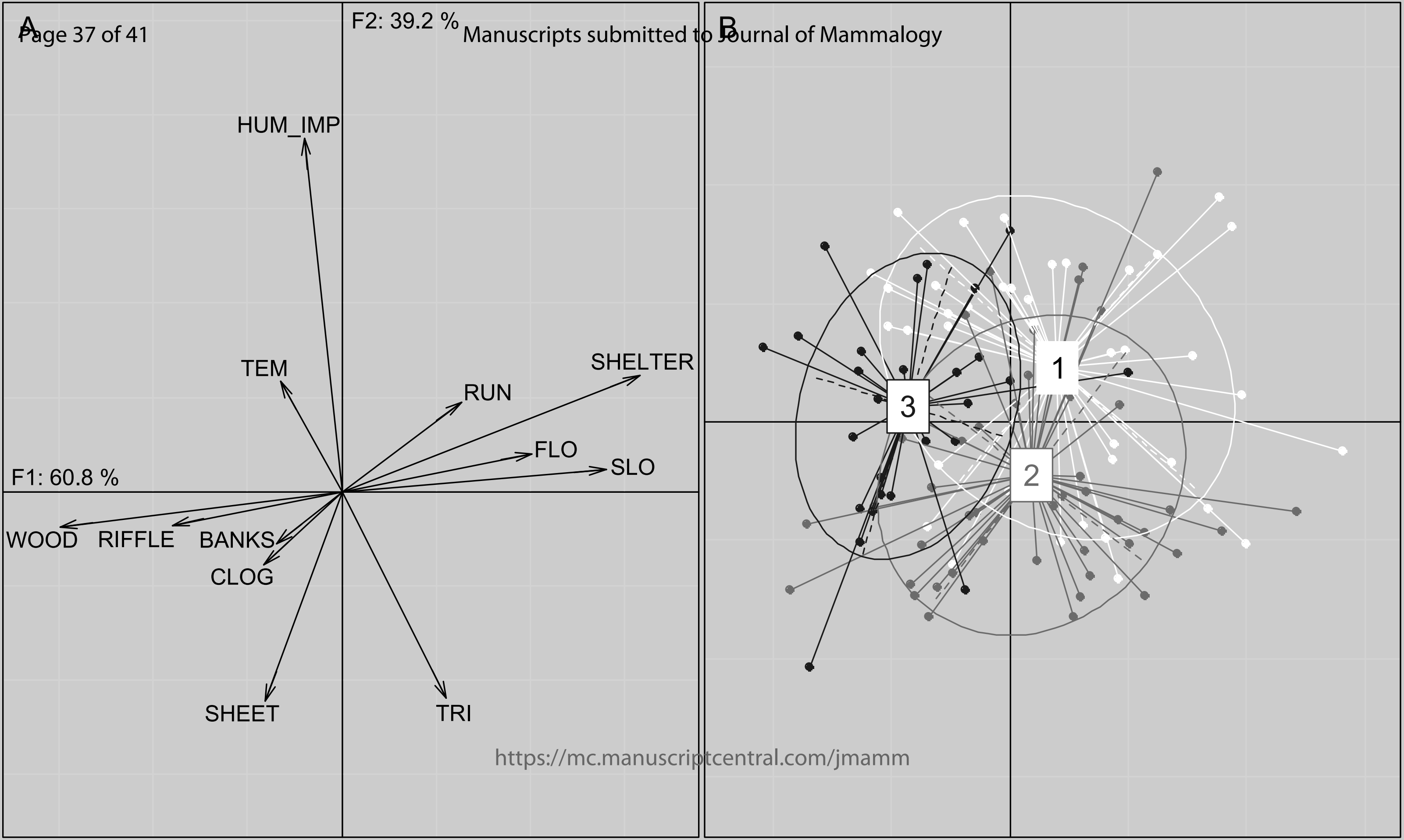
Table 4.— Mean and standard deviation (%) of the 12 environmental variables for the three summer diet groups of the Pyrenean desman in the French Pyrenees. See Table 1 for variables description.

Environmental variables	Groups		
	1	2	3
SHELTER	74.42 ± 18.33	67.79 ± 17.16	59.42 ± 21.76
CLOG	11.89 ± 12.61	15.43 ± 16.79	19.71 ± 16.23
SHEET	4.81 ± 6.20	6.60 ± 11.08	4.64 ± 5.12
RUN	23.39 ± 16.04	20.75 ± 11.91	21.24 ± 12.77
RIFFLE	35.77 ± 23.52	37.26 ± 18.82	44.43 ± 22.60
BANKS	0.35 ± 0.25	0.47 ± 0.23	0.52 ± 0.28
WOOD	50.50 ± 23.03	44.51 ± 17.63	53.47 ± 17.74
HUM_IMP	28.79 ± 29.69	9.29 ± 17.49	19.79 ± 20.64
TEM	9.70 ± 1.93	8.94 ± 1.73	10.34 ± 1.48
FLO	1.32 ± 1.87	0.68 ± 0.83	1.27 ± 1.64
SLO	8.78 ± 8.35	10.79 ± 8.91	8.07 ± 10.54
TRI	3.31 ± 2.13	3.42 ± 1.93	2.68 ± 1.19


B
A
FRANCE
• Pyrenean desman sampling sites
N
French Pyrenees hydrographic regions
Western Pyrenees
Central Pyrenees
Eastern Pyrenees
Pyrenean desman diet groups
1
2
3
0
25
50
100
km
1°0'0"W
0°0'0"
1°0'0"E
2°0'0"E
3°0'0"E
44°0'0"N
43°0'0"N
42°0'0"N


Cluster Dendrogram
Height
0.0
0.5
1.0
1.5
2.0
Diet group 3
Diet group 1
Diet group 2


% of sites with prey
0
20
40
60
80
100
Hydropsyche
Perlodes
Baetis
Perlodes
Epeorus*
Ecdyonurus
Rhithrogena
Ephemerella
Dinocras
Perla
Protonemura
Leuctra
Halticoperla*
Epeorus
▬ Bembidion
Allotrichoma
Elodes
▶Gammarus
Potamophylax
Rhyacophila
Tipula
Odontocerum
Isoperla
Tvetenia
Chaetopteryx
Orthocladius
▬ Phytomyza
Sericostoma
Liponeura
▬ Philoscia
Psychoda
▬Sargus
Thraulodes*
▬ Hemiandrus*
▶Dikerogammarus
Cricotopus
Pteronarcys*
Arcynopteryx
Diplectrona
▬Helina
▬Lasiomma
▬Abragrotis*
Anopheles
▬Aporrectodea
Ephydra
▬Episyrphus
Habrophlebia
% of sites with prey
0
20
40
60
80
100
Diet groups
□ 1
▨ 2
■ 3
▶Holthuispenaeopsis*
Rheotanytarsus
▬Scaptomyza
▬Siphona
▬Tomosvaryella
Simulium
Annitella
Salamandra
Halesus
Eloeophila
Corynoneura
▬Eburia*
▬Glomeris
Synorthocladius
Thienemannia
Allogamus
Amphinemura
▬Armadillidium
▶Arrhis*
▬Blera
Calotriton
▬Ceratitis
Dicrotendipes
Kogotus*
▶Laccophilus
▬Limonia
Macropelopia
▬Opilio
▬ Orthodes*
▶Fish
Pseudodiamesa
Zapada*
▬Zaphne
Limnophyes
Paranemoura*
Drusus
Philopotamus
▬Chrysopidgc*
▬Paranchus
Paratanytarsus
▬Philodromus
▬Plagiostenopterina*
▶Platorchestia*
Polypedilum
▶Torrenticola


A
F2: 39.2 %
F1: 60.8 %
HUM_IMP
TEM
RUN
SHELTER
FLO
SLO
WOOD
RIFFLE
BANKS
CLOG
SHEET
TRI
B
1
2
3

Supplementary Data S1.— Complete list of taxa identified as Pyrenean desman prey by molecular analysis of 383 faeces collected in the French Pyrenees. Frequencies of occurrence of each prey (FO: % of faeces with taxa) are displayed. * indicates misidentified taxa whose distribution areas exclude the Pyrenees and France. Habitat type of taxa is given (aq: exclusively aquatic; aq/te: with aquatic and terrestrial stages; te: exclusively terrestrial).

Class	FO (%)	Order	FO (%)	Family	FO (%)	Genus	Habitat	FO (%)
Insecta	99.7	Ephemeroptera	86.7	Heptageniidae	59.0	*Rhithrogena*	aq/te	36.6
						Ecdyonurus	aq/te	35.3
						Epeorus	aq/te	9.1
						Electrogena	aq/te	0.5
				Baetidae	56.7	*Baetis*	aq/te	56.4
						Acentrella	aq/te	0.3
				Ephemerellidae	37.1	*Ephemerella*	aq/te	37.1
				Leptophlebiidae	4.7	*Thraulodes**	aq/te	3.9
						Habroleptoides	aq/te	0.5
						Habrophlebia	aq/te	0.3
		Plecoptera	84.9	Nemouridae	49.4	*Protonemura*	aq/te	47.3
						Nemoura	aq/te	1.8
						*Paranemoura**	aq/te	1.3
						Amphinemura	aq/te	0.3
						*Zapada**	aq/te	0.3
				Perlodidae	32.6	*Perlodes*	aq/te	30.6
						Isoperla	aq/te	3.1
						Arcynopteryx	aq/te	0.5
						*Kogotus**	aq/te	0.5
				Leuctridae	29.8	*Leuctra*	aq/te	29.5
						*Perlomyia**	aq/te	0.3
				Perlidae	25.9	*Perla*	aq/te	15.7
						Dinocras	aq/te	15.1
				Notonemouridae	5.5	*Halticoperla**	aq/te	5.2
						*Spaniocerca**	aq/te	0.3
				Pteronarcyidae	5.2	*Pteronarcys**	aq/te	5.2
				Taeniopterygidae	2.6	*Brachyptera*	aq/te	2.1
						Taeniopteryx	aq/te	0.5
		Trichoptera	64.0	Hydropsychidae	46.7	*Hydropsyche*	aq/te	46.2
						Diplectrona	aq/te	1.3
				Limnephilidae	19.1	*Potamophylax*	aq/te	8.1
						Chaetopteryx	aq/te	3.4
						Allogamus	aq/te	2.6
						Halesus	aq/te	2.4

				Annitella	aq/te	2.1
				Drusus	aq/te	1.8
		Rhyacophilidae	6.5	*Rhyacophila*	aq/te	6.5
		Odontoceridae	3.4	*Odontocerum*	aq/te	3.4
		Sericostomatidae	2.1	*Sericostoma*	aq/te	2.1
		Philopotamidae	1.3	*Philopotamus*	aq/te	1.0
				Wormaldia	aq/te	0.3
		Goeridae	0.3	*Goeracea**	aq/te	0.3
		Uenoidae	0.3	*Thremma*	aq/te	0.3
Diptera	45.7	Simuliidae	15.4	*Simulium*	aq/te	14.9
				*Stegopterna**	aq/te	0.5
		Chironomidae	14.6	*Limnophyes*	aq/te	2.4
				Orthocladius	aq/te	2.4
				Synorthocladius	aq/te	1.6
				Cricotopus	aq/te	1.0
				Tvetenia	aq/te	1.0
				Brillia	aq/te	0.8
				Corynoneura	aq/te	0.8
				Polypedilum	aq/te	0.8
				Thienemannia	aq/te	0.8
				Diamesa	aq/te	0.5
				Dicrotendipes	aq/te	0.5
				Microspectra	aq/te	0.5
				Smittia	aq/te	0.5
				Cardiocladius	aq/te	0.3
				Conchapelopia	aq/te	0.3
				Macropelopia	aq/te	0.3
				Microtendipes	aq/te	0.3
				Parametriocnemus	aq/te	0.3
				Paratanytarsus	aq/te	0.3
				Paratrichocladius	aq/te	0.3
				Pseudodiamesa	aq/te	0.3
				Rheotanytarsus	aq/te	0.3
				Zavrelimyia	aq/te	0.3
		Stratiomyidae	10.7	*Sargus*	te	10.7
		Tipulidae	4.7	*Tipula*	aq/te	4.7
		Ephydridae	3.7	*Allotrichoma*	aq/te	2.6
				Ephydra	aq/te	0.8
				Scatophila	aq/te	0.3
		Anthomyiidae	2.6	*Lasiomma*	te	1.3
				Zaphne	te	1.3
		Psychodidae	2.6	*Psychoda*	aq/te	2.6
		Pediciidae	1.8	*Dicranota*	aq/te	1.6
				Pedicia	aq/te	0.3
		Agromyzidae	1.3	*Phytomyza*	te	1.3
		Limoniidae	1.3	*Eloeophila*	aq/te	1.0

						Limonia	te	0.3
				Calliphoridae	0.8	*Lucilia*	te	0.8
				Muscidae	0.8	*Helina*	te	0.5
						Thricops	te	0.3
				Platystomatidae	0.8	*Plagiostenopterina**	te	0.8
				Sciomyzidae	0.8	*Pteromicra*	aq/te	0.8
				Syrphidae	0.9	*Blera*	te	0.3
						Episyrphus	te	0.3
						Spilomyia	te	0.3
				Tachinidae	0.8	*Euthera**	te	0.3
						Siphona	te	0.3
						*Tachinid**	te	0.3
				Tephritidae	0.8	*Anastrepha**	te	0.5
						Ceratitis	te	0.3
				Blephariceridae	0.5	*Liponeura*	aq/te	0.5
				Drosophilidae	0.5	*Scaptomyza*	te	0.5
				Anisopodidae	0.3	*Sylvicola*	te	0.3
				Athericidae	0.3	*Atherix*	aq/te	0.3
				Chloropidae	0.3	*Epichlorops*	te	0.3
				Culicidae	0.3	*Anopheles*	aq/te	0.3
				Hybotidae	0.3	*Platypalpus*	te	0.3
				Pipunculidae	0.3	*Tomosvaryella*	te	0.3
				Sarcophagidae	0.3	*Sarcophaga*	te	0.3
				Tabanidae	0.3	*Haematopota*	te	0.3
		Plecoptera_ Ephemeroptera	30.8	Perlodidae_ Heptageniidae	30.8	*Perlodes_Epeorus**	aq/te	30.8
		Coleoptera	14.1	Carabidae	7.1	*Bembidion*	te	6.5
						Abax	te	0.3
						Paranchus	te	0.3
				Scirtidae	6.3	*Elodes*	aq/te	6.3
				Cerambycidae	0.5	*Eburia**	te	0.5
				Dryopidae	0.3	*Dryops*	aq/te	0.3
				Dytiscidae	0.3	*Laccophilus*	aq	0.3
				Staphylinidae	0.3	*Lesteva*	te	0.3
		Orthoptera	6.5	Anostostomatidae	6.5	*Hemiandrus**	te	6.5
		Neuroptera	1.8	Chrysopidae	1.8	*Chrysopidgc**	te	1.8
		Hemiptera	1.3	Aphididae	0.5	*Rhopalosiphum*	te	0.5
				Pentatomidae	0.5	*Pentatoma*	te	0.5
				Cercopidae	0.3	*Philaenus*	te	0.3
		Lepidoptera	1.0	Geometridae	0.3	*Lycia*	te	0.3
				Noctuidae	0.8	*Abragrotis**	te	0.3
						*Lacinipolia**	te	0.3
						*Orthodes**	te	0.3
		Odonata	0.5	Calopterygidae	0.3	*Calopteryx*	aq/te	0.3
				Gomphidae	0.3	*Ophiogomphus*	aq/te	0.3
		Hymenoptera	0.3	Formicidae	0.3	*Lasius*	te	0.3
Malacostraca	18.0	Amphipoda	16.7	Gammaridae	16.5	*Gammarus*	aq	14.6

						Dikerogammarus	aq	2.6
				Oedicerotidae	0.5	*Arrhis**	aq	0.5
				Talitridae	0.3	*Platorchestia**	aq	0.3
		Isopoda	1.6	Philosciidae	0.5	*Philoscia*	te	0.5
				Armadillidiidae	0.3	*Armadillidium*	te	0.3
				Oniscidae	0.3	*Oniscus*	te	0.3
				Porcellionidae	0.3	*Porcellio*	te	0.3
				Scyphacidae	0.3	*Haloniscus**	aq	0.3
		Decapoda	0.3	Penaeidae	0.3	*Holthuispenaeopsis**	aq	0.3
Diplopoda	3.7	Glomerida	2.4	Glomeridae	2.1	*Glomeris*	te	2.1
				Glomeridellidae	0.3	*Glomeridella*	te	0.3
		Polydesmida	0.8	Polydesmidae	0.8	*Polydesmus*	te	0.8
		Julida	0.5	Julidae	0.5	*Allajulus*	te	0.3
						*Pteridoiulus**	te	0.3
		Chordeumatida	0.3	Craspedosomatidae	0.3	*Ochogona*	te	0.3
Lissamphibia	2.9	Urodeles	2.9	Salamandridae	2.9	*Calotriton*	aq/te	1.8
						Salamandra	aq/te	1.0
Arachnida	2.4	Araneae	0.8	Philodromidae	0.5	*Philodromus*	te	0.5
				Tetragnathidae	0.3	*Metellina*	te	0.3
		Opiliones	0.5	Phalangiidae	0.5	*Opilio*	te	0.5
		Sarcoptiformes	0.5	Ceratozetidae	0.3	*Fuscozetes*	te	0.3
				Oribatulidae	0.3	*Zygoribatula*	te	0.3
		Trombidiformes	0.5	Torrenticolidae	0.5	*Torrenticola*	aq	0.5
Clitellata	0.5	Haplotaxida	0.5	Lumbricidae	0.3	*Aporrectodea*	te	0.3
				Megascolecidae	0.3	*Metaphire*	te	0.3
Collembola	0.3	Entomobryomorpha	0.3	Tomoceridae	0.3	*Tomocerus*	te	0.3
Eurotatoria	0.3	Bdelloida	0.3	Habrotrochidae	0.3	*Habrotrocha*	aq	0.3
Eutardigrada	0.3	Parachela	0.3	Isohypsibiidae	0.3	*Thulinius**	aq	0.3
Gastropoda	0.3	Littorinimorpha	0.3	Tateidae	0.3	*Potamopyrgus*	aq	0.3
Unidentified fish							aq	0.5